12-1995

DNA amplification fingerprinting of chrysanthemum cultivars

Mary Catherine Scott

To the Graduate Council:

I am submitting herewith a thesis written by Mary Catherine Scott entitled "DNA amplification fingerprinting of chrysanthemum cultivars." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Landscape Architecture.

Robert N. Trigiano, Major Professor

We have read this thesis and recommend its acceptance:

Gustavo Caetano-Anollés, Brad Reddick, Sue Hamilton

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council:

I am submitting herewith a Thesis written by Mary Catherine Scott entitled "DNA Amplification Fingerprinting of Chrysanthemum". I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Ornamental Horticulture and Landscape Design.

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Accepted for the Council:

Associate Vice Chancellor and
Dean of the Graduate School

DNA AMPLIFICATION FINGERPRINTING OF CHRYSANTHEMUM CULTIVARS

A Thesis

Presented for the

Master of Science Degree

The University of Tennessee, Knoxville

Mary Catherine Scott

December 1995

DEDICATION

This thesis is dedicated to the memory of my Grandma

Mrs. Eleanora Peduto Capozzoli
1907-1985

and Aunt

Mrs. Elaine Capozzoli Eicher
1943-1993

they gave me the love support and the confidence in my own abilities to achieve everything that made this possible.

ACKNOWLEDGMENTS

To my major professor, Dr. R.N. Trigiano, for his support, guidance, patience friendship, for taking such good care of Mary Ann's Daughter and for his extremely warped sense of humor.

To my other committee members, Drs. Gustavo Caetano-Anollés, Brad Reddick and Sue Hamilton, for their time, ideas and suggestions.

The Department of Ornamental Horticulture and Landscape Design for the use of laboratory facilities, materials and equipment. The OHLD secretaries Angie, Joy, Nikki, Kathy and Angela for their always smiling faces.

Dr. Don Williams, Sue, Hanna and Sandra for their friendship, infinite wisdom and support.

Dr. Effin Graham and Vivian for being Bethany Alumni and proud.

My parents and family, especially Ellie, for their endless support encouragement and always being there when I needed them.

Fellow graduate students and labmates for their companionship and helping to maintain my sanity, Lisa Bell, Heather Osborn, Ledare Habare, Olga Ruiz-Marin, Craig Green, Kevin Carter, Tracy Crouse, Terisa Cerny, Randy Krieg, Nicole Cardwell and Carol Dabney. Most importantly to Amy MacKenzie, Kathy, Missy and Gina Malueg for being my surrogate family.

To my betrothed, Dan Williams, for his love, faith in me and unwavering moral support.

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CHAPTER 1

INTRODUCTION

Chrysanthemums, mentioned in the writings of Confucius, were probably cultivated before 500 BC. They were introduced to Europe in 1789 and to the United States after the nineteenth century. These earlier plants bare little resemblance to the multitude of chrysanthemum cultivars available today. Chrysanthemums have become an important horticultural crop through careful breeding and selection for desirable traits such as cold tolerance, flower shape and color, and disease resistance (Smith, 1975).

Chrysanthemum [*Dendranthema grandiflora* Tzelev. or *Chrysanthemum morifolium* Ramat. (synonym) (Anderson, 1987)] belongs to the Asteraceae family because its blooms contain two separate, but closely related floral systems. In the center of the inflorescence is a yellow or green disk composed of both staminate and pistilate flowers. Around the central disk are long pistilate florets of varying color and form that are often wrongly referred to as petals (Smith, 1975).

The National Chrysanthemum Society has developed a detailed classification system for chrysanthemum cultivars based on the characteristics of the inflorescence. First, the florets are segregated into two divisions. Division A contains ray florets with strap like or lingulate corolla, and disk florets are vase shaped and relatively short. Division A has two sections. In Section I, the disk flowers are conspicuous and made up of unmodified florets surrounded by one of more rows of ray florets at right angles to the stem. Section I

includes Single, Semidouble, Regular Anemone, Irregular Anemone and Spoon Anemone Infloresences. In Section II, the disk may be present and concealed or absent. Section II includes Pompon, Regular Incurve or Chinese Incurve, Irregular Incurve, Reflexed or Decorative Pompon, Decorative or Aster-flowered Reflex, Regular or Chinese Reflex and Irregular Japanese Reflex. Division B is based on the presence of tubular flowers. Division B includes Single Spoon, Semidouble and Double Spoon, Quill, Thread and Spider Chrysanthemums (Cumming, 1964).

