The use of flow cytometry in the biosystematics, ecology and population biology of homoploid plants

Využití průtokové cytometrie při studiu homoploidních skupin rostlin

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Over the last decade there has been a tremendous increase in the use of flow cytometry (FCM) in studies on the biosystematics, ecology and population biology of vascular plants. Most studies, however, address questions related to differences in genome copy number, while the value of FCM for studying homoploid plant groups has long been underestimated. This review summarizes recent advances in taxonomic and ecological research on homoploid plants that were made using FCM. A fairly constant amount of nuclear DNA within each evolutionary entity together with the often large differences between species means that genome size is a useful character for taxonomic decision-making. Regardless of the number of chromosomes, genome size can be used to delimit taxa at various taxonomic levels, resolve complex low-level taxonomies, assess the frequency of interspecific hybridization or infer evolutionary relationships in homoploid plant groups. In plant ecology and evolutionary biology, variation in genome size has been used for prediction purposes because genome size is associated with several phenotypic, physiological and/or ecological characteristics. It is likely that in the future the use of FCM in studies on taxonomy, ecology and population biology of homoploid plants will increase both in scope and frequency. Flow cytometry alone, but especially in combination with other molecular and phenotypic approaches, promises advances in our understanding of the functional significance of variation in genome size in homoploid plants.

K e y w o r d s: biosystematics, ecology, flow cytometry, genome size, homoploid species, hybridization, nuclear DNA content, population biology, taxonomy

Introduction to flow cytometry

Flow cytometry (FCM) is a fast and effective way of simultaneously analysing several optical properties (fluorescence, light scatter) of single particles in suspension as they move in a narrow liquid stream through a powerful beam of light (Shapiro 2004). The recorded optical signals can be used to infer the chemical and/or physical composition of the particles. This technology was originally developed in the late 1950s for rapid counting and analysing of blood cells in clinical research and practice (Shapiro 2007). However,

it took two more decades before FCM started to be used in various fields of biological science, including experimental and field botany, with the advent of user-friendly and versatile bench-top instruments, discovery of new fluorochromes and development of convenient protocols (Doležel et al. 2007a). A major breakthrough in plant FCM was the development of a rapid, easy and reliable method for isolating nuclei from solid tissues (Galbraith et al. 1983), which triggered the use of cytometry in plant laboratories worldwide and resulted in an ever-increasing number of scientific papers (Loureiro et al. 2007). Despite this the various disciplines of plant biology, such as plant population biology, taxonomy, ecology and evolutionary biology, have still exploited only a small part of the full potential of FCM. Nevertheless, over the last decade the use of this method has resulted in significant advances in our understanding of the patterns and processes in many plant systems (Kron et al. 2007). The great majority of FCM applications in plant science are based on recording fluorescence intensities of nuclei stained with DNA-selective dyes, which provide estimates of genome size (nuclear DNA content) with a high level of precision. The results can either be expressed in absolute units (picograms of DNA or numbers of base pairs; for the conversion factor see Doležel et al. 2003) or in relative terms as an indication of the level of ploidy. Other parameters (e.g. forward and side scatter and particle volume) are rarely recorded in plant studies despite their great potential for certain purposes (Loureiro et al. 2006a, b).

The popularity of FCM over other methods for characterizing cells and other particles lies in its advantages. First, a wide range of parameters can be simultaneously recorded for each and every particle, avoiding the risk of biased measurements due to sample heterogeneity and allows complex particle populations to be de-convoluted. Furthermore, high numbers of particles can be analysed quickly, making the results highly representative and statistically robust. Other advantages relate specifically to DNA-based assays. Flow cytometry mostly provides analyses of unsurpassed accuracy and resolution, which facilitates the detection of tiny differences in the amount of nuclear DNA. In addition, sample preparation is usually easy and convenient (i.e. chopping the sample and an internal reference standard in an appropriate buffer, filtration to remove tissue debris and staining the isolated intact nuclei using a DNA-selective fluorochrome) and usually does not take more than a few minutes. Dozens of samples can therefore be assayed within a working day, which makes it easy for large-scale screening of ploidy and genome size variation at the individual, population and landscape levels. In some cases, the number of analysed individuals can further be increased by pooling several samples, as for instance when a rapid check of ploidy/genome size homogeneity is the major aim. Another feature that needs to be highlighted is that very little plant tissue is required for this analysis, which makes FCM a non-destructive tool. The material subjected to FCM can also be used for other analyses or cultivated. Thus it is possible to investigate samples from the very early ontogenetic stages of plants or perform thorough analysis of rare or endangered species with little effect on an individual's fitness or population density. It should be added that a broad variety of tissues of most plant species can be analysed: in addition to leaf laminas, which are usually the first choice, high-resolution histograms can often be achieved by analysing petioles, stems, roots, sepals, petals, seeds and other tissues. Of paramount importance is the fact that the plant tissue does not need to be mitotically active (in contrast to conventional karyological analyses). Because FCM records the total amount of nuclear DNA irrespective of the number of chromosomes, it can distinguish between plants with the same

number of chromosomes (homoploid taxa) but with different amounts of nuclear DNA, a task that is difficult to achieve as quickly and cheaply using other contemporary cytogenetic tools. Last but not least, the low operating costs of FCM is likely to have been a major contributing factor to the recent increase in the popularity of this method in plant sciences.

