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# Identification of chrysanthemum cultivars and stability of DNA fingerprint patterns

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**Abstract** Several techniques of DNA analysis were applied to identify chrysanthemum cultivars. Unrelated cultivars could be distinguished by using RAPDs (random amplified polymorphic DNAs), inter-SSR (simple sequence repeat) PCR (polymerase chain reaction), hybridization-based DNA fingerprinting, as well as RFLPs (restriction fragment length polymorphisms). Cultivars with different flower colours and belonging to one family, i.e. vegetatively derived from 1 cultivar, appeared to have the same DNA fragment patterns, whichever technique was applied. The absence of polymorphisms between different accessions of the same cultivar indicated a high stability of the observed patterns.

**Key words** Chrysanthemum · RAPD · DNA fingerprint · RFLP · Stability

## Introduction

Chrysanthemum (*Dendranthema grandiflora* Tzvelev or *Chrysanthemum morifolium* Ramat.) is a popular cut flower and pot plant of high economic value. It has been grown in China for more than 2000 years, and over the last 200 years it has also been bred in the USA, Japan, and several countries in Europe (Broertjes and Van Harten 1978). Chrysanthemum is part of a hexaploid species complex and has on average 54 chromosomes (Dowrick and El-Bayoumi 1966). The complex nature of chrysanthemum hampers progress in the genetic analysis of many of its traits. The species has a strong sporophytic self-incompatability system and, therefore, many crosses between related or unrelated cultivars are not successful. Inbred lines can seldom be made: those that are made suffer from a strong inbreeding depression (Anderson et al. 1992).

Chrysanthemum is propagated vegetatively by taking cuttings. New commercially interesting cultivars can be bred in two ways (Broertjes and Van Harten 1978). The first is to make a cross between two existing cultivars. The offspring of a biparental cross is highly variable for many characters, and new cultivars can be selected from the $F_1$ population. The second way is to obtain "sports" of a single successful cultivar. Sports are cultivars derived vegetatively that differ from the original cultivar in some characteristics, e.g. flower colour or leaf shape. Sports originating from one cultivar are called a family. Sporting can occur spontaneously or by artificual induction using X-rays as agents (Broertjes and Van Harten 1978).

Several mechanisms have been described explaining why some cultivars have a less stable phenotype than others (Dowrick and El-Bayoumi 1966). Abnormalities in the distribution of chromosomes during mitosis or meiosis may result in a loss or gain of (part of) a chromosome in one of the resulting cells. All clonally derived cells would then be different from those derived from normal cells. This means that chimaera may be formed. In plants, cells differentiate into three different cell layers. Leaves, stems and buds that form side branches consist of all three layers, the petals only have the two outermost layers, i.e., layers I and II, whereas the generative cells are formed from layer II. In chrysanthemum it is often observed that the cell layers are geneti-

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cally different from each other. This is called pericline chimera (Bush et al. 1976). Consequently, the layers may differ in the expression of a certain trait, e.g., flower colour. Moreover, in a periclinical chimera, a bud originating from a cell of one layer may lead to a shoot with a different flower colour than if the bud originated from a cell of another, genetically different, layer (Stewart and Dermen 1970). This is also the reason why in chrysanthemum a high number of variants can often be obtained by regeneration from diverse parts of the plant (Malaure et al. 1991a, b). Those cultivars that are chimeric are less stable in phenotype than those that have genetically uniform cell layers.

The identification of cultivars or breeding lines is extremely important in all horticultural and agricultural species in order to protect plant breeders' rights. Until recently, new varieties have usually been described on the basis of their phenological and morphological characteristics. This necessitates large-scale growth experiments, often with fully mature plants, under conditions that are as uniform as possible. The application of protein and allozyme electrophoresis has largely improved the identification of cultivars, however, the level of polymorphism obtained is often not high enough to distinguish cultivars, and again the growth conditions may influence the quality and quantity of proteins.

Many new possibilities for the identification of cultivars has opened up with the development of techniques that allows DNA polymorphism to be analysed. Both restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR) - based techniques are now widely used in many fields in biology. The PCR methods using arbitrary primers have become especially popular among researchers recently (Williams et al. 1990; Caetano-Anollés et al. 1991; Welsh and McClelland 1990). Of these techniques random amplified polymorphic DNAs (RAPDs) is applied the most widely. RAPDs have been used for cultivar identification in, amongst others, broccoli and cauliflower (Hu and Quiros 1991), cocoa (Wilde et al. 1992), buffalo grass (Wu and Lin 1994) and potatoes (Demeke et al. 1993). Mulcahy et al. (1993) showed that apple cultivars could be identified using RAPDs and that sports, which originated from one cultivar, were all identical to that original cultivar. RFLP analysis using species-specific single-locus probes was performed to distinguish rose cultivars (Rajapakse et al. 1992) and potato breeding lines (Gorg et al. 1992). DNA finger printing using minisatellite or microsatellite probes appeared to be successful in raspberries and blackberries (Nybom et al. 1989), *Vitis vinifera* (Striem et al. 1990; Thomas et al. 1993) and in avocado (Lavi et al. 1991). Even in tomato, which is genetically invariable with most other techniques, cultivars were distinguished using microsatellite probes (Vosman et al. 1992).

