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Cytogenetic Characterization of *Zinnia* Species and Cultivars

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ABSTRACT

Zinnia is a tropical ornamental plant, and its cultivar classification is complicated. The typical characters such as height, flower size and leaf shape are of a quantitative nature, and under strong environmental control. In some cases, unequivocal determination of cultivars may be difficult using morphological features only. So in this study, cytological, cytogenetical and molecular biological characteristics of *Zinnia* have been analyzed. The DNA content of *Zinnia angustifolia* (cv. 'Starbright'), *Zinnia elegans* (cv. 'Short Stuff', 'Dreamland', 'Jupiter', 'Border Beauty', 'Jungle', 'Piccolo', 'Sinnita'), *Zinnia haageana* (cv. 'Chippendale Daisy', 'Persian Carpet') and the F_1 hybrid cv. 'Profusion' (*Z. elegans* x *Z. angustifolia*) has been analyzed by flow cytometry, and compared to morphological features (guard cell size, number of chloroplasts per guard cell, number of chromosomes). The three analyzed species *Z. angustifolia*, *Z. haageana* and *Z. elegans* could be clearly distinguished by their DNA content. The nuclear DNA content ranged from 1 pg (2C) in *Z. angustifolia* cv. 'Starbright' up to 5 pg in the tetraploid *Z. elegans* cv. 'Jungle'. Genetic diversity among and between Zinnia varieties was accessed by RAPD, confirming a higher genetic identity in *Z. elegans* cv. 'Dreamland' when compared to *Z. haageana* cv. 'Persian Carpet'.

Keywords: cytogenetics, flow cytometry, RAPD, *Zinnia* spp.

INTRODUCTION

Zinnia is a beautiful ornamental plant. Novel flower colors of this plant are of great commercial and aesthetic value. Many new cultivars are created either by hybridization (cv. 'Profusion', *Z. elegans* x *Z. angustifolia*) or by X-ray mutagenesis (Venkatachalam 1991). Zinnias are used as ethnobotanical plant in some regions of North America, e.g. *Z. angustifolia* as a poison, and *Z. elegans* as an anodynic and menstrual medicinal herb. Interestingly, zinnia is a good model system to study the *in vitro* interactions of different cell types and the consequences for commitment to a particular cell fate such as tracheary elements (TE) (Pesquet *et al*. 2006).

The identification of cultivars or breeding lines is very important in all horticultural and agricultural species in order to protect the rights of plant breeders (Wolff *et al*. 1995). In *Zinnia* cultivars are usually identified by flowering trials as well as by morphological characteristics (e.g. flower shape and size, leaf and growth morphology). However, those characteristics may be affected by environmental factors. To overcome these problems, in the present study we analyzed *Zinnia* species and cultivars on the genotypic level (DNA content, DNA sequence).

MATERIALS AND METHODS

Plant materials

Seeds of all plants analyzed in the present study were provided by the AFM Flower Seed Co., Ltd. (Chiang Mai). The following 11 cultivars were studied: *Z. angustifolia* cv. 'Starbright'; *Z. haageana* cvs. 'Persian Carpet', 'Chippendale Daisy'; *Z. elegans* cvs. 'Short Stuff', 'Dreamland', 'Jupiter', 'Border Beauty', 'Jungle', 'Giant', 'Sinnita'; and a hybrid (*Z. elegans* x *Z. angustifolia*) cv. 'Profusion'. *Raphanus sativus* cv. Saxa seeds used as internal standard for flow cytometry were kindly provided by Dr. Jaroslav Dolezel, Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Olomouc, Czech Republic and were grown in the greenhouse under natural conditions.

Flow cytometry

Nuclei were isolated from fresh, young leaves (of *Zinnia* species or cultivars, and of *Raphanus sativus* at comparable developmental stages) by chopping with a sharp razor blade, and stained for flow cytometry with either propidium iodide (PI, CyStain® PI Absolute P, PARTEC GmbH, Münster, Germany) or 4, 6-diamidino-2-phenylindole (DAPI, CyStain® UV Precise P, PARTEC GmbH, Münster, Germany). In every sample a minimum of 10,000 nuclei was analyzed with a Partec PAS flow cytometer (Partec GmbH, Münster, Germany). The coefficient of variation (CV) was estimated with the WinMDI software (Version 2.0.4); only samples with a CV <5% were accepted.