The genus *Chrysanthemum* contains of over 160 species. The tremendous differences in size, form and color of the familiar chrysanthemum scarcely reveals the origins of this hybrid group. The literature suggests that ornamental chrysanthemum cultivars are the progeny of crosses between *C. indicum* (L.), *C. morifolium* (Ramat.), *C. sibiricum.* (Fisch. ex F. Forbes & Hemsl.), *C. ornatum* (Hemsl.), *C. japonense* (Mak.) and *C. makinoi* (Matsum. & Nakai) (Dowrick, 1953), are daisy-like flowers endemic to Asia (Cumming, 1964).

Cultivated chrysanthemums constitute an important portion of the floral in the United States. In 1991, the combined wholesale value of potted flowering mum, cut mum and garden mum was over one-hundred and fifty million dollars (Agricultural Statistics Board, USDA, 1992). Specialist propagators around the world have provided growers with over one billion cuttings per annum. Knowledge of the photoperiod requirements for blooming chrysanthemums for short days (long nights) has enabled it to be a year-round crop and can now be forced to flower on any targeted date at any latitude throughout the world.

Chrysanthemum is a hexaploid species, and cultivated chrysanthemums are a polyploid hybrid complex comprising six or seven species (Dowrick, 1953). The chromosome

number of cultivated chrysanthemum varieties chromosome numbers vary between 36-75 with most having 54 (Dowrick 1953; Endo, 1969; Nazeer and Koshoo, 1983). Meiotic analysis has shown that bivalents are normally formed suggesting an allopolyploid origin (Dowrick, 1953; Wantanabe, 1977; Nazeer and Khoshoo, 1985). However these types of studies of the species are difficult due to stable self incompatibility reaction and lack of progeny (Drelow et al., 1973). Genetic studies are important in determining if a trait is passed to the next generation in a Mendelian manner. Knowledge of how traits are passed is fundamental to breeding and molecular genetic studies. Whether most characters in chryanthemum are inherited in a disomic (selective chromosome pairing) or a hexasomic way (random chromosome pairing) is not known. There has been evidence for disomic transmission because of bivalent formation, however carotenoid pigmentation appears to be transmitted in a hexasomic way (Langton, 1989).

Due to chrysanthemums allopolyploid origin and apparent self and cross incompatibility, breeding is a process based on patience and luck. New cultivars originate either from seed or from sports (i.e., somatic mutations). However, garden chrysanthemums do not reproduce true to type from seed. The polyploid condition of chrysanthemum and their hybridity make possible numerous combinations of chromosomes and genes (Cumming, 1964).

> "William Duffett, the eminent hybridizer at Yoder Brothers, once hoped to build up pure blood lines of chrysanthemums as breeding aids. By seed reproduction of types true to color, habit, and floral class, he planned to develop a reservoir of talented parents. These would then presumably, pass on their desirable traits to less gifted cultivars. Also, by intercrossing inbred, pure lines, he hoped for notable increase in vigor and size. This technique, successful with corn and host of annuals, has given rise to the noted F1 hybrids. But the complex ancestry of chrysanthemums has stood in the way of pure line-breeding, and Mr. Duffett has been disappointed in his quest." (Cumming, 1964)

Sports arise spontaneously, and while many varieties produce sports continually from the time of their origin, others do so infrequently or never. The sports may in turn give rise to further mutations and in this way whole families or series of sports may be built. Mutations have also been induced with x-ray and gamma radiation. The mutants had changes in chromosome number, loss of pigmentation and deeper flower colors (Dowrick and El-Bayoumi, 1965).

Chrysanthemum Pests and Diseases

From traditional breeding and selection of sports there has arisen forms of chrysanthemum differing in size, shape, color and hardiness. However, little attention has been paid to that of disease and insect resistance. Chrysanthemums are plagued with aphids, spider mites, thrips, rusts, leaf-spot, mildew, bacterial blight and viruses. Disease is a costly problem that can ruin whole crops of chrysanthemums. White Rust, caused by the fungus, *Puccinia horiana* (Henn.), and is an example of a devastating malady of mum that can potentially cause great economic loss. However, there are cultivars resistant to the fungus and it may be possible to develop new resistant cultivars using DNA fingerprinting, conventional breeding techniques and/or genetic transformation.