Genome size: a fundamental parameter

Genomes represent a distinct and legitimate level of biological organization, with their own unique evolutionary histories and genome size as one of their inherent properties (Gregory 2005a). The amount of nuclear DNA characteristic of a particular genotype is usually referred to as the C-value. The symbol C (= Constant) was first introduced by Swift (1950), but it was only recently that the terminology for genome size was standardized by Greilhuber et al. (2005). They introduced the adjectives "holoploid" and "monoploid" (with acronyms C-value and Cx-value, respectively) to distinguish between the size of the complete chromosome complement (with chromosome number n) and that of the monoploid chromosome set (with chromosome number x).

In the plant kingdom, genome sizes of approximately 4,500 species are known (~ 1.8% of the species of land plants; Bennett & Leitch 2005a). The variation in holoploid genome size is amazing and spans nearly a 2000-fold range, with *Genlisea margaretae* (*Lentibulariaceae*, 1C = 0.065 pg; Greilhuber et al. 2006) and *Fritillaria assyriaca* (*Liliaceae*, 1C = 127.4 pg; Bennett & Leitch 2005a) with the smallest and the largest genomes, respectively. However, there are large differences in the variation in genome size among different taxonomic groups, which determines the value of genome size as a taxonomic and/or ecological marker in particular plant groups. For example, while it is highly variable in angiosperms and ferns, in gymnosperms and mosses there is little variability in genome size (Leitch & Bennett 2007, Temsch et al. 2010). The differences in genome size are largely caused by different amounts of non-coding repetitive DNA, which is composed of transposable elements, satellite DNA, introns and pseudogenes (Bennett & Leitch 2005a).

The evolution of genome size is a highly dynamic and bidirectional process. According to current views, the amount of DNA an organism has is a result of a dynamic balance between expansion and contraction forces (Bennett & Leitch 2005a). In homoploid plants, major mechanisms responsible for genome expansion include amplification and insertion of transposable genetic elements (Vitte & Bennetzen 2006) and evolution and amplification of satellite repeats (Lim et al. 2006). It is known that environmental conditions may modulate the transcriptional activity of (retro)transposons (Kalendar et al. 2000). The loss of DNA and thus the reduction in the size of the genome is associated with mechanisms like unequal intrastrand homologous recombination, illegitimate recombination and/or higher overall rate of nucleotide deletion over insertion (Bennetzen et al. 2005). Despite the dynamic nature of the evolution of genome size (Leitch et al. 2008, Lysak et al. 2009) its size within the same evolutionary unit (e.g. species) seems to be stabilized by internal mechanisms and as a consequence genuine intraspecific variation in genome size is quite rare (but see Schmuths et al. 2004, Obermayer & Greilhuber 2005, Šmarda & Bureš 2006, Leong-Škorničková et al. 2007, Suda et al. 2007a,b, Slovák 2009).

Practical considerations

Research on homoploid plants often requires the detection of small differences in genome size, which places high demands on the quality of FCM analysis. In contrast to the estimates of the level of ploidy, which can withstand certain relaxation of methodological standards, studies on homoploid plants require analysis with high resolution and accuracy. General recommendations for estimating the genome sizes are summarized elsewhere (Doležel & Bartoš 2005, Doležel et al. 2007b, Greilhuber et al. 2007). Of these recommendations those of particular importance to studies on homoploid plants are standardization (internal standard with genome size similar to that of the sample to be analysed should always be used) and an awareness of the effect of secondary metabolites. Previous studies have demonstrated that secondary compounds may negatively affect the fluorescence and scatter properties of isolated nuclei and thus result in aberrant results (Noirot et al. 2000, Price et al. 2000, Loureiro et al. 2006b, Walker et al. 2006). Testing for the presence of inhibitors (Price et al. 2000) or analysing scatter properties of the nuclei (Loureiro et al. 2006b) is therefore advisable. While the determination of genome size in absolute units requires the use of intercalating fluorochromes (the most popular of which is propidium iodide), fluorescent dyes with a base bias (AT- or GC-selective) may often be more suitable for the detection of small differences in the amount of nuclear DNA. DAPI (4',6-diamidino-2-phenylindole) is favoured because of its high selectivity for DNA and relative insensitivity to differences in chromatin condensation (Shapiro 2004), resulting in fluorescence histograms of unsurpassed resolution and very low background (Fig. 1).

The differences in nuclear DNA content that can be reliably discriminated with the aid of FCM largely depend on the coefficients of variation (CVs) of the resulting peaks. As a rule of thumb, differences of as low as 3–4% can be differentiated (plants with small genomes are generally more challenging to distinguish than those with large genomes). Detection of differences below 3% may also be possible in some cases, although this requires several repetitions on different days (preferably in different seasons and using different stains). To minimize the effect of secondary metabolites, plants with putative differences in genome size should be cultivated under similar conditions for a year or more. The use of two different internal reference standards (one with a smaller and the other with larger genome size than that of the sample) may also improve the resolution. In any case, the most convincing evidence of genuine variation in the amount of nuclear DNA is the presence of double peaks or a bimodal peak obtained after simultaneous analysis of samples with different genome sizes (Zonneveld 2001, Leong-Škorničková et al. 2007, Suda et al. 2007a, b, Kolář et al. 2009, Slovák et al. 2009, Šmarda & Bureš 2010). To the best of our knowledge, the most striking example of a high-resolution histogram with well separated peaks is the simultaneous analysis of female and male plants of *Silene latifolia*, which differ by 3.7% in genome size (Doležel & Göhde 1995). Basically, two distinct peaks can only be achieved if the CVs of the peaks are less than half of the difference in genome size of the simultaneously analysed samples (Fig. 1).