PI staining: Young leaves (about 1 cm^2) were chopped in 400 µl nuclei extraction buffer at room temperature. The nuclei suspension was filtered through a 42-µm nylon filter, and 1.6 ml of staining buffer (containing 100 µg PI) and 3 µl RNase were added.

DAPI staining: Nuclei were extracted and stained using the high resolution DNA Precise P kit (PARTEC). Young leaves (about 1 cm^2) were chopped in solution A of the kit. 0.5 ml of 1% PVP was added. The nuclei suspension was filtered through a 42-µm nylon filter, and 1.5 ml of solution B was added.

Two samples of 5 plants per cultivar were analyzed. The following species/cultivars were used as internal standard: *Raphanus sativus* cv. 'Saxa' for DNA content estimation (PI staining), and comparative analyses of *Z. angustifolia*, *Z. elegans* cv. 'Dreamland' and the hybrid 'Profusion' (DAPI staining); the *Z. angustifolia* cv. 'Starbright' and for comparative analyses of the DNA content in cultivars of *Z. elegans* (DAPI staining). Further on, intraspecific variation in cultivars of *Z. elegans* was studied by mixing samples in the following order 'Border'/'Dreamland', 'Dreamland'/'Giant', 'Giant'/'Sinnita', 'Sinnita'/'Jupiter', 'Jupiter'/'Short stuff'.

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Original Research Paper

Size of guard cells and number of chloroplasts

Small parts of the lower epidermis of young leaves were manually removed, placed in a drop of water on a microscopic slide following the method as described by Dorè (1986), and measured under a light microscope (DM 5000 B, LEICA, Wetzlar, Germany). The number of chloroplasts per guard cell was counted under a fluorescence microscope (DM 5000 B, LEICA, Wetzlar, Germany). Four leaves per two plants per cultivar were analyzed.

Chromosome counting

For chromosome counting root tips from greenhouse plants were used. The root tips were pre-treated for 4 hours in saturated PDB (para-dichlorobenzene), and then fixed 24 hours in 1:3 glacial acetic acids: 95% ethanol. After a passage through 75% ethanol the root tips were hydrolyzed in 1 N HCl for 15 min at 60°C, and stained with Feulgen solution for 4 hours, squashed in aceto-carmine, and analyzed under a light microscope. Five cells per cultivar were analyzed.

DNA extraction

Total DNA from leaves of young *Zinnia* seedlings was isolated following a modified method used for *Asparagus officinalis* (Eimert *et al*. 2003). Two volumes of DNA extraction buffer containing 50 mM Tris-HCl (pH 8.0), 312.5 mM NaCl, 20 mM EDTA (pH 8), 1% sarkosine and 7 M urea were added to 100-500 mg leave tissues, and ground gently at room temperature in a mortar with a pestle. The homogenate was centrifuged for 10 min at 12,500 rpm at room temperature in order to remove tissue fragments. The supernatant was extracted twice with phenol: chloroform: isoamyl alcohol (v/v 25:24:1). Half volume of 5 M NaCl was added to the supernatant and mixed. Two volumes of 95% ethanol were added and mixed gently. The homogenate was centrifuged for 10 min at 12,500 rpm at 4°C. DNA was washed with 70% ethanol and air-dried. Finally, the DNA was dissolved in 50 µl of TE. The DNA isolated from each cultivar was run in a 1.3% agarose gel to check the quality and was also scanned in a spectrophotometer and absorbances (A) were noted at 260 and 280.

