

# Rapid detection of genetic variability in chrysanthemum (*Dendranthema grandiflora* Tzvelev) using random primers

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Genetic variation in chrysanthemum (*Dendranthema grandiflora*) was studied using a recently developed technique generating Random Amplified Polymorphic DNAs (RAPDs). It appeared that variation between cultivars was high and that the cultivars used could be distinguished from each other by using only two different primers. A family of cultivars, derived from one original cultivar by vegetative propagation, had identical fragment patterns. Because of the high level of polymorphism and clonal stability RAPD fragments are useful for cultivar identification. Genetic variability among related *Dendranthema* species was too high to study genetic distances either among cultivars within chrysanthemum or among species related to chrysanthemum.

**Keywords:** chrysanthemum, cultivar identification, genetic variability, PCR, RAPDs.

## Introduction

*Dendranthema grandiflora* Tzvelev (*Chrysanthemum morifolium* Ramat.) is a polyploid belonging to a hexaploid species complex comprising six or seven species (Dowrick, 1953). On average 54 chromosomes are present, although counts from 36 to 75 are possible (Langton, 1989). Regular bivalents are formed during meiosis suggesting an allopolyploid origin (Dowrick, 1953; Watanabe, 1977). It is not known whether most characters are inherited in a disomic or a hexasomic way. Bivalent formation suggests a disomic transmission. Presence of carotenoid pigmentation, however, appeared to be transmitted in a hexasomic way (Langton, 1989). Chrysanthemum has a strong self-incompatibility system, thus causing many crosses between related or unrelated individuals to be unsuccessful. Usually only between 5 and 50 per cent of crosses between sibs in an  $F_1$  are compatible (Drewlow *et al.*, 1973; Ronald & Ascher, 1975; Zagorski *et al.*, 1983; Stephens *et al.*, 1984). The genetics of the sporophytic self-incompatibility system is not completely resolved but probably several loci are involved (more than two) and there is dominance of alleles (Zagorski *et al.*, 1983; Stephens *et al.*, 1984). Polyploidy, unknown

origin of the species and the self-incompatibility system caused many genetic analyses to be unsuccessful in chrysanthemum and, therefore, little is known about the genetics of the species.

Commercially chrysanthemum is mainly asexually propagated by terminal, vegetative cuttings (Dowrick & El-Bayoumi, 1966; Teynor *et al.*, 1989). Propagation via seeds is usually used only to develop new cultivars since biparental crosses give an extremely variable  $F_1$  differing in many morphological characters (Dowrick & El-Bayoumi, 1966).

In this study we examine genetic variability in the species *Dendranthema grandiflora* and among related species. We expect the variability among cultivars within chrysanthemum to be large considering the out-crossing nature of the mating system (Brown, 1979; Loveless & Hamrick, 1984; Wolff, 1991). We further expect that many loci are in a heterozygous state and these loci may, therefore, show segregation in the offspring of a biparental cross. Our future goal is to use molecular markers for cultivar identification and in mapping studies. The present study is the first one exploring molecular variability in chrysanthemum.

During recent decades several techniques have been introduced that detect molecular variability within and among several species. Three of these widely applied techniques are the use of restriction fragment length polymorphisms (RFLPs) (e.g. Helentjaris *et al.*, 1986),

DNA fingerprinting (Jeffreys *et al.*, 1985) and specific amplification of polymorphic DNA fragments with PCR (Weining & Langridge, 1991). Prior knowledge of the DNA composition of the species and/or the presence of useful probes is, however, required. Recently a new and rapid technique to detect polymorphisms was developed using the polymerase chain reaction (PCR) and random primers to amplify DNA (Welsh & McClelland, 1990; Williams *et al.*, 1990). The technique is named RAPD after Random Amplified Polymorphic DNAs (Williams *et al.*, 1990). The DNA fragment patterns generated can discriminate between individuals, cultivars or species (Arnold *et al.*, 1991; Caetano-Anolles *et al.*, 1991; Hu & Quiros, 1991; Quiros *et al.*, 1991; Hadrys *et al.*, 1992). In some cases the transmission of polymorphic fragments and the localization of the amplified DNA fragment on the genome was determined (Williams *et al.*, 1990; Martin *et al.*, 1991). RAPDs have already been used successfully in mapping studies in *Arabidopsis* (Reiter *et al.*, 1992) and in tomato (Aarts *et al.*, 1991; Martin *et al.*, 1991; Klein-Lankhorst *et al.*, 1991). Micheltore *et al.* (1991) showed that the RAPD markers are extremely useful in a bulked segregant analysis to detect disease-resistance genes in lettuce.