In chrysanthemum, cultivars are identified in flowering trials, and breeders' rights are presently described by cultivar characteristics like flower, leaf and growth morphology. Fiebich and Hennig (1992) showed that chrysanthemum cultivars are genetically highly variable and that allozyme electrophoresis could be helpful in distinguishing cultivars. In a preliminary study of Wolff and Peters-Van Rijn (1993) the discriminating power of DNA analysis (RAPDs) was assessed: 20 cultivars gave different fragment patterns using two or three RAPD primers. Cultivars belonging to one family could, however, not be distinguished by their RAPD fragment pattern. RFLP probes from a *Pst*I library showed high levels of polymorphism between the four cultivars tested (Wolff et al. 1994b).

If, in future, DNA techniques are to complement or replace cultivar identification by growth trials, then the stability and reproducibility of fingerprints or PCR-generated patterns of a strain or cultivar is very important. Strains or cultivars that are kept or propagated in tissue culture or vegetatively must have the same DNA fragment pattern, even after many years of cultivation.

The aim of the study presented here was to show that chrysanthemum cultivars can be identified with DNA techniques and that DNA patterns are stable in families, in tissue culture and after years of vegetative propagation. We used RFLP analysis, microsatellite DNA fingerprinting and two PCR-based techniques, namely, RAPDs and inter-SSR (simple sequence repeat) PCR (Ziekiewicz et al. 1994), and compared the results obtained.

## Materials and methods

The plant material was obtained from the breeders, Fides, CBA and Hoek Breeding. Table 1 shows the different groups of cultivars and their code numbers. Two families, family A and B, were represented by 17 and 7 individuals, respectively. These individuals differ in one or more characters, e.g., flower colour, flower size or growth rate. Four cultivars, C, D, E and F, were represented by material that had been maintained in tissue culture for 4 years and by material that was vegetatively propagated and kept in greenhouses. The last two groups, cvs. G and H, represent cultivars that had been kept by different companies for many years (different accessions of one cultivar). These cultivars keep the same cultivar name but may have a slightly different morphology, flower colour or leaf colour.

Leaf material was ground in liquid nitrogen, freeze-dried and kept at −20 °C. DNA was extracted according to Wolff and Peters-Van Rijn (1993).

RAPD analysis was generally performed as described by Wolff and Peters-Ven Rijn (1993), with minor modifications. Families or groups of cultivars were always compared within one PCR run. The Mg concentratioin was either 3 m*M* or 2 m*M*. The thermocyclers used were a Perkin Elmer 9600, a Perkin Elmer 480 and an MJ Research PTC-100. All cyclers gave good results and almost identical patterns. The DNA was preheated for 4 min at 94 °C. Forty-five cycles were run of either 1 min at 94 °C, 1 min at the annealing temperature and 2 min at 72 °C, or 15 s at 94 °C, 45 s at the annealing temperature and 1.5 min at 72 °C. In most cases the annealing temperature was 36 °C, but with a few primers it was set at a higher temperature to reduce the number of bands and the background (see Table 2). The ramping between the annealing temperature and 72 °C was set at $0.3 - 0.4\,^{\circ}C\,s^{-1}$. The primers used for the different groups of samples are given in Table 2.

RFLP analysis was done using 18 random cultivars, 6 from each of the three breeders mentioned earlier (the cultivars are listed in Fig. 3), and with one group mentioned in Table 1, namely family A. The restriction enzymes used were *Eco*RI, *Eco*RV and *Hin*dIII. Probes and procedures are described in Wolff et al. (1994b).

**Table 1** Description and origin of the plant material used

| Number | Origin | Description | |
|---|---|---|---|
| CH 36–62 | CBA | Family A | |
| CH 59 | Fides | Family B (1979) | Original |
| CH 60 | Fides | Family B (1988) | From CH64 spontaneously |
| CH 61 | Fides | Family B (1989) | From CH59 spontaneously |
| CH 62 | Fides | Family B (1983) | From CH59 spontaneously |
| CH 63 | Fides | Family B (1984) | From CH59 spontaneously |
| CH 64 | Fides | Family B (1987) | From CH63 spontaneously |
| CH 65 | Fides | Family B (1987) | From CH63 spontaneously |
| CH 66 | CBA | Cultivar C | From tissue culture (4 years) |
| CH 67 | CBA | Cultivar C | Vegetatively propagated |
| CH 68 | CBA | Cultivar D | From tissue culture (4 years) |
| CH 69 | CBA | Cultivar D | Vegetatively propagated |
| CH 70 | CBA | Cultivar E | From tissue culture (4 years) |
| CH 71 | CBA | Cultivar E | Vegetatively propagated |
| CH 72 | CBA | Cultivar F | From tissue culture (4 years) |
| CH 73 | CBA | Cultivar F | Vegetatively propagated |
| CH 74 | V. Ruiten | Cultivar G | |
| CH 75 | Chryselo | Cultivar G | |
| CH 76 | Hilvo | Cultivar G | |
| CH 77 | Dekker | Cultivar G | |
| CH 78 | Straathof | Cultivar G | |
| CH 79 | Fides | Cultivar H | |
| CH 80 | Brasil | Cultivar H | |
| CH 81 | Lyraflor | Cultivar H | |
| CH 82 | Straathof | Cultivar H | |

DNA fingerprinting with the $(GATA)_4$ microsatellite probe was performed on the following groups: family A members (CH 36–CH 52, with the exceptioin of CH 42 and CH 48), family B members (CH 59–CH 65), 5 accesions of cv G (CH 74–CH 78), 4 accessions of cv H (CH 79–CH 82) and CH 66–CH 71. Seven µg of DNA was digested with the methylation-insensitive *RsaI* (5 U/µg DNA), electrophoresed and blotted on Hybond N membranes according to the manufacturer's descriptions. Hybridization and washing conditions are described in Wolff et al. (1994a).