Puccinia horiana originated in Asia and subsequently spread to Europe and North America beginning in the early 1960s. It is a microcyclic rust with a very short life cycle. After a leaf becomes infected by basidiospores, a visible lesion is formed and teleutospores are produced. These spores germinate in situ and form more basidiospores capable of infecting other chrysanthemum leaves (Dejong and Radermaker, 1986), therefore, crop devastation can quickly escalate.

White rust was first reported in North America in 1978 and was successfully contained to amateur growers in New Jersey (Peterson et al., 1978). It was not reported in a commercial greenhouse in North America until 1992. In January of this year, potted chrysanthemums worth 2.3 million dollars, were destroyed because of white rust in California (Klassen, 1992). These same greenhouses were quarantined for six weeks, and during that time, beds were steam pasteurized and then inspected by the USDA for white fungus before reopening (Neal, 1992).

White rust spores can remain viable on chrysanthemum leaves for up to eight weeks and can also survive on plant debris. Basidiospores are disseminated by wind and may be transported on clothing. Because of this mode of dispersal, white rust was discovered in many greenhouses near to the original site of infection in California (Klassen, 1992). Nurseries that had white rust were required to spray with Eagle™, a new pesticide, once a week for five weeks, to keep white rust from spreading outside the California counties. The nurseries, which had infected plants, were also required to pass USDA inspections prior to any shipping. Growers in the same county, who did not have white rust, were also required to spray with Eagle™ for three consecutive weeks and were inspected prior to shipping plants (Anonymous, 1993). These costly measures were undertaken only after the disease had caused extensive damage. Growers believe that more preventive measures should have been addressed, and the USDA should have inspected imports more closely, especially from countries known to have white rust (Neal, 1992). Other alternative measures would have include using fungicides and resistant cultivars, to prevent the establishment of white rust.

Fungicides can be used each time an outbreak occurs or as preventative treatment. Use of resistant species, however, could have long term benefits. Resistant species can slow or

stop the spread of the disease. When a resistant chrysanthemum cultivar is infected with white rust, the invaded cells die very rapidly and pustules do not develop (hypersensitive response) (Rademaker and DeJong, 1987). New resistant cultivars could be developed by breeding with known resistant cultivars. Unfortunately, it is difficult to transfer this trait into other cultivars using conventional breeding techniques (Rademaker and DeJong, 1987). Advances in genetic engineering have opened new avenues for crop improvement. Genes can now be incorporated into chrysanthemum cells by direct gene transfer or *Agrobacterium*- mediated transformation (Pavingerová et. al., 1994). For example chrysanthemum flower color has already been altered through genetic engineering using an "antisene" chalcone synthase gene (Mol et al., 1990).

DNA Fingerprinting

Advances in molecular techniques have provided means to accurately identify individuals or groups of plants regardless of phenotypic variation that may be present due to environmental conditions (Hubbard, 1992). Restriction Fragment Length Polymorphisms (RFLP) (Botstein et al, 1980), Arbitrarily-Primed Polymerase Chain Reaction (AP-PCR)(Welsh and McClelland, 1990), Random Amplified Polymorphic DNA (RAPD)(Williams et al., 1990), and DNA Amplification Fingerprinting (DAF)(Caetano-Anollés et al., 1991) have all been successful in mapping plant genomes.

RFLP mapping is based on restriction enzyme digestion of DNA. This results in DNA fragments of different lengths which can be separated by electrophoresis and resolved using radioactive labeled or biotintylated DNA probes. While the RFLP method is useful in providing markers for many organisms, the technique has several disadvantages. RFLP

requires a large amount of clean, non-degraded genomic DNA. Prior knowledge of the DNA composition of the species and/or the presence of useful probes is required. Probes are radioactively labeled DNA sequences that anneal to DNA template that has been restricted with enzymes. The time needed to obtain a DNA profile is relatively long, and as with most fingerprinting techniques, the results can be uninformative. Finally, radioactivity and ethidium bromide are hazardous and caution is needed for safe handling and disposal.