Applications of flow cytometry in the study of homoploid plant groups

The use of FCM in research on homoploid plants is largely based on exploiting and interpreting differences in genome size (either absolute or relative) among analysed samples. There are three principal ways in which this genome size data can be used: (i) for taxonomic

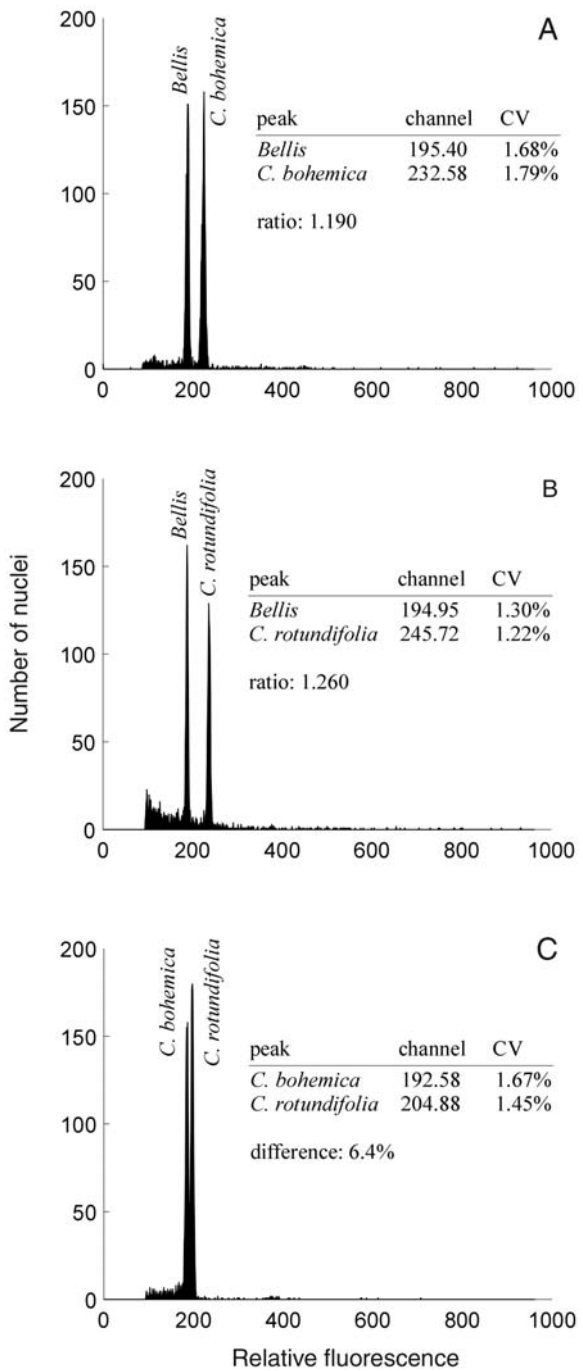

Fig. 1. – High-resolution histograms showing the difference in the genome size of tetraploid plants (2n = 4x = 68) of *Campanula bohemica* and *C. rotundifolia*. Panels A and B present results for separate analyses of plants, using *Bellis perennis* as an internal reference standard. The difference was confirmed by the results of a simultaneous analysis of both species (panel C). Nuclei were isolated in Otto's buffers and stained with DAPI.

purposes as a taxon-specific marker; (ii) for prediction, as genome size is associated with several phenotypic, physiological and/or ecological characteristics (i.e. the nucleotypic effect; Bennett 1972); and (iii) for understanding the dynamics of genome evolution (by studying the extent of inter- and intraspecific variation in genome size). The latter topic will not be discussed in this review because it is the subject of another paper in this special issue of Preslia (Šmarda & Bureš 2010).

Plant taxonomy and biosystematics

While chromosomal data is widely used for decision making in taxonomy (Stace 2000, Ekrt et al. 2009), the taxonomic and evolutionary significance of variation in genome size has only recently been acknowledged (Kron et al. 2007). The value of genome size lies in the fact that it may vary dramatically among different (though often closely related) homoploid species (see Bennett & Leitch 2005a) but is mostly constant within the same species/evolutionary unit (Greilhuber 1998, 2005, Bennett et al. 2000, Murray 2005). As a result, genome size can be used as a supportive character to circumscribe taxa at various taxonomic ranks (mainly species) and can help to resolve complex low-level taxonomies. Moreover, variation in genome size can be viewed as an indicator of taxonomic heterogeneity, incipient speciation, or complex evolutionary history (Obermayer & Greilhuber 2005, Leong-Škorničková et al. 2007, Suda et al. 2007a,b, Slovák et al. 2009). Such groups are in need of taxonomic revision, which may result in re-defining of species boundaries (Greilhuber & Speta 1985), detection of an hitherto undescribed taxon (Maxted et al. 1991), or revelation that a species has been misidentified (Yeater et al. 2004). Because FCM is a rapid and precise method for screening variation in genome size in large population samples and across large spatial scales, it may also be used as an exploratory tool in studies on taxonomically challenging plant groups.