Inter-SSR PCR and polyacrylamide gel electrophoresis were performed as described by Zietkiewicz et al. (1994). The oligonucleotide primers (used one at a time) were: BDB $(CA)_7C$, DBDA $(CA)_7$, VHVG$(TG)_7$ and HVH $(TG)_7T$, where B, D, H and V stand for C or G or T, A or G or T, A or C or T, and A or C or G, respectively. The cultivars that were used are: family A members CH 36, CH 37, CH 38, CH 39, CH 42 and CH 45; family B cvs CH 59, CH 60, CH 61 and CH 62; duplicate accessions CH 66–CH 73; cv G accessions CH 74–CH 76 and cv H accessions CH 79–CH 81. Each cultivar is represented twice on each autoradiogram, with the amplifications being done separately for each.

Photographs of the RAPD patterns and autoradiograms of the SSR-PCR, RFLP and DNA fingerprint analysis were compared for variation in patterns within and among groups. Similarity between two randomly chosen cultivars is based on twice the number of bands in common, divided by the sum of the bands in both lanes. The probability of obtaining identical fragment patterns in 2 cultivars is the average similarity to the power of the average number of fragments per lane. In all cases only fragment patterns of cultivars on one gel were compared. Similarly RFLP patterns were only scored for pairs of cultivars that were in neighbouring lanes.

## Results

RAPD analysis was performed with 26 primers on 13 cultivars from family A (CH 36–CH 48) and with 15 primers on 7 members of family B and the other groups of accessions (CH 59–CH 78), except for the cultivar H accessions, which were tested with 9 primers (Table 2). All of the RAPD primers used resulted in the amplification of 2–9 bands on average over the cultivars (Table 3, Fig. 1). The cultivars that did not belong to one family showed distinct patterns for almost all of the primers. Some differences are based on strong intensity differences (more than 6 times) of a band of the same size. Similarity of RAPD patterns between 2 random cultivars depended strongly on the primer and ranged from 3% to 80% for a single primer. Two primers (numbers 19 and 42) gave irregular polymorphic patterns within a cultivar family or group. However, when the procedure was repeated with the same primers no polymorphism or different polymorphic patterns were recorded. Therefore, these primers (numbers 19 and 42) that gave non-reproducible polymorphic patterns in different runs were not used in further experiments with chrysanthemum. The probability of obtaining an identical fragment pattern in 2 randomly chosen cultivars is on average 6.7%, although some primers have a much higher discrimination power (down to a 0.5% probability, Table 3). We concluded that a combination of two or three RAPD patterns is sufficient to discriminate all cultivars tested.

Polyacrylamide gel electrophoresis of inter-SSR PCR products revealed complex patterns with 26–27 bands per lane for all of the primers used [Table 3, Fig. 2; only the results with DBDA$(CA)_7$ and HVH $(TG)_7T$ primers are shown]. Each of the primers allowed discrimination between the 8 different cultivars not belonging to one family; no variation between members of a family or different accessions of 1 cultivar could be seen. Similarity between random cultivars was 36% on average. The probability of identical fragment patterns was $62 \times 10^{-12}$. Although 1 primer was enough to see the pattern variation between cultivars, it should be noted that many differences were based on the intensity of the bands. Therefore, testing of the cultivars should be performed using more than 1 primer to increase the reliability of the results.

DNA fingerprinting based on hybridization with the microsatellite probe $(GATA)_4$ also showed highly polymorphic patterns between the cultivars, but not within families or accessions of 1 cultivar (results not shown). Each lane had approximately 12 discernable bands with only a 28% similarity between cultivars.

The RFLP probes from a genomic *PstI* library were selected on the basis of results from a preliminary study on 4 different cultivars (Wolff et al. 1994b). Probes that gave good banding patterns with a specific restriction enzyme were chosen. Of the 44 probes used 37 gave good banding patterns in the present analysis, with on average 3.7 scorable bands in each lane (see example in Fig. 3). All 18 cultivars had a different banding pattern with 33 of the 37 successful probes. The average similarity of banding patterns was 53% (Table 3).

No polymorphic patterns appeared upon RFLP analysis of family A with all 16 probes used on the

**Table 2** RAPD primers and the annealing temperature used, together with the groups of accessions for which the primers were used