RAPD analysis

DNA amplification for the RAPD analysis was performed in a MJR PTC 100 thermo cycler (MJR, Martinsried, Germany) in volumes of 25 µl consisting of 1x reaction buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 2 units of Taq DNA polymerase, 0.2 µM primer, 2.5 µg BSA and 25 ng of genomic DNA. All the reagents mentioned were provided by MBI Fermentas (St. Leon-Rot, Germany). Following an initial denaturation step at 95°C for 5 min, the amplification program was set to 35 cycles of denaturation at 95°C for 1 min, annealing at 35°C for 15 sec, 40°C for 15 sec. and extension at 72°C for 2 min with a final extension at 72°C for 5 min. Amplified fragments were resolved in 1.3% agarose gels followed by staining with ethidium bromide (0.5 µg/ml) and gels were viewed and photographed under UV trans illumination (2UV Transilluminator, UVP, Cambridge, UK). All primers used were 10-mer random primers (University of British Columbia, Canada) and the DNA samples were amplified with 5 random primers. Only primers producing reproducible bands were considered for analysis.

Distance matrix analysis of the RAPD data was performed using Bionumerics v. 3.0 (Applied Maths, Belgium). The distance matrix based on RAPD data sets was graphically represented as a dendrogram using the UPGMA method. The genetic identity and genetic distance were calculated with POPGen v. 1.32 (Yeh and Boyle 1997).

Statistical analysis

Statistical analyses were conducted using SPSS© v. 10 ($P<0.05$).

RESULTS

The DNA content (2C) of the species and cultivars of the genus *Zinnia* varies between 1.00 to 4.94 pg (**Table 1**). The flow cytometric analyses could clearly show that there is a significant difference between the DNA content of *Z. angustifolia*, *Z. haageana* and *Z. elegans*. No significant difference could be found between the cultivars of *Z. elegans* (**Fig. 1**) with only one exception; cv. 'Jungle' has about two times the amount of DNA of the other analyzed *Z. elegans* cultivars. By analyzing mixed samples of two different cultivars in *Z. elegans* we could show that the DNA contents in this group are identical; the histograms always consist of one peak only (**Fig. 2A** shows the mixed sample of the cultivars 'Dreamland'/'Giant' only; the other combinations are identical, and therefore, they are not shown). The DNA content of the interspecific hybrid 'Profusion' represents approximately the sum of the DNA contents of both its parents (*Z. angustifolia* and *Z. elegans*, **Fig. 2B**).

The species and cultivars analyzed by flow cytometry have also been studied with regards to the size of their guard cells of the lower leaf epidermis, and the number of chloroplasts per guard cell as well (**Fig. 3**; **Table 2**). Chromosomes were counted in the three *Zinnias* as well as in the putative tetraploid *Z. elegans* cv. 'Jungle' (**Table 3**).

Five primers, comprising UBC 65, 87, 88, 89 and 95, were selected for RAPD analysis. The rational behind the choice is explained in the discussion. A typical RAPD profile is shown (**Fig. 4**). The percentage of polymorphic loci of *Z. elegans* cv. 'Dreamland' is 41.84 and of *Z. haageana* cv. 'Persian Carpet' is 65.31. Thus, diversity within the population of *Z. haageana* cv. 'Persian Carpet' was higher than within the population of *Z. elegans* cv. 'Dreamland' (**Fig. 5**).

DISCUSSION

Zinnia has been used as a model system for analyzing tracheary element differentiation as well as for analyzing programmed cell death as a part of xylem differentiation (Pesquet *et al*. 2006). However, data about the DNA content in this genus have not been published so far. Compared to other species of the family Compositae (e.g. *Leontodon longorostis* = 0.8 pg; *Leucanthemum subglaucum* = 49.66 pg; PlantDNA C-values Database, Royal Botanical Gardens, Kew), *Z. angustifolia* (2C: 1 pg), *Z. haagena* (2C: 1.7 pg) and *Z. elegans* (2C: 2.6 pg) have a rather low DNA content. There are significant differences in the DNA content between the three analyzed *Zinnia* species. Therefore, the flow cytometric estimation of the DNA content can be used as a tool to distinguish between the species at early developmental stages, when other, morphological markers are not yet available (e.g. seedlings). However, the discrimination of cultivars of *Z. elegans* by flow cytometry proved unfeasible. When mixed samples of two different cultivars of *Z. elegans* were analyzed, only a single peak could be detected. This clearly demonstrates that the DNA contents of these (diploid) cultivars are identical. The only exception

Table 1 DNA contents of species and cultivars in the genus *Zinnia*.