Resolving homoploid taxonomies

The nearly 2000-fold variation in the genome size of angiosperms indicates it may be possible to use genome size data in making taxonomic decisions. Indeed, variation in genome size across angiosperms is much higher than is the variation in the traditional and widely used cytogenetic marker, i.e. the number of somatic chromosomes (2n = 4–640; Stace 2000). A survey of the Plant DNA C-values database (Bennett & Leitch 2005b) revealed a high incidence of intra-generic variation in genome size in species with the same number of chromosomes. At least two-fold variation in monoploid genome size is recorded for more than one third of the genera for which there is sufficient coverage of homoploid species (Suda et al. 2006). Genera *Bulnesia*, *Crepis*, *Cypripedium*, *Dendrobium*, *Lonicera*, *Oxalis*, *Phalaenopsis*, *Scilla*, *Senecio*, *Sisyrinchium*, *Tradescantia*, *Vaccinium* and *Vicia* are some of the most best examples of genera with a large between-species divergence in genome size that is not accompanied by changes in the number of chromosomes.

By providing extensive data on genome size, FCM has made significant contributions to the detection and delineation of taxa, revealing cryptic diversity and resolving complex homoploid taxonomies (Kron et al. 2007). The use of FCM is particularly rewarding in plant groups with phenotypic similarities, low numbers of distinct morphological characters (such as geophytes, graminoids or parasites), continuous morphological variation and/or in groups

prone to inter-specific hybridization or with complex evolutionary histories (e.g. allopolyploids). One of the first FCM studies on homoploid groups with some taxonomic relevance dealt with the genus *Helleborus* (Zonneveld 2001). All *Helleborus* species have 32 chromosomes in their somatic cells but the largest genome is nearly twice as large as that of the smallest. Genome size can be used to distinguish several species and the variation also corresponds well with the sectional division. This study was followed by several others, which examined the taxonomic value of genome size in a representative set of samples of an herbaceous perennial *Hosta* (Zonneveld & Van Iren 2001), geophytes *Agapanthus* (Zonneveld & Duncan 2003), *Galanthus* (Zonneveld et al. 2003) and *Nerine* (Zonneveld & Duncan 2006) and a succulent *Gasteria* (Zonneveld & van Jaarsveld 2005). Dozens of studies aimed at assessing the variation in genome size of particular plant groups were published over the last decade and the list is continuously growing. Some of these works intentionally searched for, and found species-specific genome sizes, as in the case of *Lactuca* (Koopman 2000), *Petunia* (Mishiba et al. 2000), *Elytrigia* (Mahelka et al. 2005) and *Curcuma* (Leong-Škorničková et al. 2007). Others primarily addressed different questions and inter-specific differences in genome size were detected as a by-product, as is the case for *Hydrangea* (Cerbah et al. 2001), *Artemisia* (Torrell & Vallès 2001), *Echinops* (Garnatje et al. 2004), *Carthamus* (Garnatje et al. 2006) and *Trifolium* (Vižintin et al. 2006).

Although less thoroughly explored, genome size is a useful marker of taxonomic ranks below and above the species level. The value of genome size for distinguishing between different subspecies is documented by Loureiro et al. (2007), based on a study of Iberian fescue species. Despite their karyotype constancy, *Festuca ampla* subsp. *ampla* is easily distinguished from subsp. *transtagana* due to its ~5% smaller genome. Genome size also supports the subgeneric division in the genus *Equisetum*: taxa from subg. *Equisetum* have significantly smaller values than their counterparts from subg. *Hippochaete* (Obermayer et al. 2002). Similarly, an analysis of nuclear DNA content provided information on the sectional classification of the genus *Taraxacum* (Záveský et al. 2005). In addition, different plant families can show distinct variation in genome size, graminoids being an illustrative example. While *Cyperaceae* and *Juncaceae* mostly have small genomes (mean/median 1C-values: 1.14 pg/0.40 pg and 1.22/1.08 pg, respectively), those of *Poaceae* are much larger (mean/median 1C-values: 5.60/4.73 pg) (Bennett & Leitch 2005b).

A practical application of species-specific genome sizes in field research is the determination of non-flowering or poorly developed individuals, plant fragments, young seedlings or subterranean parts (roots, rhizomes). Unlike the conventional phenotype-based approach genome size can be used to reliably assign a sample to a particular species. Flow cytometry is particularly useful if taxonomically identified samples from a given locality were previously analysed for their genome sizes (it is important, however, that the genome size estimations are made in the same laboratory due to the potential for variation in the estimates obtained by different laboratories; Doležel et al. 1998). For example, it is possible to distinguish roots of the dominant species of plants in a mountain meadow by means of the inter-specific differences in their genome sizes (J. Suda et al., unpublished results).

Incidence of homoploid hybridization

Identification of homoploid hybrids using genome size is currently a challenging but at the same time fascinating aspect that can be studied using FCM. Provided the parental taxa

differ sufficiently in genome size (by at least 7%), it is possible to detect homoploid hybrids using FCM. The need for high quality of analyses has not deterred researchers from pursuing such studies and the number of publications documenting the successful use of FCM in assessing the frequency of homoploid hybridization has increased in recent years (Loureiro et al. 2008).