| Number | Nucleotide sequence | Annealing temp. | Family A | CH 59 to CH78 | Cultivar H |
|---|---|---|---|---|---|
| 1 | 5′-TGG TCA CTG A-3′ | 36 | + | – | – |
| 2 | 5′-AGG TCA CTG A-3′ | 36 | + | – | – |
| 3 | 5′-TCG TCA CTG A-3′ | 36 | + | – | – |
| 4 | 5′-TGC TCA CTG A-3′ | 36 | + | – | – |
| 5 | 5′-TGG ACA CTG A-3′ | 36 | + | – | – |
| 6 | 5′-TGG TGA CTG A-3′ | 36 | + | – | – |
| 7 | 5′-TGG TCT CTG A-3′ | 36 | + | + | + |
| 8 | 5′-TGG TCA GTG A-3′ | 36 | + | – | – |
| 9 | 5′-TGG TCA CAG A-3′ | 36 | + | – | – |
| 10 | 5′-TGG TCA CTC A-3′ | 36 | + | – | – |
| 11 | 5′-TGG TCA CTG T-3′ | 36 | + | – | – |
| 12 | 5′-CGG TCA CTG T-3′ | 36 | + | – | – |
| 13 | 5′-CGG CCA CTG T-3′ | 40 | – | + | – |
| 14 | 5′-CGG CCC CTG T-3′ | 40 | – | + | – |
| 15 | 5′-CGG CCC CGG T-3′ | 40 | + | + | – |
| 16 | 5′-TGG TGA GTG T-3′ | 36 | + | – | – |
| 17 | 5′-TCA CGA TGC A-3′ | 36 | + | – | – |
| 18 | 5′-GCA AGT AGC T-3′ | 36 | + | + | + |
| 19 | 5′-GGA ATA AGC G-3′ | 36 | + | – | – |
| 20 | 5′-AGG AGA ACG G-3′ | 36 | + | – | – |
| 21 | 5′-GCT CGT CGC T-3′ | 40 | + | – | – |
| 22 | 5′-GCA TGT CGT G-3′ | 36 | + | + | + |
| 23 | 5′-GCC TGT CGA T-3′ | 36 | + | + | + |
| 25 | 5′-TGG TCC TGC G-3′ | 40 | + | + | – |
| 26 | 5′-TGC TGG GCG G-3′ | 40 | + | + | + |
| 27 | 5′-GGC TCG CGG G-3′ | 42 | – | + | + |
| 29 | 5′-GGG CGG GGC G-3′ | 42 | + | – | – |
| 30 | 5′-GGG AGG TGG C-3′ | 40 | + | – | – |
| 31 | 5′-GAG TGA TGA G-3′ | 36 | + | – | – |
| 37 | 5′-AAC GGC CAA C-3′ | 36 | – | + | + |
| 41 | 5′-AGG TCT GTG AG-3′ | 42 | – | + | – |
| 42 | 5′-GTT CCG TTC CG-3′ | 44 | – | + | + |
| 43 | 5′-TGG TCT TCC GTG-3′ | 44 | – | + | – |
| 44 | 5′-TCG CTT GCC T-3′ | 36 | – | + | + |

**Table 3** Analysis of fragment patterns obtained with RAPD analysis, inter-SSR PCR, RFLP analysis and $(GATA)_4$ DNA fingerprinting. The mean of the number of fragments per lane ($n$), the similarity of fragment pattern (S) and the probability of identical fragment patterns ($S^n$) as well as the standard deviation (between brackets) and range are given. The standard deviation is the deviation between primers or probes, except for $(GATA)_4$ fingerprinting, where the deviation among cultivars or pairs of cultivars is given

| Technique | $n$ | S | $S^n$ |
|---|---|---|---|
| RAPD | 5.40 (1.47) | 0.513 (0.200) | 0.067 (0.100) |
| analysis | 2.29–8.28 | 0.281–0.843 | 0.005–0.376 |
| Inter-SSR | 26.8 (0.3) | 0.356 (0.098) | $62 \times 10^{-12}(7 \times 10^{-12})$ |
| PCR | 26.4–27.2 | 0.349–0.363 | $2.4–122 \times 10^{-12}$ |
| RFLP | 3.75 (1.06) | 0.530 (0.155) | 0.150 (0.168) |
| | (2.47–6.38) | 0.193–0.861 | 0.002 – 0.690 |
| $(GATA)_4$ fp | 12.33 (2.42) | 0.279 (0.082) | $14.5 \times 10^{-9}$ |

*Eco*RV-digested DNA. Polymorphic patterns were, however, present with 5 of the 13 probes used on *Eco*RI-digested DNA, and 6 out of 8 probes used on *Hin*dIII-digested DNA. This means that a total of 11 probes gave a slightly different pattern for some of the family A cultivars (Fig. 4). If the family A cultivars were ordered according to their ancestral derivation, the deviating RFLP patterns were irregularly placed in the scheme. Two or more cultivars having the deviating pattern were not always within one line of inheritance and were not always directly derived from CH 36, but via another family member that did not have the deviating pattern (see Fig. 5 for the probes used on the *Eco*RI blots). The variation observed does not seem to be of genetic origin but rather an artefact of the method used. *Eco*RI and *Hin*dIII are methylation-sensitive enzymes, while *Eco*RV, which did not reveal any polymorphisms, is not. Therefore, we concluded that differential methylation may have caused the variable patterns among family members that was obtained on the *Eco*RI and *Hin*dIII blots.

## Discussion

In order to apply molecular techniques for cultivar identificatioin one needs to know the levels of polymorphism revealed by the different techniques in the species under study. Poulsen et al. (1993) showed that each microsatellite probe had a different level of discriminatioin in *Brassica napus* and related species. Some probes could distinguish species, others breeding lines and again others individuals. Tomato cultivars are generally indistinguishable with RFLPs or RAPDs, but they