AP-PCR, RAPD and DAF utilize the enzymatic amplification of specific DNA sequences. This is accomplished by using a primer that targets a DNA region and initiates DNA synthesis. The DNA region is copied many times by using a temperature cycle that produces DNA fragments. Different primers are used to target different regions of DNA. Nucleotide substitutions, deletions, insertions or inversions can change a priming site or a segment between priming sites. Consequently, the DNA sites may be unable to support amplification. This could abolish, create or change the fragment length, which is known as a polymorphism or Amplification Fragment Length Polymorphism (AFLP) (Caetano-Anollés et al., 1991). The DNA fragments resulting from amplification can be separated using gel electrophoresis. After the gel is developed, the fragments of different lengths can be differentiated as bands. Polymorphisms are detected as missing bands or new bands when compared to preparation from other cultivars, species or organisms. AP-PCR and RAPD use relatively long arbitrary primers and do not produce many bands compared to DAF. DAF, however, uses primers as short as five nucleotides in length that produce up to 100 bands. The more complex banding pattern of DAF yields more information than either AP-PCR or RAPD. Another advantage of DAF is that it uses silver staining, rather than a radioactivity (AP-PCR) or ethidium bromide (RAPD) for detection of amplified products. Silver stained polyacrylamide gels have very high resolution and can be store for

years(Caetano-Anollés et al., 1992).

DAF has been employed to detect genetic differences among animals, plants, fungi and prokaryotic organisms. In plants, DAF has been used to characterize several turfgrasses, soybean cultivars, varieties of rice and inbred lines of maize (Caetano-Anollés et al., 1991) as well as Petunia [*Petunia* spp. Juss.] (Cerny and Starman, 1995) and dogwood (Caetano-Anollés and Trigiano, 1996). DAF is well-suited to identifying chrysanthemum cultivars because they are asexually propagated, i.e., there is no exchange of genetic information between individuals. All vegetative plants or clones in a cultivar are genetically identical, consequently the genetic differences within individual plants will not interfere with the genetic separation of cultivars.

DAF of entire and endonuclease digestion and genomic DNA has been used to identify a markers tightly linked to the supernodulation locus in soybean. By restricting the DNA, either before of after amplification, a more complex and informative banding patterns can be attained. Endonulease digestion is accomplished with restriction enzymes that cut DNA in a specific nucleotide sequences. Many new AFLPs are generated from DAF of digested DNA (Caetano-Anollés et al., 1993). Mini-hairpin primers which are a loop of 3-4 nucleotides and a stem of only 2 terminal nucleotides, can also be used to obtain unique profiles. This structure allows for an extremely short primer of 5 nucleotides, while offering the stability of longer oligonucleotide primers. These new primers produce complex and highly reproducible amplified DNA profiles useful for genome analysis and identity testing (Caetano-Anollés and Gresshoff, 1994).

Rationale

Molecular genetics has only recently been applied to the field of horticulture science. Applications of molecular genetics include variety protection, phylogenic studies, gene mapping and eventually gene transfer.

Cultivars of chrysanthemum have traditionally been distinguished on the criteria of flower type, color, as well as cultural and physiological attributes (flowering requirements, etc.). Although these criteria are invaluable and extensively employed in cultivar identification, they assess only the phenotypic parameters. These parameters are flexible, highly variable and subject to change due to cultural or environmental ambient conditions. Therefore, these measures of differences may not be very accurate for assessing genetic differences or relationships between cultivars.

The ability to identify cultivars genetically is important to the horticulture industry because many significant varieties can be patented. In the past, it has been difficult to prove patent infringement on phenotypically similar plants. Genetic DNA techniques are currently used to identify rose [*Rosa* spp.] cultivars for patent protection (Hubbard et al., 1992; Rajapakse et al., 1992; Torres et al., 1993). Recently, genetic variation in chrysanthemum was studied using RAPD. In this study cultivars could be distinguished from each other, whereas related cultivars within a series could not be distinguished (Wolff and Peters-Van Rijn, 1993). A series is a group of chrysanthemum cultivars generated from an primary cultivar by sports.

Knowledge of the genetic relationships among individuals or populations enables nurseries

to better organize germplasms and to more efficiently sample genotypes. It is important at the inception of a breeding program to possess knowledge of the genetic relationships among the breeding genotypes. This knowledge can be used to complement phenotypic information in the development of breeding populations. Knowledge of the genetic similarity between genotypes may some day facilitate the choice of individuals to cross in hybrid combinations to optimize expression of heterosis (Skroch, 1992). This is especially important in chrysanthemums because they are very difficult to breed. DNA fingerprints can also be used in breeding to see if hybrids are produced or if a specific marker is present in the new individual. This is crucial in breeding long living species such as trees that could take long years to show phenotype.