Jeschke et al. (2003) determined the genome sizes of two species of *Amaranthus* with 2n = 32 (*A. hybridus*, 2C = 1.04 pg and *A. tuberculatus*, 2C = 1.34 pg) and suggested that FCM can be used to detect inter-specific hybrids that other techniques fail to reveal. This prediction was later confirmed by Trucco et al. (2006) who found that putative hybrids had genome sizes intermediate to and non-overlapping with that of the parental species. In New Zealand hybrids between two introduced species of *Hieracium* subg. *Pilosella* were identified using FCM (Morgan-Richards et al. 2004). The authors report that while the karyotypes of the putative parental species were not sufficiently different to be distinguished using conventional karyological studies, the sizes of the haploid genomes differed markedly. The most parsimonious explanation for intermediate genome size values observed in some individuals is the incidence of inter-specific hybridization. There are many taxonomically complicated plant groups among the grasses; the tribe *Triticeae* is particularly challenging due to widespread hybridization and polyploidy. Mahelka et al. (2005) used FCM to assess the frequency of interspecific hybridization between hexaploid *Elytrigia repens* and *E. intermedia*. Surprisingly, they found a high proportion of hybrid plants, indicating that only weak reproductive barriers exist between the species. In previous studies, crosses were most likely overlooked or misidentified as they are weakly morphologically differentiated. Yet another example of a hybrid-prone plant group is the *Dryopteris carthusiana* complex. This fern group consists of one diploid (*D. expansa*) and two allotetraploid (*D. carthusiana* and *D. dilatata*) species in Central Europe. Regardless of ploidy level, all species differ in genome size, which allows not only species boundaries to be delimited but also the easy and reliable recognition of all hybrid combinations (L. Ekrt et al., unpublished results). There are several other homoploid plant groups in which genome size values intermediate between the parent values indicate the presence of homoploid crosses, including *Alstroemeria* (Buitendijk et al. 1997), *Oxalis* (Emshwiller 2002) and *Cucurbita* (Šiško et al. 2003).

Despite the fact that hybrid plants mostly have genomes of intermediate size between their putative parents, the few exceptions make the identification of hybrids more difficult. One such example concerns the genus *Cirsium*. After analysing the amount of nuclear DNA in twelve species and the same number of *Cirsium* hybrids, Bureš et al. (2004) concluded that the genome size of crosses may not always be located exactly halfway between the values of their putative parents, but closer to the species with the smaller genome. Elimination of certain parts of the genome shortly after hybridization (possibly entailing some evolutionary advantage) is proposed as the reason for the observed discrepancy. Nevertheless, as there is a high incidence of inter-specific hybridization in *Cirsium* it is possible that some of the plants are backcrosses rather than F1 hybrids. Another interesting outcome of the cited study is the negative relationship between genome size and the incidence of hybrids; i.e. species with small genomes produced inter-specific hybrids more frequently. A similar situation to that in *Cirsium,* but with the genome size of the hybrids more similar to that of the large-genome parent is recorded in *Helianthus* (Baack et al. 2005). No changes in genome size were detected in the first six generations following

hybridization. However, established homoploid hybrid species of *Helianthus* have about 50% more nuclear DNA than the parents, indicating that their genomes must have undergone a massive increase in size during their evolutionary history (Baack et al. 2005). The evolutionary forces responsible for the repeated and independent increases in genome size in hybrid *Helianthus* species remain to be discovered.

Inference of evolutionary history

Data on genome size can also provide insights into the evolutionary history of some plant groups, such as allopolyploids (i.e. polyploids combining genomes from at least two parental species; Leitch & Bennett 1997).

Early attempts to characterize ancestral genomes of allopolyploids using FCM date back to 1997, when Lee and co-workers analysed the nuclear DNA contents of 21 monosomic wheat lines. Although it was not possible to easily distinguish all of the monosomic lines, the authors found that the D genome (2C = 5.05 pg) contained markedly less DNA than both the A (2C = 6.15 pg) and B (2C = 6.09 pg) genomes (Lee et al. 1997). Similarly, Lysák et al. (1999) report a twelve percent difference in the amount of nuclear DNA between genomes A (donated from *Musa acuminata*) and B (donated from *M. balbisiana*) present in triploid banana cultivars, and proposed that comparative analysis of genome size may be helpful in identifying the diploid progenitors of cultivated triploid *Musa* clones.

Studies on wild allopolyploid species are still scarce. For a high-mountain allotetraploid *Androsace brigantiaca*, genome size provides independent support for putative parental species that were previously identified using molecular markers (Dixon et al. 2009). Inter-specific differences in genome size observed among some hawkweed species (Bräutigam & Bräutigam 1996) stimulated a detailed investigation of this taxonomically complicated group (Suda et al. 2007a). The evolutionary history of *Hieracium* subg. *Pilosella* was shaped by frequent inter-specific hybridization and genome duplication, and as a result two kinds of species, 'basic' and 'intermediate' (i.e. hybrid), are usually recognized. Although their phenotypes are similar, genome sizes can be used for inferring evolutionary relationships and genome constitution (i.e. putative parental combinations) of hybrid species of hawkweed (Suda et al. 2007a). In the related *Hieracium* subg. *Hieracium* the nuclear genome size was analysed in a phylogenetic framework to assess the relationships between genome size and ploidy, breeding system and selected ecogeographic features (Chrtek et al. 2009). Phylogeny was found to be the most important factor explaining the pattern of variation in genome size, outweighing any correlation with ecogeographic variables. Species of western European origin had significantly smaller genome sizes than their eastern European counterparts. Genome size also seems to be a useful marker for inferring evolutionary history in the *Melampyrum nemorosum* group (M. Štech et al., unpublished data). Small but reproducible differences in the amount of nuclear DNA were observed among populations from different geographic regions in Central Europe. In addition, ancient hybridization is suspected because of the existence of intermediate genome sizes and phenotypic variation in some taxa.