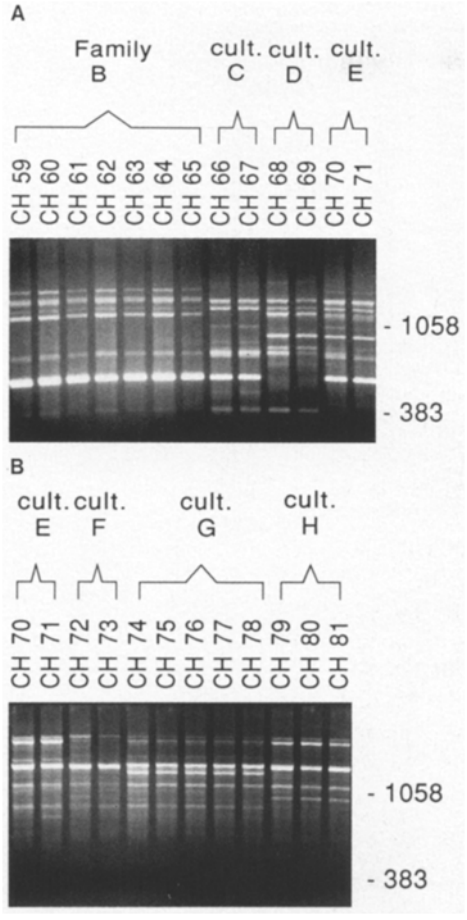


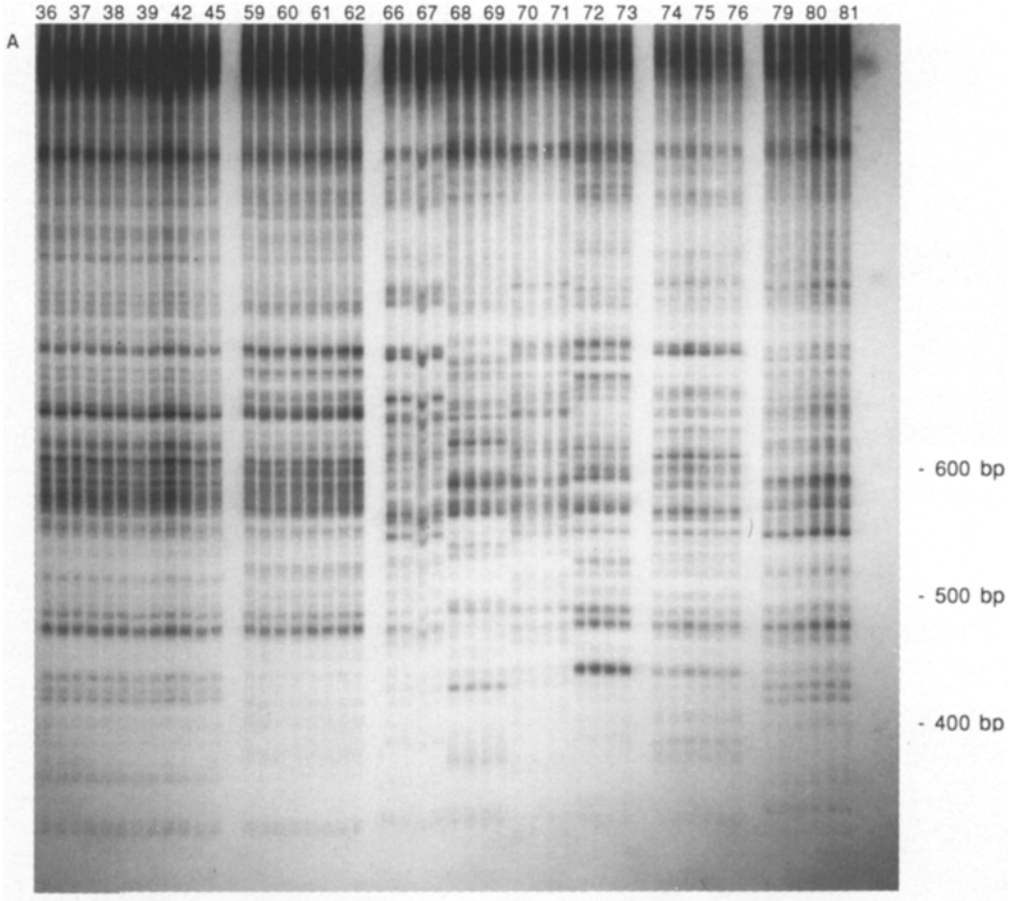

**Fig. 1** RAPD patterns of **A** the family B (*lanes 1–7*:CH 59–CH65) and 2 accessions each of cvs C, D and E (*lanes 8–13*: CH 66–CH 71) obtained with primer 41, and **B** 2 accessions each of cvs E and F (*lanes 1–4*: CH 70–CH 73), 5 accessions of cv G (*lanes 5–9*: CH 74–CH 78) and 3 accessions of cv H (*lanes 10–12*: CH 79–CH 81) obtained with primer 25

could be told apart using $(GATA)_4$as a probe (Vosman et al. 1992). For highly inbreeding plants or crops with a narrow germ plasm it is also more difficult to find probes or primers that will discriminate the genotypes. In the outcrossing *Plantago lanceolata* the M13 probe could discriminate all 32 individuals, while in 40 plants of the inbreeding *Plantago major* only a few different banding patterns were discerned (Wolff et al. 1994a).

Chrysanthemum cultivars appear to be extremely polymorphic, whichever technique is applied. This is caused by (1) the fact that breeders purposely intercross their cultivars with unrelated material from other breeders or even from other countires and (2) the strong sporophytic self-incompatibility system, which prevents successful crossing of related genotypes. The level of variation between chrysanthemum cultivars is too high to successfully analyse the degree of relatedness between cultivars if these are further apart than the parents and their offspring; cultivars taken from one breeding company have just as many polymorphic fragment patterns as randomly chosen cultivars (Wolff and Peters-Van Rijn 1993).