Both RAPD and DAF have been used to identify a specific locus. RAPD has been used to identify a marker for rust resistance in common bean. Using RAPD on a resistant bean cultivar, as compared to a non-resistant bean cultivar, the AFLP was identified as an marker (Haley et al., 1993). DAF in a similar experiment was used to identify a marker linked to the supernodulation locus (Caetano-Anollés et al., 1993). Theoretically the same approach, using DAF, applies to chrysanthemum and for instance, the white rust locus. White rust resistance is thought to be controlled by a single dominant gene (DeJong and Rademaker, 1985). Chrysanthemums have six copies of each chromosome (hexaploid). If one or more copies contain the dominant gene for resistance, the plant is completely resistant to the white rust fungus (Rademaker and DeJong, 1987). Consequently DAF of resistant chrysanthemum cultivars compared to non-resistant chrysanthemum cultivars might produce AFLPs linked to white rust resistance. However, this is a lengthy and tedious process especially in a plant where no molecular markers are available.

Two approaches to doing marker studies are either by using Near Isogenic Lines (NILs)

(Martin et al., 1991; Reiter et al., 1992) or Bulked Segregant Analysis (BSA) (Michelmore et al., 1991). Near isogenic lines are made when a donor line P1, is crossed to a recipient line, P2. The resulting F1 hybrid is then back crossed to the P2 recipient. From the backcrossed generation, individuals are chosen that contain the gene of interest based on phenotype and then those individuals are backcrossed to the P2 for several additional generations. Eventually an individual is produced with most of the genes derived from P2, except for a small segment carrying the gene of interest. Fingerprinting techniques can be used to find a marker that is linked to the gene of interest. Bulked segregant analysis requires a breeder to hybridize a P1 and P2, the resulting F2 generation will segregate for alleles from both parents. The F2 population is then divided into two pools of contrasting individuals based on the phenotype of interest. The two pools should only differ in their allelic content only at loci contained in the chromosome region close to the target gene (Tanksley et al., 1995). Again that gene can be located using DNA fingerprinting techniques. Since chrysanthemum produce sports and cultivars only differ in color, comparison of cultivars within a series may produce markers linked to color. However, it is not known if the differences arise from point mutation, deletions or inversions (Wolff and Peters-Van Rijn, 1993).

The first step to applying DNA fingerprinting to the chrysanthemum industry in a practical and profitable way, is to separate closely related cultivars for cultivar identification and marker analysis. Twenty-one cultivars divided into six series were selected for DAF screening. Furthermore, DNA from all cultivars within a series was mixed together in equal amounts to create banding profiles useful in marker analysis. The mixed DNA was called bulked DNA and may provide unique banding profiles that are characteristic of a series.

CHAPTER 2

DNA AMPLIFICATION FINGERPRINTING (DAF) IDENTIFIES CLOSELY RELATED CULTIVARS IN SIX SERIES OF CHRYSANTHEMUM

Abstract

The genetic distance of closely related cultivars of chrysanthemum (*Dendranthema grandiflora)* was assessed using DNA Amplification Fingerprinting (DAF). Twenty-one cultivars of chrysanthemum included in the study were members of the following series: Anne (3), Blush (3), Boaldi (2), Charm (5), Davis (4), and Pomona (4). The genetic variability of these cultivars within and between series were evaluated using eleven arbitrary octamer primers. A few polymorphic loci were found that uniquely identified closely related cultivars within a series. In contrast, many polymorphisms were observed between members of different series. Genetic distances between cultivars were evaluated using UPGMA (Unweighted Pair Group Cluster Analysis Using Arithmetic Means) and Principal Coordinate Analysis. The average genetic distance between series was ten fold greater than between cultivars within a series. DNA from all cultivars belonging to a series were also bulked to generate DNA profiles containing unique amplified products for each series. Polymorphic loci that were generated by the DAF technique can possibly be utilized for patent protection, phylogenic studies and for identification of useful markers in breeding.