Species	Cultivar	2C nuclear DNA content (pg) Mean ±S.D.
Z. angustifolia	Starbright	1.00 ± 0.03 [a*]
Z. haageana	Persian Carpet	1.73 ± 0.05 [b]
	Chippendale Daisy	1.64 ± 0.07 [b]
Z. elegans	Short Stuff	2.51 ± 0.07 [c]
	Dreamland	2.49 ± 0.08 [c]
	Jupiter	2.52 ± 0.08 [c]
	Border Beauty	2.49 ± 0.06 [c]
	Giant	2.52 ± 0.09 [c]
	Sinnita	2.53 ± 0.08 [c]
	Jungle	4.94 ± 0.06 [e]
Z. elegans x *Z. angustifolia*	Profusion	3.54 ± 0.07 [d]

* Estimated by using *Raphanus sativus* cv. 'Saxa' as internal standard.

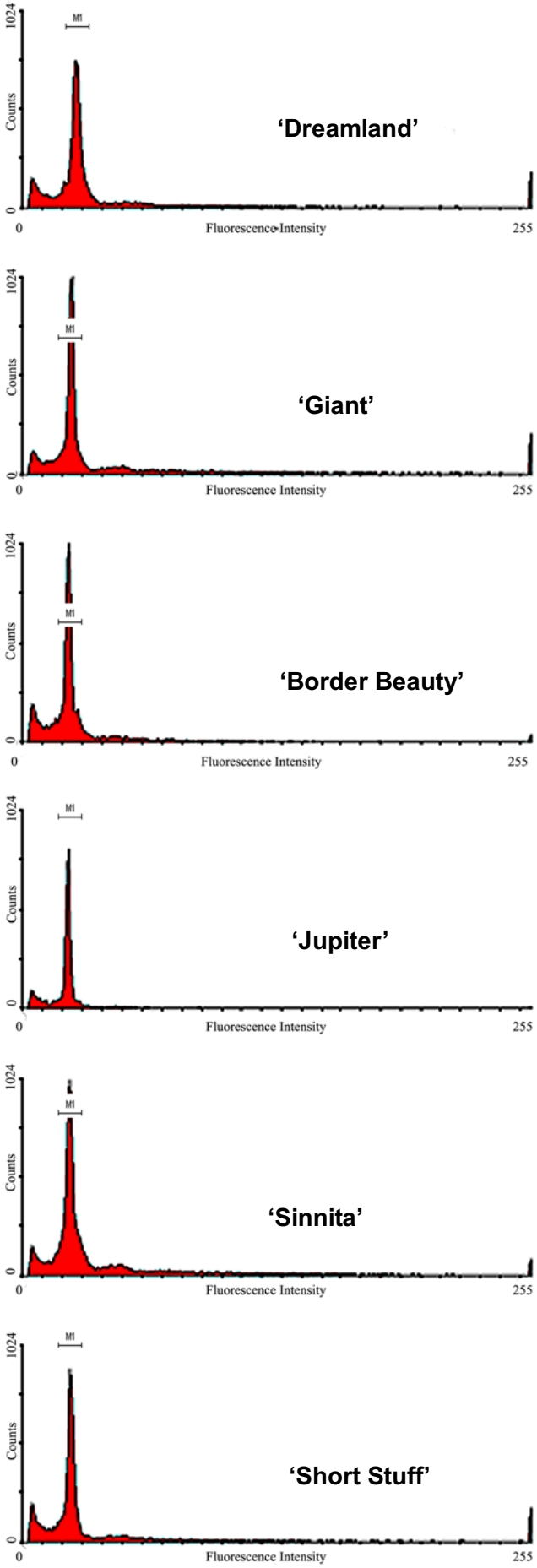

Fig. 1 FCM histograms of *Zinnia elegans* cultivars clearly demonstrating that all of those cultivars have the same DNA content.