The newly developed technique of RAPD seems suitable for studying genetic variability in chrysanthemum and related species as species-specific probes for studying RFLPs are not present at this moment. In a preliminary study we observed that the polymorphic RAPD markers have a genetic basis: all fragments present in the offspring of a biparental cross were clearly transmitted from parents to offspring (Wolff, unpublished results). Only one non-parental band was seen in one of the offspring out of 43 polymorphic fragments scored using nine primers. Variability within *Dendranthema grandiflora* will be studied by looking at genetic variability among cultivars, and at the same time determine whether RAPDs are useful for identifying cultivars. Furthermore, we can test the stability of fragment patterns in mutant members of a clonal family of cultivars that differ in specific morphological and physiological characters. Genetic variability among related chrysanthemum species will be analysed by studying several species from this polyploid species complex.

## Materials and methods

### Plant material and experimental setup

The plant material was kindly provided by three Dutch chrysanthemum breeding companies as leaf material, freshly cut from adult plants. In chrysanthemum breeding it is often the case that from a successful cultivar

other cultivars are obtained by (somatic) mutation with, amongst others, different flower colour. These new cultivars are made by irradiating the successful original cultivar with gamma-rays or by cloning the original cultivar into thousands of clonal derivatives and looking for a spontaneous somatic mutation. In this study we used one specific family, the family derived from CH36. The original cultivar is pink flowered and by now at least 14 different coloured cultivars have been obtained by spontaneous mutation and four derived by irradiation. To study clonal stability 13 members of this family and 27 primers (numbers 1 to 26 and 29) were used.

For cultivar variability 18 different cultivars from three breeders and eight primers (numbers 1, 13, 14, 15, 18, 21, 22 and 23) were used. Designation of the cultivars used and their origin is given with Fig. 1.

Variability among species was studied using six primers (numbers 3, 4, 15, 21, 22 and 23) and 15 individuals, representing 13 different species. Names of the species and their ploidy level, as far as information is available from the literature, are given with Fig. 3.

### DNA extraction

Chrysanthemum leaves were ground in liquid nitrogen and freeze dried under vacuum. The dry powder was kept at  $-20^{\circ}\text{C}$ . DNA was extracted from the dry powder according to Saghai-Marooof *et al.* (1984) with the modifications of a double chloroform/isoamyl-alcohol (24:1) treatment, the use of cold absolute ethanol to precipitate the DNA, two washes in 76 per cent ethanol/0.2 M NaAc prior to the wash in 76 per cent ethanol/10 mM  $\text{NH}_4\text{OAc}$  and the dissolving of the DNA in 10 mM Tris/1 mM EDTA (pH 8.0). Yields were usually 70  $\mu\text{g}$  DNA per 300 mg of powder per extraction.

### Primer synthesis

Oligodeoxynucleotide primers were synthesized using a DNA synthesizer 381A from Applied Biosystems. After deprotection of the oligos, two extractions with phenol/chloroform (1:1) and two extractions with chloroform were done. The primers were numbered according to the order of synthesis (Table 1); numbers one to eleven are identical to the primers published by Williams *et al.* (1990).