However, it should be noted that the pattern of variation in genome size in allopolyploid derivates (e.g. the additivity of parental genome sizes) might be obscured by different post-polyploidization processes, in particular, genome downsizing (Leitch & Bennett

2004). For example, Hendrix & Stewart (2005) estimated the nuclear DNA content of *Gossypium* species and found that tetraploids with A and D genomes have less DNA than expected from the sum of the C-values of their putative parents. Even more complex picture of the variation in genome size is reported for the allopolyploid *Nicotiana* species (Leitch et al. 2008). Comparison of the actual (= determined by FCM) and expected (= sum of genome size of species identified as either a parent or most closely related to the diploid progenitor) revealed downsizing of the genome in four polyploids and an increase in five. The difference between the actual and expected values generally increases with increase in evolutionary age (Leitch et al. 2008). These data indicate that the value of FCM for inferring evolutionary history in ancient (allo)polyploids may be limited and such inferences should always be compared with those indicated by more robust phylogenetic markers.

Plant ecology, reproduction and evolutionary biology

Accurate identification of organisms is as important in ecology and evolutionary biology as in plant taxonomy and biosystematics. However, ecological research has different objectives, such as seeking for affinities between organisms that have similar functions or exhibit parallel responses in contemporary ecosystems (Grime 1998). Despite the long standing evidence that the amount of DNA in the genome may have considerable biological effects on organisms independent of the encoded genetic information (the so-called nucleotypic theory; Bennett 1972), variation in genome size has only rarely been integrated into ecological predictions and models. There are many characters that are more or less tightly correlated with genome size, ranging from the sub-cellular, cellular and tissue levels up to the whole plant development and physiological processes (see Leitch & Bennett 2007 for a review). Of particular interest because of their potential ecological significance are the relationships between genome size and cell volume (Jovtchev et al. 2006), cell size and stomatal density (Knight & Beaulieu 2008), duration of cell division (Bennett 1977), pollen volume (Bennett 1972), seed size (Bennett 1972) and seed mass (Beaulieu et al. 2007). Better understanding of functional implications of small versus large genomes is one of the challenges facing contemporary ecological and evolutionary research. Since meaningful comparative studies require the collection of genome sizes for many species, this task is particularly amenable using FCM. Homoploid plant groups with similar evolutionary histories and sufficiently different in their content of nuclear DNA are particularly useful model systems, as the effect of genome size is not blurred by other types of karyological variation (e.g. differences in genome copy number or number of somatic chromosomes).

Ecological implications

Perhaps the most universal correlation across eukaryotes is the link between genome size and cell size (Gregory 2005b). In plants, genome size accounts for nearly 60% of the total variation in guard cell length and size of epidermal cells (Beaulieu et al. 2008). These authors also found that stomatal density – although growth form-dependent – scales negatively with genome size. Because the size and frequency of stomata dramatically influence carbon fixation and water use efficiency, changes in genome size may alter plant physiol-

ogy and thus determine the ecological and life-history strategy of a species. Therefore, the link between genome size – stomata size/density – plant physiology – plant ecology may be utilized for predicting the natural distribution of a species. For example, large genomes are never associated with a high frequency of small stomata, which signify adaptation to dry environments (Beaulieu et al. 2008). Consequently, species with large genomes are under-represented in environments characterized by low precipitation and high temperatures (Knight & Ackerly 2002).

There are studies that have focused on searching for correlations between genome size and various environmental parameters (such as latitude, altitude, temperature and precipitation). While pioneering studies report rather straightforward relationships (e.g. a positive correlation between genome size and sensitivity to frost; MacGillivray & Grime 1995), later works reveal a more complex picture, with actual relationships being often variable and dependant on the plant group studied and environmental parameters (see Knight et al. 2005 for a review). It seems plausible that the effect of ecological variables may be masked by other evolutionary forces or the results may be biased due to non-representative sampling (e.g. coverage of only a part of the ecological range, or of a limited number of species, etc.) or incorrect data analysis (e.g. the use of linear models only, omission of a phylogenetic correction, etc.).

Genome size-related processes at the cellular level (in particular, the duration of meiosis and mitosis) translate into the rate of ontogenetic development at the organismal level. An example is the minimum generation time, i.e. the minimum period of time from germination until the development of the first mature seed (Leitch & Bennett 2007). On average, ephemerals have the smallest genomes, whereas obligate perennials have the largest. Moreover, while herbaceous plants with small genomes (< 6 pg/2C) may have many developmental lifestyles, those with large genomes have fewer (e.g. species with a genome size above 50 pg/2C can only be perennials). This relationship, however, does not apply to woody plants that usually have much smaller genomes, perhaps as a consequence of developmental constraints related to the size of their cambial cells (Ohri 2005). Also, differences in plant phenology may be related to changes in genome size through concomitant influences on the duration of cell division. Grime et al. (1985) demonstrate that species with small genomes tend to grow later in the year than species with large genomes. Development in early spring involves mainly a sudden expansion of cells formed during the previous summer, while the growth later in the season is predominantly realized by quick cell divisions.