Cultivar identification in chrysanthemum can be done with any of the techniques used in this study. The similarity of fragment patterns of different cultivars with RAPDs, inter-SSR PCR, RFLPs and microsatellite fingerprinting is comparable. However, the high numbers of fragments per lane with $(GATA)_4$ fingerprinting and inter-SSR PCR gives a much lower probability of finding an identical fragment pattern by chance alone. Microsatellite fingerprinting is technically demanding. Inter-SSR PCR gives the highest number of bands and the lowest probability of identical fragment patterns. RFLPs will discriminate most cultivars easily, without having to cope with intensity differences. However, RAPDs seem to be the quickest and easiest technique for which no radioactivity is needed, in contrast to the other techniques used. Two or three RAPD primers used on the same cultivars have the same discrimination power as one $(GATA)_4$ fingerprint. RAPDs have been applied for identification in many other plants, like in the vegetatively propagated apples (Koller et al. 1993) and raspberry cultivars (Graham et al. 1994), in the sexually derived rape seed (Mailer et al. 1994) and in somatic and sexual hybrids (Baird et al. 1992; Marsolais et al. 1993).

There are, however, also some disadvantages of RAPDs compared to for example, species-specific RFLPs or PCR with specific primers. Reaction conditions, such as the $Mg^{2+}$ concentration, DNA concentration, the cycling programme and the thermocycler used may influence RAPD patterns to different degrees (Weising et al. 1994). Also, if a different polymerase is used, e.g. Stoffel fragment instead of Amplitaq (both Perkin Elmer), a totally different pattern may be obtained (Weising et al. 1994). Therefore, it does not seem to be the best solution to describe cultivars and protect breeders' rights by using RAPD patterns. For this specific purpose a technique that is 100% transferable between laboratories should be used. RFLP analysis showed highly differentiating patterns between the cultivars, but it may not be efficient to transport probes between laboratories. A defined set of probes could be used at a central point, e.g. Assinsel or UPOV. It would be more efficient to have specific primers and send primers or the nucleotide sequences to other laboratories. One could think of inter-SSR PCR (this paper and Zietkiewicz et al. 1994) or PCR-based fingerprinting with specific primiers complementary to the DNA flanking simple sequence repeats (Weising et al. 1994; Wolff et al. in prep). In the former technique genomic segments flanked by simple-sequence repeats are amplified, whereas in the latter technique genomic segments that are known to contain simple-sequence repeats are amplified with specific primers. In both techniques no hybridization is necessary as in RFLPs and microsatellite DNA fingerprinting, but the use of polyacrylamide gels and radioactive labelling is recommended. RAPD analysis seems to be, however, the


36 37 38 39 42 45 59 60 61 62 66 67 68 69 70 71 72 73 74 75 76 79 80 81
A
- 600 bp
- 500 bp
- 400 bp

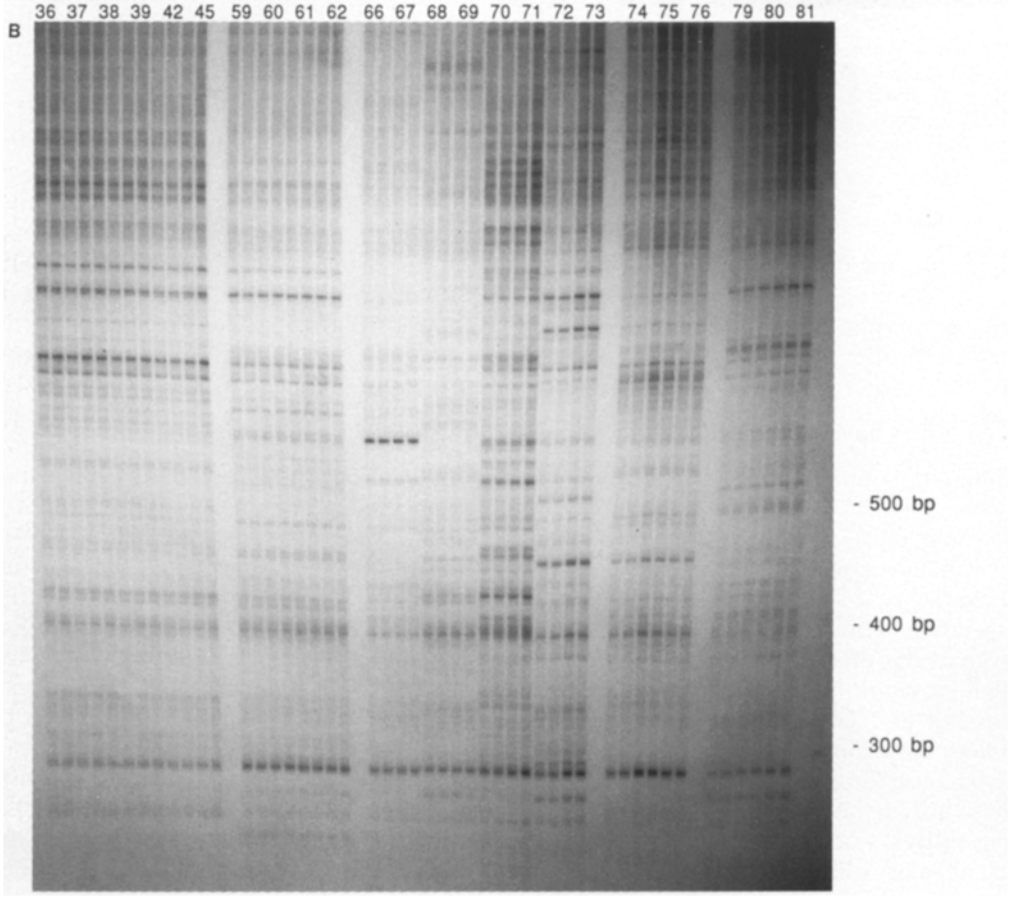

36 37 38 39 42 45 59 60 61 62 66 67 68 69 70 71 72 73 74 75 76 79 80 81
B
- 500 bp
- 400 bp
- 300 bp