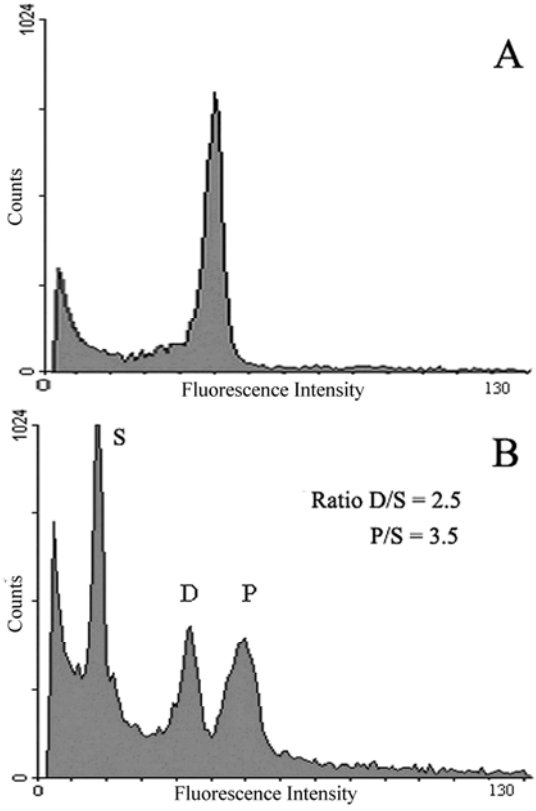

Fig. 2 FCM histogram showing combination peaks of (**A**) 'Dreamland'/ 'Giant', (**B**) 'Dreamland'/'Profusion'. Internal standard = S.

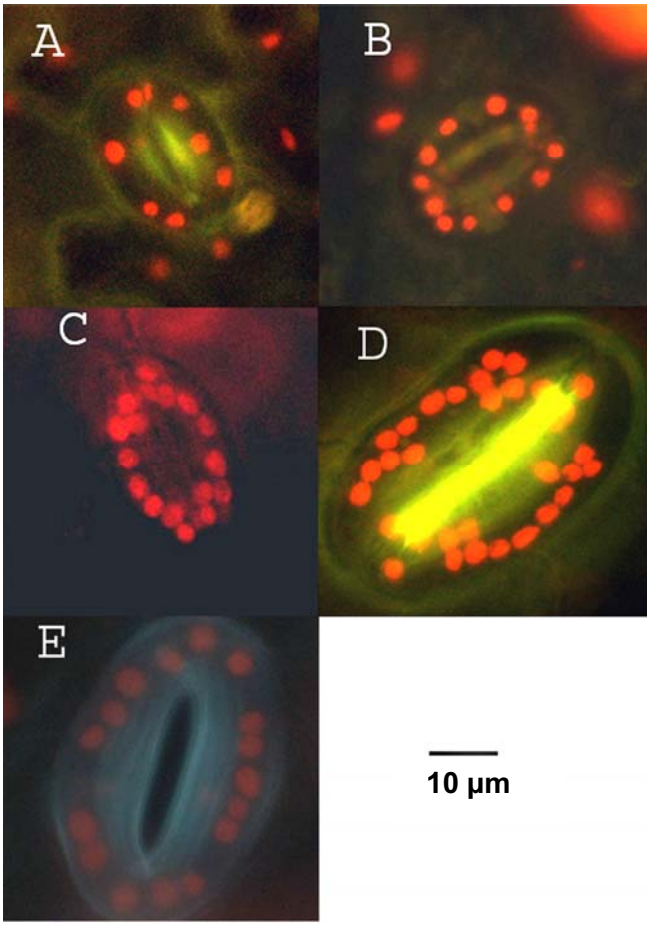

Fig. 3 Fluorescence microphotographs of guard cells in *Z. angustifolia* (**A**), *Z. haageana* cv. 'Persian Carpet' (**B**), *Z. elegans* cv. 'Dreamland' (**C**), *Z. elegans* cv. 'Jungle' (**D**) and the interspecific hybrid cv. 'Profusion' (**E**).

Table 2 Differences in the number of chloroplasts, and in the size of guard cells in zinnias (mean ± SD).