Another thoroughly explored and ecologically important trait constrained by genome size is seed mass. Seed characteristics (such as size and weight) are among the least plastic components of plant structure (Silvertown 1989). A number of ecological papers have documented a strong link between seed mass and habitat, with shade and drought being among the most important environmental parameters (e.g. Baker 1972, Foster & Janson 1985). Whereas seed size is negatively correlated with seed number, there is a positive relationship between germination date and seed size (Silvertown 1981). Other plant traits correlated with seed size include mode of dispersal, seed persistence in soil bank, seedling survival, plant growth form and specific leaf area, etc. (Westoby et al. 1996). As a result, seed mass is a critical component of a plant's life history that affects its fitness, population dynamics and interactions with other trophic levels (e.g. seed predators). Collectively, seed mass can be used as an indicator of the quality of the site to which a particular species

is adapted. Interestingly, seed characteristics are closely related to genome size – comparative analysis of independent contrasts shows that differences in genome size are positively correlated with differences in seed mass (Beaulieu et al. 2007). However, the relationship between both variables is quite complex and non-linear. In general, very small seeds are hardly ever associated with large genomes, possibly because of a developmental constraint. Within the set of morphological and ecological parameters considered by Beaulieu et al. (2007), genome size was found to be the second most important character for explaining the variation in seed mass, after plant growth form. Considering the relationship between genome size and seed size, the amount of nuclear DNA can itself be used as a predictor of plant traits, plant life strategy (e.g. the Grime's C-S-R scheme) and/or the width of ecological niche. For example, rapid establishment and fast production of many small seeds are prerequisites for being a successful weed or an invasive plant and as expected, such species have mostly small genomes (Bennett et al. 1998, Kubešová et al. 2010). It is, therefore, not surprising that Rejmánek et al. (2005) list genome size among the eight best predictors of invasiveness.

In summary, the ecology of plants is determined by sets of traits, many of which are constrained by genome size. The amount of nuclear DNA therefore determines the range of conditions in which a plant can evolve ("windows of opportunity") while its genetic composition defines its actual phenotypic expression.

Implication for reproduction

Not only seed number and mass but also the mode of reproduction (whether sexual or apomictic, outcrossing or selfing) can be predicted from genome size, although the success rate varies between different plant groups, and/or the relationship may, at least partly, also reflect other traits (e.g. the common association of an annual life history with autogamy). Perhaps the most convincing evidence for an association between genome size and apomictic reproduction in homoploid plants comes from a study of *Hypericum* sect. *Ascyreira* (Matzk et al. 2003). In this evolutionary old section, apomicts have significantly larger genomes than their sexual counterparts with the same number of chromosomes (2n = 32). The authors postulate that the smaller genomes in sexuals resulted from a gradual decrease in nuclear DNA content by elimination of retroelements. Nevertheless, it should be noted that in most plant groups with versatile breeding systems, ploidy level (rather than genome size) is the key determinant of the mode of reproduction (see Krahulcová & Rotreklová 2010). Similarly, Albach & Greilhuber (2004) report a significant correlation between selfing and small genome size in *Veronica,* however there is only a weak association when species with the same number of chromosomes are compared (our analysis based on data in Table 1 of Albach & Greilhuber 2004).

In some dioecious plants (e.g. *Cannabis*, *Coccinia*, *Humulus*, *Phoenix*, *Rumex* sect. *Acetosa, Silene* and *Viscum*) the male and female individuals are chromosomally heteromorphic (i.e. they differ in the size or number of sex chromosomes). Flow cytometry provides a means of determining gender in such systems regardless of their ontogeny (Vagera et al. 1994, Doležel & Göhde 1995). Because male and female plants may differ in growth characteristics and the sex ratio in a population greatly influences demographic parameters, FCM data may provide useful insights into the population dynamics of dioecious plants. Recently, the identification of male- and female-determin-

ing pollen grains became possible using FCM (Stehlik et al. 2007). This opens new possibilities for determining sex ratios at the earliest ontogenetic stages (gamete production) and assessing potential differences in temporal dynamics between both sexes (e.g. stage-specific survival).

In addition to sex-related differences in the amount of nuclear DNA, *Silene latifolia* exhibits variation in genome size, which is independent of sex (Meagher & Costich 1994). Interestingly, this variation is phenotypically associated with differences in calyx diameter. Because the size of the parts of flowers often play an important role in reproductive performance and fitness (e.g. different attractiveness for pollinators, protection from seed predation, etc.), variation in genome size in this dioecious species may be adaptive.

Evolutionary implications

Some evidence has accumulated in recent years that supports a relationship between genome size and the number of species in angiosperms (but note there is no similar comparison for homoploid taxa). Vinogradov (2003) reports a negative correlation between the average genome size and the number of species in a genus. This finding motivated Knight and co-workers to further explore the dataset using different statistical models (Knight et al. 2005) and they found that genera with large genomes were less likely to be highly speciose, suggesting that a large genome may be disadvantageous. In addition, a link was also drawn between large genomes and the threat of extinction; species that are under no threat of extinction generally have small genomes, whereas those that are have higher DNA values (Vinogradov 2003). Another indirect support for the idea that large genomes may be maladaptive comes from a study of the Macaronesian insular flora (Suda et al. 2003, 2005). Very small genomes clearly prevail in species-rich genera that underwent adaptive radiation, providing support for a relationship between the content of nuclear DNA and bursts of speciation in insular populations. In summary, the effects of genome size on plant evolution are extremely interesting; however, additional tests (using homoploid groups) are required to evaluate the generality of the above conclusions.