### Amplification reaction conditions

The use of RAPDs was developed by Williams *et al.* (1990). The DNA was amplified under conditions similar to normal PCR, with the exception that only a

**Table 1** Nucleotide sequences of the primers used for generating RAPDs

1	5'-TGG TCA CTG A-3'
2	5'-AGG TCA CTG A-3'
3	5'-TCG TCA CTG A-3'
4	5'-TGC TCA CTG A-3'
5	5'-TGG ACA CTG A-3'
6	5'-TGG TGA CTG A-3'
7	5'-TGG TCT CTG A-3'
8	5'-TGG TCA GTG A-3'
9	5'-TGG TCA CAG A-3'
10	5'-TGG TCA CTC A-3'
11	5'-TGG TCA CTG T-3'
12	5'-CGG TCA CTG T-3'
13	5'-CGG CCA CTG T-3'
14	5'-CGG CCC CTG T-3'
15	5'-CGG CCC CGG T-3'
16	5'-TGG TGA GTG T-3'
17	5'-TCA CGA TGC A-3'
18	5'-GCA AGT AGC T-3'
19	5'-GGA ATA AGC G-3'
20	5'-AGG AGA ACG G-3'
21	5'-GCT CGT CGC T-3'
22	5'-GCA TGT CGT G-3'
23	5'-GCC TGT CGA T-3'
24	5'-GCG TGA CTT G-3'
25	5'-TGG TCC TGC G-3'
26	5'-TGC TGG GCG G-3'
29	5'-GGG CGG GGC G-3'

single primer was used and that the nucleotide order of the primer was random, with the only requirement that the G + C content be at least 50 per cent (Williams *et al.*, 1990). In a previous experiment the reaction conditions were optimized (Wolff *et al.*, 1993). Reactions were performed in a volume of 50  $\mu$ l containing 20 mM Tris-HCl, pH 8.3, 50 mM KCl, 3 mM MgCl<sub>2</sub>, 0.001 per cent gelatin, 100  $\mu$ M each of dATP, dCTP, cGTP and dTTP, 0.2  $\mu$ M primer, 25 ng of chrysanthemum genomic DNA, and 1 U of *Taq* polymerase (Amplitaq, Perkin Elmer Cetus) using a Perkin Elmer 9600 thermal cycler. After 5 min heating at 94°C 45 cycles were run. Each cycle consisted of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C. Ramp time between annealing temperature and 72°C was set at 0.3–0.4°C s<sup>-1</sup> (a total of 2 min). A ramp time shorter than 1.5 min led to low or no DNA amplification. In a preliminary experiment the influence of DNA and polymerase concentration was tested with three primers and two different DNA templates.

Amplified DNA fragments were separated by electrophoresis in a 1.4 per cent agarose gel with a TBE buffer system (Sambrook *et al.*, 1989). The total reaction volume was loaded on the gel. Gels were

stained with ethidium bromide and fragment patterns were photographed for further analysis.

### Observations

Different fragments produced with each primer were numbered sequentially and presence or absence of fragments in each individual was scored. Individuals from the same row on one gel were compared with each other. Fragments with a medium or strong signal were taken into account as these fragments are fully reproducible. Fragments with the same mobility on the gel but with different intensities were not distinguished from each other when cultivars or species were compared with each other.

### Variability

Variability among cultivars and among species was expressed as the similarity *S*. This is calculated as:

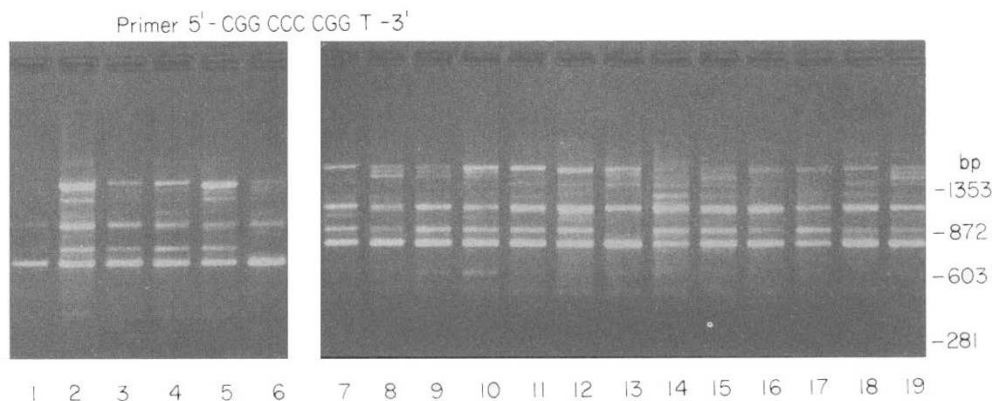
$$S = \frac{2 \times N_{AB}}{N_A + N_B},$$