**Fig. 2** Autoradiogram after inter-SSR PCR with several members of families A and B and different accessions of several cultivars. **A** primer DBDA $(CA)_7$ **B** primer HVH $(TG)_7T$

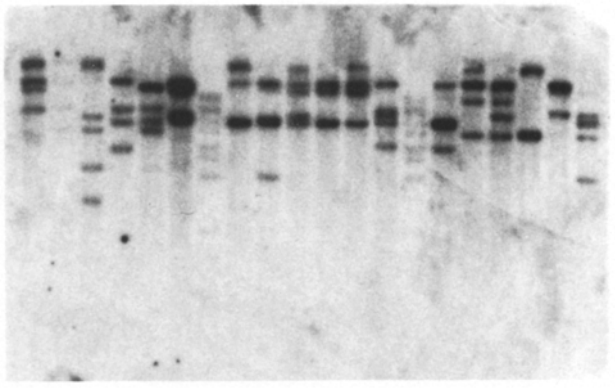


**Fig. 3** RFLP patterns of 18 different cultivars digested with *Eco*RI after hybridization with probe WR325

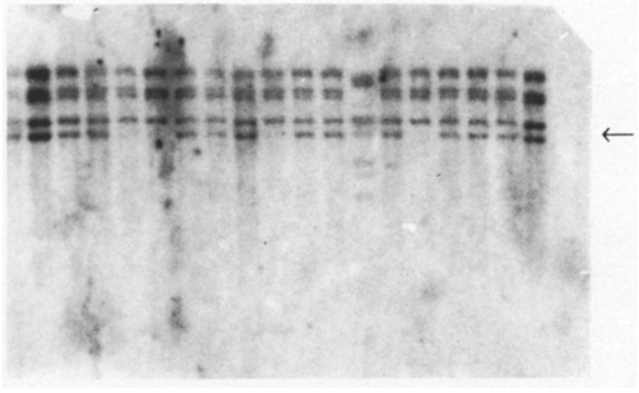


**Fig. 4** RFLP pattern of 17 members of family A after digestion with *Eco*RI and hybridization with probe WR325. The polymorphic band is indicated with an *arrow*

easiest and quickest option for within-laboratory comparisons.

If DNA techniques have to be used for identification purposes the patterns have to be stable within cultivars. Several studies were designed to test this. No genetic variation was revealed after studying RAPD patterns from plants regenerated from protoplast cultures or from in vitro-propagated lant material in *Picea mariana* (Isabel et al. 1993), meadow fescue (Vallés et al. 1993) and tomato (Vosman et al. 1992). Parent et al. (1993) tested the stability of RAPD patterns in raspberry and showed that no differences were found in different periods of the growing season or in different phases of the certification programme. However, Nelke et al. (1993) found one somaclonal varient in red clover using the Jeffreys probes 33.15 and 33.6. This variant was genetically stable in later generations. Brown et al. (1993) also found several differences in regenerated protoplasts of *Triticum* with RAPDs.

In chrysanthemum, no polymorphisms were apparent between members of a family, between DNA from material kept in tissue culture or vegetatively propagated, or between different accessions from 1 cultivar. The RFLPs in family A are an exception, and these will be discussed in the next paragraph. We mainly used PCR techniques, RAPDs and inter-SSR PCR for this purpose. It can be concluded that with these techniques it can be reliably determined whether two unknown samples are samples from different cultivars, with the exception of cultivars that are vegetatively derived from each other.

Lee and Phillips (1988) have given an extensive review on somaclonal variation that may shed some light on the process of sporting. They state that, especially in species with a lot of heterochromatin, a late or delayed replication of the heterochromatic parts may take place. This can result in breakage and a loss of (a part of) a chromosome. This may indeed be the case in chrysanthemum since polyvalents and chromosomal bridges have, although rarely, been observed in polyploid taxa closely related to chrysanthemum (Watanabe 1977). Another reason for phenotypic differences may be differential methylatioin of the DNA (Brown and Lörz 1986). We have shown that RFLP patterns of members of family A were slightly different in some cases, irregularly distributed over the descent scheme. Polymorphisms were only found with the 2 methylation-sensitive restriction enzymes. Therefore, differential methylation may have caused the RFLPs. Consequently, we conclude that in all studies in which restriction enzymes are used for identifying genotypes or cultivars methylation-insensitive restriction enzymes should be used. The differences in, for instance, flower colour are, however, not based on differential methylation since these differences are relatively stable over environments.

There were no RAPD or SSR-PCR differences between phenotypically different members of a family. The different flower colours may have been caused by differences in only the outermost layer or the two outermost layers. The DNA used was obtained from leaf material, which contains all three layers. If phenotypically different members of a family differ genetically in, for example, layer I or layer II, this may go unnoticed if the DNA is extracted from leaves.

Another reason for the absence of polymorphisms in phenotypically diverse families or between different accessions of one cultivar may be that the part of the genome studied was too small and that more primers should have been used. If a certain number of RAPD primers is used and no differences are found, what does that mean in respect to the size of the genome, or in other words which part of the genome is tested for homology? Because chrysanthemum has a large genome ($3 \times 10^9$ bp) we expect that primer-template homology must be close to 100%. The first eight nucleotides from the 3′ end are important for primer annealing (Williams et al.