Species	Cultivar	Length (µm)	Chloroplast/guard cell
Z. angustifolia	Starbright	28.7 ± 2.6 a	4.6 ± 0.7 a
Z. haageana	Chippendale Daisy	27.5 ± 2.2 a	4.8 ± 0.7 a
	Persian Carpet	27.8 ± 2.1 a	5.6 ± 0.7 b
Z. elegans	Short Stuff	36.2 ± 3.4 cde	7.3 ± 1.0 ef
	Dreamland	36.6 ± 3.0 de	7.5 ± 1.1 fgh
	Jupiter	35.4 ± 2.8 cde	7.8 ± 1.0 gh
	Border Beauty	35.0 ± 3.8 bcd	7.9 ± 1.0 h
	Giant	34.6 ± 3.2 bcd	7.2 ± 0.9 def
	Sinnita	34.2 ± 3.0 bc	6.8 ± 0.7 cde
	Jungle	44.6 ± 3.5 f	11.9 ± 1.3 j
Z. elegans x *Z. angustifolia*	Profusion	43.1 ± 3.9 f	8.7 ± 0.9 i

Table 3 Chromosome numbers of *Zinnia* spp.

Species	Cultivar	Chromosome number (2n)
Z. angustifolia	Starbright	22
Z. haageana	Persian Carpet	24
Z. elegans	Dreamland	24
	Jungle	48

is 'Jungle' that could be characterized as a tetraploid cultivar by flow cytometry and by counting its chromosomes (see below).

By analyzing the DNA content of the interspecific hybrid 'Profusion' (*Z. angustifolia* x *Z. elegans*) and of its parents we could show that 'Profusion' is an allodiploid hybrid (Narayan 1998). By analyzing the morphology of guard cells of the three *Zinnia* species, we could demonstrate that there is a positive and significant correlation between the DNA content and the average guard cell size. The same positive correlation has been observed between the DNA content and the number of chloroplasts per guard cell. Those findings are in accordance with the reports about other species in angiosperms (e.g. *Capsicum annuum*, haploids: 9.3 choroplasts per cell, diploids: 18.4 chloroplasts per cell – Qin and Rotino 1995; on average the duplication of the nuclear DNA amount entails an increase of the plastid number by about 70%, for review see Butterfass 1973). Consequently, these morphometrical characteristics can be used to discriminate species in the genus *Zinnia* if flow cytometry is not available. However, flow cytometry is the preferable method because it is able to detect chimeras in contrast to the morphometrical methods which are restricted to the epidermal cell layer.

The chromosome numbers of *Z. angustifolia* (2n = 22, Keil *et al*. 1988; Razaq *et al*. 1988) *Z. haageana* (2n = 24, Lane and Li 1993; Jose and Mathew 1995) and *Z. elegans* (2n = 24, Husaini and Iwo 1990; Nirmala and Rao 1990; Zhao *et al*. 1990; Razaq *et al*. 1994; Huang and Zhao 1995) have been previously investigated. Our own observations support those previous reports. However, the *Z. elegans* cv. 'Jungle' has a chromosome number of 48, and thus it is a tetraploid cultivar. We could show that the DNA content of *Z. haageana* and *Z. elegans* is significantly different while the number of chromosomes is identical. It might be caused by the evolutionary accumulation of repetitive DNA sequences and/or retrotransposons. Those sequences are generally considered to have an inherent tendency towards amplification, and thereby, increasing the DNA content and the size of chromosomes, respectively (Kubis *et al*. 1998).

Intraspecific variation in the *Z. elegans* cultivars could not be detected by flow cytometry, by counting the number of chloroplasts in guard cells, or by analyzing the size of guard cells. Therefore, we tried to establish whether RAPD fingerprinting is a suitable method to investigate intraspecific variation. Initially, a total of ten arbitrary decamer primers giving good and reliable amplification in other plant species (*Vitis*, *Prunus*, *Asparagus*; unpublished results) were tested for good amplification, clear and distinct bands and a sufficient number of amplicons with *Zinnia* DNA. All these primers are of dinucleotid (microsatellite-like) composition with a higher (60%) GC content allowing for better reproducibility due to higher annealing temperatures. Out of these primers five satisfying the above mentioned prerequisites best (UBC 065, 087, 088, 089, 095; sequences available at www.michaelsmith.ubc.ca/services/NAPS/Primer_Sets/) were chosen for further use in the RAPD analysis.