in which  $N_{AB}$  is the number of bands shared by individuals A and B, and  $N_A$  and  $N_B$  are the number of bands in individuals A and B, respectively. The similarity measure can also be called band sharing. Distance can be calculated as  $D = 1 - S$  (Swofford & Olsen, 1990). The chance of finding two individuals with the same fragment pattern can be calculated as the mean similarity ( $\bar{S}$ ) to the power of the mean number of bands ( $\bar{N}$ ) (Nyblom & Hall, 1991).

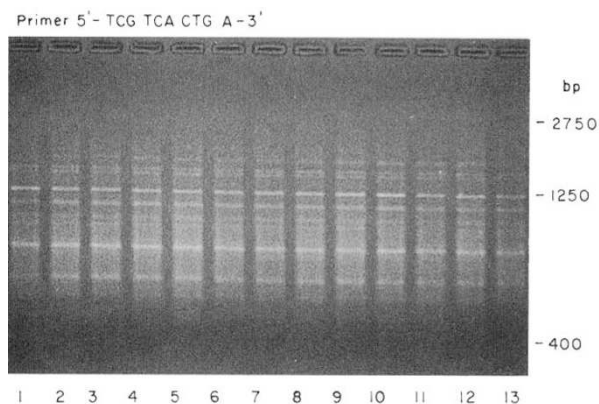
### Results

The Dutch cultivars showed strongly differing fragment patterns (Fig. 1). On average, band sharing was 0.66 (range 0.49–0.77). Only a few primers (1–3), depending on the number of polymorphic fragments generated by the primers chosen, were needed to distinguish all cultivars in this study. Each primer gave on average seven bands per individual. This, together with a mean similarity of 0.66, gives a chance of finding two identical patterns in two individuals of  $5 \times 10^{-2}$  for one primer,  $3 \times 10^{-3}$  when using two primers and  $1.6 \times 10^{-4}$  when using three primers. Similarity among cultivars was calculated for within breeding company and a between company comparison (only the two largest groups, from Fides and from CBA were compared). Cultivars obtained from CBA were slightly more similar (*t*-test;  $P = 0.028$ ) to each other ( $S = 0.71$ ) than they were to cultivars from Fides ( $S = 0.66$ ). Cultivars from Fides were as similar to each other





**Fig. 1** RAPD fragment pattern of several Dutch chrysanthemum cultivars. From lane 1 to lane 18 cultivars with the following numbers are used: CH1, CH2, CH3, CH4, CH5, CH6, CH7, CH8, CH9, CH10 (all from Fides), CH11, CH12, CH14, CH17 (from CBA), CH53, CH54 (from Fides), CH55, CH57 (from Hoek Breeding).



**Fig. 2** RAPD fragment of a family of cultivars derived from number CH36.

( $S=0.66$ ) as to cultivars from CBA ( $S=0.66$ ). The cultivars belonging to one family, all derived from CH36, were extremely similar (Fig. 2). Only one primer (number 19) out of the 27 tested gave slightly different patterns for these 13 mutant cultivars. In this case there was marginal variation for the fainter bands only.

The different *Dendranthema* species were studied with six primers. Genetic variability among the species was high (Fig. 3). Some primers yielded extremely different banding patterns in each species; many bands were present in only one of the species. Mean similarity among species was 0.49 (range 0.22–0.77). This is significantly lower than similarity among chrysanthemum cultivars ( $P<0.001$ ,  $t$ -test). Two of the species were represented by two individuals from a different origin. In both cases the two individuals of one species were as different from each other as from individuals from other species. Therefore, the high variability

within chrysanthemums seems to be present within other related species as well. The ploidy level of some of the species is known from the literature (see Fig. 3 and Kawata, 1978). There was no relationship between the ploidy level and the number of RAPD fragments generated (*c.f.* Fig. 3:  $4\times$  species in lanes 1 and 5 and  $10\times$  species in lanes 2 and 3).