Future directions

Over the years, the application of FCM has had a marked effect on studies in many different fields of plant research, including plant biosystematics, ecology and population biology. The majority of the studies, however, addressed questions related to differences in genome copy number (Ekrt & Štech 2008) and tended to disregard the value of FCM for studying homoploid plant groups. Nonetheless, the last decade has seen a significant increase in the use of FCM for resolving complex low-level taxonomies, detecting interspecific hybrids and inferring the evolutionary histories of several homoploid plants. In addition, variation in genome size is now acknowledged as an important parameter in plant ecology and evolution, with consequences for an individual's behaviour, ecological preferences and population demography. As a result, FCM is now a well-established analytical tool in an ever-growing number of plant laboratories worldwide and the number of accesses to genome size databases has steadily increased. Despite significant advances in recent years, much work still needs to be done to realize the full value of FCM in plant research.

The expectation is that genome size will soon become an integral part of any taxonomic study and the extent of its variation will be screened across large spatial scales, representative populations and at different ontogenetic stages. Flow cytometry is likely to be used in studies in other disciplines, such as conservation biology, where this technique will provide valuable data on, for example, the extent and frequency of inter-specific hybridization and thus help in the evaluation of the risk of a breakdown of species integrity. To assess the dynamics of homoploid hybridization, the analysis of pollen grains (which is still rare because of methodological difficulties) is likely be particularly rewarding. While some questions can be addressed using FCM alone, more robust results will be achieved by combining FCM with other techniques, such as molecular methods, multivariate morphometrics, ecological modelling, etc. In plant ecology, it is likely that more accurate relationships between genome size and morphological, physiological or biochemical traits or sets of characters will be established and pave the way for more precise predictions of plant distribution and response of plants to changes in environmental factors. It is hoped that this will result in the development of predictive models that can be used to assess the effect of human activities on the abundance of plants and the functioning and sustainability of ecosystems. A recent interesting example of the selective significance of genome size in a plant community subjected to heavy metal stress, which has far-reaching implications, is provided by Vidic et al. (2009). In addition, biological questions regarding the extent of inter- and intra-specific variation in genome size also deserves more attention. Such studies will provide deeper insights into the functional significance of small versus large genomes and will further our understanding of the driving forces that govern the evolution of genome size in plants. It should be noted that many of the comparative studies on the ecological interpretation of the variation in genome size in plants did not take into consideration their ploidy level or number of chromosomes, which may lead to misinterpretations. Homoploid plant groups with a sufficient difference in genome size may, therefore, be particularly useful for critically evaluating the role of non-genic DNA in determining a plant's phenotype, physiology, development and interactions with other trophic levels.

Another aspect of the future use of FCM in research on homoploid plants is a methodological one. The need for high-resolution and rapid screening of large samples means that current protocols need to be improved (e.g. to ameliorate the disturbing effect of secondary metabolites) and the analysing process automated. In addition, the development of a method for the routine analysis of non-fresh samples merits consideration. While DNA ploidy levels of dehydrated plant tissues have been successfully estimated (e.g. Šmarda & Stančík 2006, Suda & Trávníček 2006) the detection of small differences in genome size in dry samples may not always be sufficiently reliable (but compare Suda et al. 2007b) and therefore the methodology needs to be improved. It is also likely that a new generation of low-cost, more compact and more stable field flow cytometers will be developed within the next few years. This will undoubtedly accelerate taxonomic and ecological research on homoploid groups in geographically remote areas or in regions where current settings may still be prohibitive.

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Souhrn

V posledním desetiletí došlo ke značnému nárůstu aplikací průtokové cytometrie v biosystematice, ekologii a populační biologii cévnatých rostlin. Valná většina studií se však zabývala polyploidními skupinami, zatímco studium druhů se stejným počtem chromozómů dosud zůstávalo stranou zájmu. Review shrnuje soudobé pokroky v poznání taxonomie, ekologie a reprodukční biologie homoploidních skupin rostlin s využitím průtokové cytometrie. Stabilní velikost genomu u většiny druhů spolu s často výraznými mezidruhovými rozdíly umožňují využít množství jaderné DNA pro taxonomické účely. Na základě velikosti genomu je možné: (i) vymezit taxony na různých hierarchických úrovních, (ii) řešit taxonomické otázky v komplikovaných skupinách, (iii) stanovit četnost mezidruhových hybridů nebo (iv) vyvozovat pravděpodobné evoluční vztahy. V rostlinné ekologii, evoluční a reprodukční biologii je velikost genomu využívána především pro prediktivní účely, díky korelaci mezi obsahem jaderné DNA a různými fenotypovými, fyziologickými a/nebo ekologickými vlastnostmi. V následujících letech očekáváme další nárůst ve využívání průtokové cytometrie při studiu homoploidních rostlin, a to jak v četnosti, tak v rozsahu aplikací. Zejména ve spojení s dalšími metodickými přístupy (molekulárními či fenotypovými) představuje průtoková cytometrie silný metodický nástroj pro řešení otázek týkajících se variability velikosti genomu a jejího funkčního významu.

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