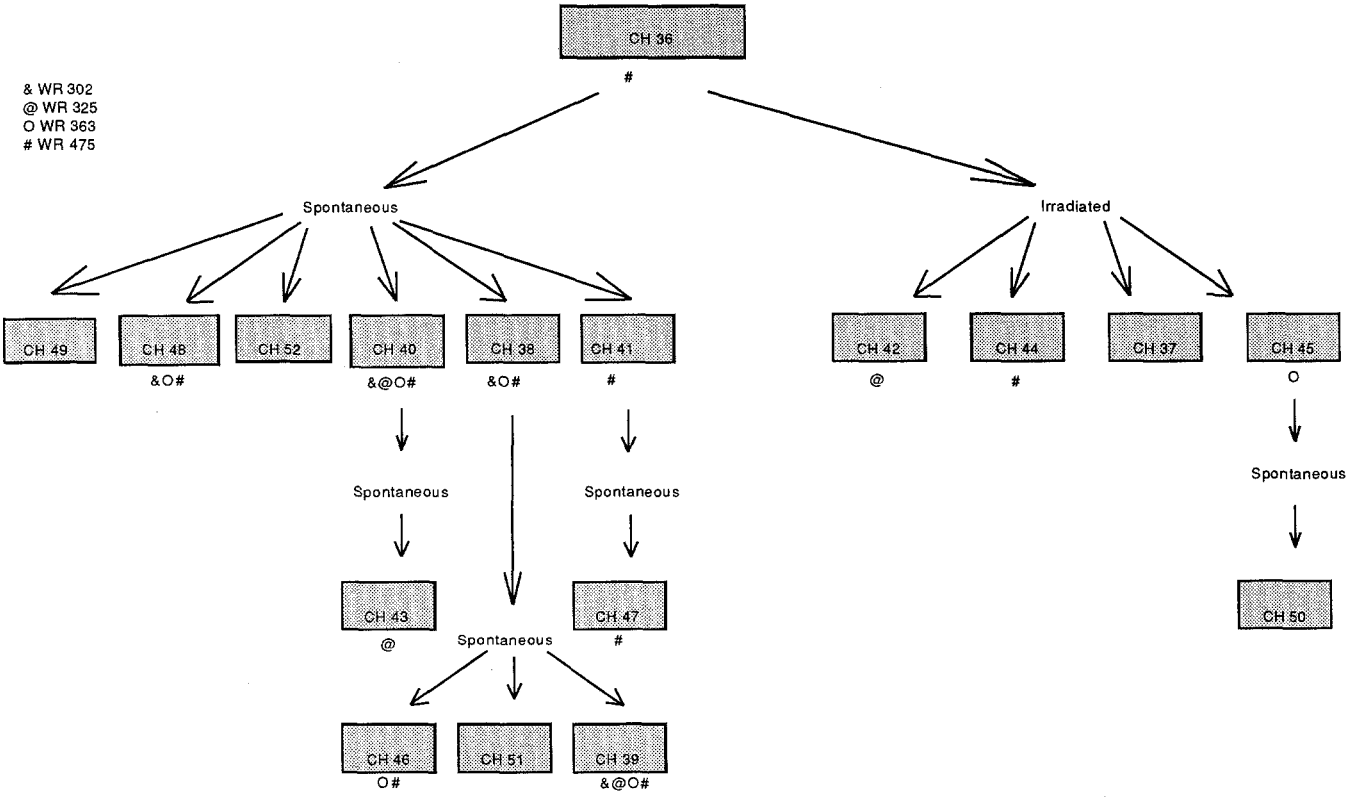



**Fig. 5** The descent scheme of family A and the RFLPs found on the *Eco*RI blots

1990). Each primer gave on average 8 fragments, which means that with 26 primers $8 \times 2 \times 26$ annealing sites (416) and $8 \times 416$ bp (3328 bp) were tested. In this way only a small part of the genome was tested for nucleotide substitution. The average size of the fragments was 1800 bp. Therefore, $3.7 \times 10^5$ bp ($8 \times 26 \times 1800$ bp) was tested for additions and deletions. This again indicates that only a relatively small part of the genome was analysed. Isabel et al. (1993) state that the number of markers needed to test the genetic integrity of clonal material is as many or more markers than would be required for a saturated map. In chrysanthemum with 54 chromosomes that would mean 200–300 markers, which could be reached using 25–35 primers. Therefore, the 26 RAPD primers used for family A adequately covers the genome for detecting deletions and additions. On the other hand, the number of RAPD primers used for the other groups of cultivars is not enough to cover the whole genome. However, in combination with the DNA fingerprinting and the SSR-PCR we used enough markers to test the genetic stability of the cultivars.

It can safely be said that members of a family will have a DNA fragment pattern that is very similar, or even identical, although more primers and fingerpring probes could be tested to sample a larger part of the genome. The keeping of mother plants in the greenhouse or stock material in tissue culture will normally lead to plants with identical DNA fragments patterns.

Although it has been shown that molecular techniques are extremely valuable for identification purposes in many species, also in chrysanthemum, our tests will not replace the traditional identification growth trials within a short time. But studies like the one presented here, and the fact that it is shown that phenological and morphological characters are in some cases influenced by the environment (Teynor et al. 1989), will at least allow the molecular techniques to complement the growth trials.

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