Because of the initial character of the RAPD analysis we decided to restrict our samples to two cultivars of two species. We chose the accessions *Z. haageana* cv. 'Persian Carpet' and *Z. elegans* cv. 'Dreamland', expecting that their assumingly higher genetic distance should give us a clear picture of the potential value of the RAPD method. The RAPD data should reflect that distance in accordance with the morphological characteristics.

Intracultivar variation was investigated among twenty samples of *Z. haageana* cv. 'Persian Carpet' and *Z. elegans* cv. 'Dreamland', each. The *Zinnia* cultivars studied here showed moderate levels of intercultivar genetic variability, as shown by a genetic identity of 0.76, but considerable intracultivar variability. Interestingly, the intracultivar genetic identity is considerably lower for *Z. haageana* cv. 'Persian Carpet' (47.99%) than in *Z. elegans* cv. 'Dreamland' (81.49%). This reflects the situation of the morphological characters (e.g. in flower color and color ratios), where *Z. haageana* cv. 'Persian Carpet' also shows a higher variation than *Z. elegans* cv. 'Dreamland'. Generally, extensive morphological diversity is found among cultivars of *Z. elegans* (Boyle and Stimart 1982). That is not surprising since *Z. elegans* is self-incompatible (Pollard 1939), although, the intensity of the self-incompatibility has later been reported to vary among clones and lines (Boyle and Stimart 1986). That too, is in accordance with the observation that the degree of morphological diversity varies between the cultivars. The actual level of genetic diversity probably depends, among other factors, on the intensity of self-incompatibility in the cultivar in question. Thus, although a male sterile line

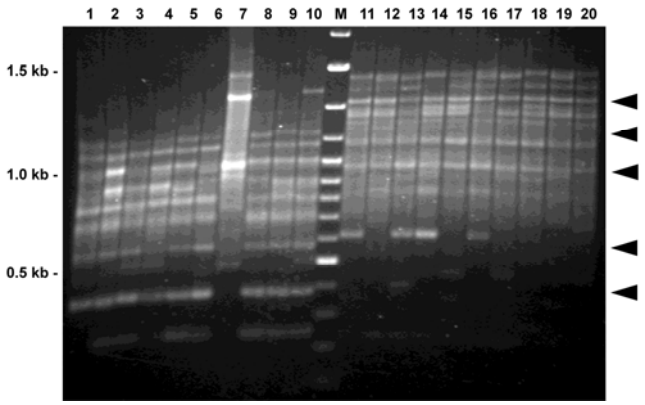

Fig. 4 RAPD profiles of 20 individual zinnia plants belonging to *Z. haageana* cv. 'Persian Carpet' (lanes 1-10) and *Z .elegans* cv. 'Dreamland' (lanes 11-20) with primer UBC 087; M: molecular-weight markers. Arrow heads indicate the position of possible discriminating bands.