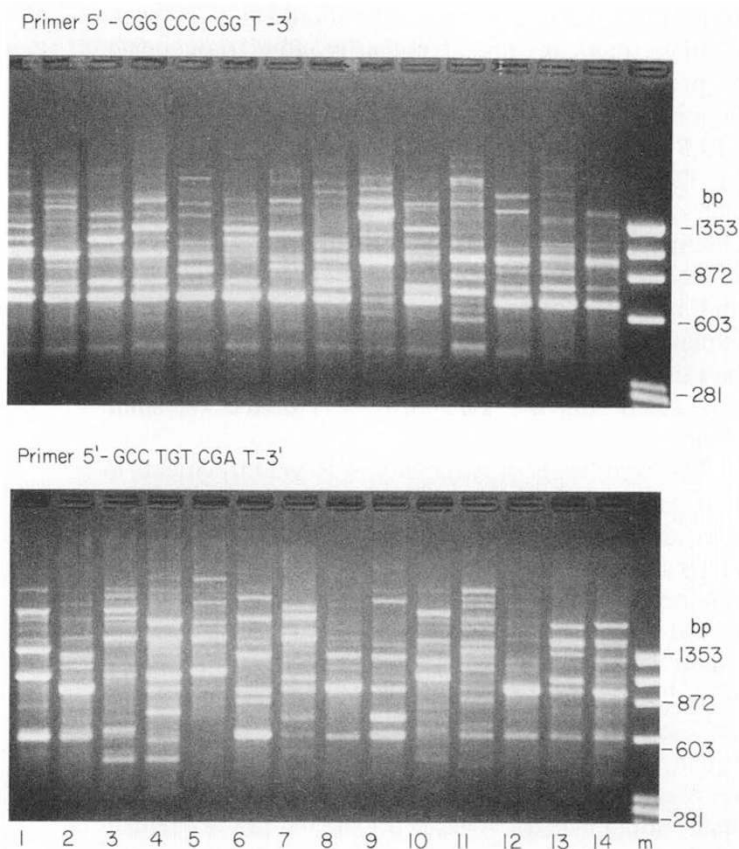
## Discussion

### *Genetic variability in chrysanthemum*

The chrysanthemum varieties show a high level of genetic variability as was expected from a plant possessing a mating system of strict outcrossing. Apparently the regular introduction of material from Japan and other countries, which is considered a good breeding practice, has resulted in the maintenance of high levels of genetic variability in the gene pool. Levels of variability for RAPD in this species are comparable to some other species studied (Carlson *et al.*, 1991; Hu & Quiros, 1991; Reiter *et al.*, 1992; Van Heusden & Bachmann, 1992; Welsh *et al.*, 1991). Some of the wild and agricultural species studied show far lower levels of variability, probably because of their mating system and the breeding strategy (Arnold *et al.*, 1991; Halward *et al.*, 1992).

Cultivar identification and cultivar relatedness are important issues for horticultural breeders. The application of RAPDs seems very valuable in this regard. If primers are chosen that are known to give highly polymorphic banding patterns in chrysanthemum, only a few primers are needed to distinguish cultivars, as long as these cultivars are not mutants of each other. Therefore, cultivars derived vegetatively from one original cultivar cannot be distinguished from each other using

**Fig. 3** RAPD fragment pattern of related *Chrysanthemum* and *Dendranthema* species. The species' names and ploidy level, if known, are from lane 1 to 14: 1. *D. yoshinaganthum* Makinoi ex Kitamura (4 ×) (CPRO) 2. *D. arcticum* L. Tzvelev (10 ×) (8 × Dowrick, 1952) (CPRO) 3. *D. pacificum* (Nakai) Kitamura (10 ×) (CPRO) 4. *D. shiwogiku* (Kitamura) Kitamura (8 ×, 10 ×) (CPRO) 5. *D. indicum* L. Des Moul (4 ×, 6 ×) (CPRO) 6. *D. zawadskii* (Herb.) Tzvelev (8 ×, Krogulevich, 1978) (CPRO) 7. *C. nan-kingense* Handel-Mazetti (Sharman) 8. *C. zawadskii* Herbich ssp. *latilobum* (maxim.) Kitagawa 'Pink Procession' (4 ×) (4 ×, 6 × Lee, 1967, 1975) (USDA) 9. *C. yezoense* T. Maek. (10 ×, Dowrick, 1952) 10. *D. pacificum* (Nakai) Kitamura (10 ×) (Sharman) 11. *D. weyrichii* (Maxim.) Tzvelev (8 ×) (6 × Shimotomai, 1932) (CPRO) 12. *D. xrubellum* Sealy 'Clare Curtis' (2n = 63 Dowrick, 1952) (Sharman) 13. *C. wakasaense* Shimot. ex Kitamura (4 ×, Dowrick, 1952) (CPRO) 14. *D. grandiflora* Tzvelev. (6 × Dowrick, 1952). Chromosome counts are from Kawata (1978), except if noted otherwise.