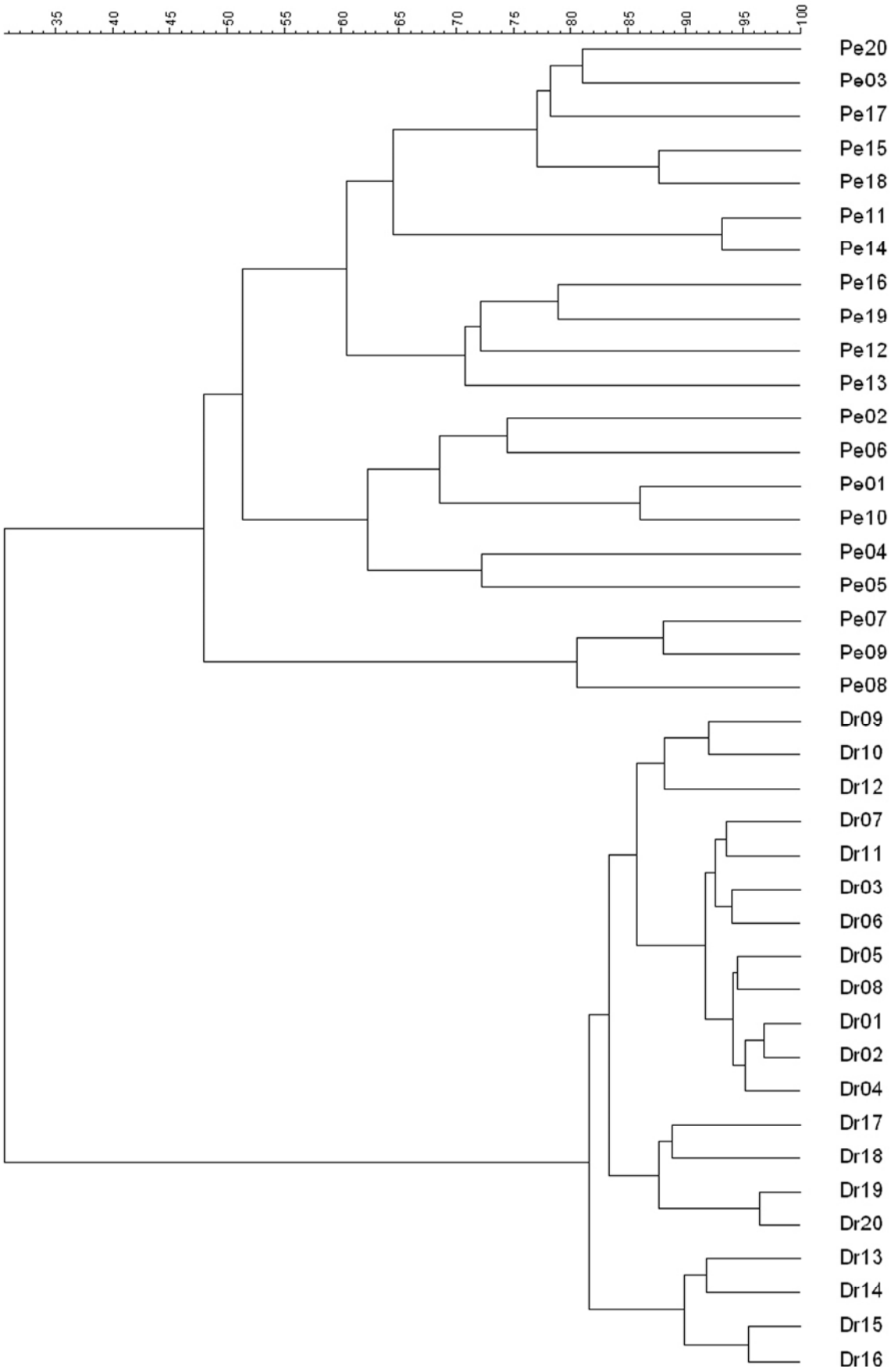

Fig. 5 Pedigree analysis in two populations of *Z. elegans* cv. 'Dreamland' and of *Z. haageana* cv. 'Persian Carpet'.

has been introduced for the creation of new uniform varieties (Cowen and Ewart 1990), a relatively high heterozygosity is expected in *Z. elegans*, especially within commercial (open-pollinated) cultivars. Little information is available on *Z. haageana* breeding and propagation. Given that all members of the *Zinnia* genus seem to exhibit at least a certain amount of self-incompatibility, it is reasonable to assume an accordingly high level of heterozygosity in *Z. haageana*, too. Especially, since all commercial *Z. haageana* cultivars are open-pollinated (National Plant Germplasm System, USDA, ARS, 1995).

Nonetheless, we found several molecular markers coup-

led to the genetic background of the tested varieties (**Fig. 4**) with the use of just 5 randomly picked primers. This suggests a potential to find such bands for varieties even closer related to each other. The possibility to define distinguishing bands in closely related varieties of other plants has been reported previously (Tessier *et al*. 1999; Wolf *et al*. 1999; Ipek *et al*. 2003). Thus, a more thorough analysis of this approach is intended and will likely allow for accurate assessment of *Zinnia* accessions using RAPD markers.

In general, our results demonstrate that flow cytometry accurately distinguishes the ploidy levels of diploid, allopolyploid and tetraploid plants in the genus *Zinnia*. The combination of RAPD analyses and flow cytometry techniques can provide an efficient screening tool of cultivars, assessing the genetic origin of aberrant plants, and quantifying the contribution of parental genomes.

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