this technique. The members of these families originate from each other by mutations. It is not known whether these are point mutations or inversions, deletions or even loss of chromosomes. Observations by Dowrick & El-Bayoumi (1966) that morphological mutations often coincide with a difference in chromosome number, seem not to be confirmed by our observation as no differences in RAPD fragment pattern among somaclonal cultivars are observed in this study. The mutations causing the morphological differences may be localized on a small part of the genome.

Similarity (band sharing) can be used as a measure of relatedness (Nyblom & Hall, 1991; Welsh *et al.*, 1991). Hu & Quiros (1991) studied genetic variability in *Brassica oleracea*, and found higher similarities among cultivars from one company than between different company. We found that for one company the cultivars may be slightly more related to each other than to cultivars from another companies. The number of cultivars in this study is, however, too low for definitive conclusions. More cultivars are needed to be conclusive about relatedness within and among these groups.

#### *Genetic diversity among Dendranthema and Chrysanthemum species*

Genetic variation among *Dendranthema* and *Chrysanthemum* species, related to the cultivated chrysanthemum, is extremely high, with a very low similarity among species. Besides that, genetic variation within species is also very high. Therefore, RAPDs do not seem useful for determining distances between these species. The usefulness of RAPDs in phylogenetic studies may be different in species that have a low genetic variability within species and where the species are more closely related. In Louisiana irises variation within species was low and natural hybrids among the species were identified because they possessed fragments of both species (Arnold *et al.*, 1991). In *Arachis* species dendrograms were generated using RAPD fragments as characters (Halward *et al.*, 1992). These dendrograms showed relationships among *Arachis* species that were in concordance with species relationships derived from allozymes, RFLPs and morphological characters. Although RAPDs may be useful in studying genetic relationships of closely related species the general use of RAPD fragment patterns for taxo-

onomic purposes is debatable. One should at least check whether fragments are identical by either sequencing or, probably easier, by using the fragments as a probe. In general, other methods, like sequencing or cpDNA RFLP analysis, are preferred in taxonomic studies (see e.g. Dowling *et al.*, 1990). In a forthcoming study we will determine copy number of fragments and test identity to similar fragments from different individuals.

The absence of a correlation between ploidy level and numbers of fragments generated can easily be explained by the fact that PCR reactions are largely based on competition processes among molecules and not solely on the presence or absence of some sequences.

The high levels of variability of RAPD fragments in chrysanthemum renders these RAPD fragments good candidates for cultivar identification and genetic analysis. For specific segregation analyses large numbers of offspring and offspring from several types of crosses are needed; this will be pursued in a subsequent study. The preliminary data on the transmission of polymorphic RAPD fragments seems to point at a diploid-like inheritance; 25 out of 43 polymorphic fragments, present in only one of the parents, segregated in a 1:1 (presence:absence) fashion (Wolff, data unpublished). These fragments are extremely valuable in studies in which markers are used to localize QTL. Furthermore, these polymorphic markers are analogous to single-dose restriction fragments (SDRFs) as described by Wu *et al.* (1992). They describe how SDRFs are extremely useful in mapping studies in polyploids and how they can be used to distinguish allopolyploidy from autopolyploidy. We have to explore the problems that the hexaploidy of the organism and the dominance of the fragments may cause in future research.

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