Introduction

Floriculture is a billion dollar industry and chrysanthemum [*Dendranthema grandiflora* Tzelev or *Chrysanthemum morifolium* Ramat (synonym) Anderson (1987)] is valued at more than 150 million dollars in the United States alone (Agriculture Statistics Board, USDA, 1992). Despite the economic value of floral products, the industry has not employed molecular studies of breeding and molecular techniques for variety/cultivar protection rights in many instances. The application of molecular genetics technology to characterize ornamental floriculture crops has only been tried with a few species including roses [*Rosa* spp.](Hubbard et al., 1992; Rajapakse et al., 1992; Torres et al., 1993), chrysanthemum (Wolff and Peters-Van Rijn, 1993 and Wolff et al., 1994) and petunia [*Petunia* spp. Juss.] (Cerny and Starman, 1995). Some existing applications of molecular genetics include patent protection, gene mapping, marker-assisted selection and genetic engineering.

There are at least two general techniques for molecular fingerprinting: Restriction Fragment Length Polymorphism (RFLP) and a multitude of related methods based on the Polymerase Chain Reaction (PCR). RFLP technology utilizes restriction endonucleases that are able to recognize specific DNA base pair sequences and cut the DNA at these sites. After highly purified (clean) DNA is restricted or digested by the enzyme(s), the various length fragments are separated on an agrose gel medium by electrophoresis. The fragments or markers can be diagnostic for cultivars or, in some instances, genetic traits. In contrast, the PCR technique involves using a DNA polymerase that makes multiple copies of either highly specified or arbitrary targeted areas of the genome. Among the various PCR based amplification procedures that use arbitrary primers or Multiple Arbitrary Amplicon Profiling

(MAAP) techniques are Arbitrarily-Primed PCR or AP-PCR (Welsh and McClelland, 1990) Random Amplified Polymorphic DNA or RAPD (Williams et al., 1990), and DNA Amplification Fingerprinting or DAF (Caetano-Anollés et al., 1991). All of the PCR processes target or amplicons that can be separated by gel electrophoresis as DNA fragments of specific length, and can be used in a variety of genetic studies including breeding, mapping and selection.

RFLP and RAPD fingerprinting techniques have recently been applied to the chrysanthemum species for the purpose of Marker-Assisted Selection (MAS). RFLP's probes have been developed for chrysanthemums for future genetic variability studies. To simplify genetic analysis, locus-specific PCR primers were also developed for MAS (Wolff et al., 1994). However, the RFLP technique is relatively laborious and not-well suited for studies of a large number of samples (Williams et al., 1990). RAPDs have been employed to assess genetic variability in the chrysanthemum species and closely related cultivars of chrysanthemum. Cultivars, which were somatic mutants of a previous variety, could not be distinguished from the parent or other cultivars within the series using RAPD (Wolff and Peters-Van Rijn, 1993b). Many new cultivars of chrysanthemum originate via somatic mutation (Dowrick and El-Bayoumi, 1966) and consequently, there seems to be a need for a more sensitive or discriminating technique that can differentiate closely related or vegetatively propagated cultivars.

Another PCR based technique, DAF, uses very short primers to produce relatively complex DNA profiles. The primers are usually 7 or 8 nucleotides (nt) in length, but can be as short as 5 nt. These profiles can contain Amplification Fragment Length Polymorphisms (AFLP's) that may be used to identify individuals (Caetano -Anollés et al., 1991). It has been successfully employed in the identification of closely related organisms including

viruses, bacteria, fungi, plants and humans (Caetano-Anollés et al., 1993).

The ability to identify cultivars using DNA is important to the horticulture industry because many varieties are patented. In the past, it has been difficult to prove patent infringement because there was not a reliable technique that could accurately differentiate between phenotypically similar plants that were different only minimally (Hubbard et al., 1992). Fingerprint techniques have been used to identify rose [*Rosa* spp.] cultivars for patent protection (Hubbard et al., 1992; Rajapakse et al., 1992; Torres et al. 1993) and progenoration species of petunia cultivars [*Petunia x Hybrida* Hort.] (Cerny 1995).

A phylogenic analysis of cultivars of chrysanthemum would be beneficial to breeders since genetic relationships can be used to complement phenotypic information in the development of breeding populations (Skroch et al., 1992). Knowledge of the genetic relationships and phylogeny among individuals or populations enables breeders to more efficiently sample genotypes (Niehuis et al., 1993). Chrysanthemums are generally self-incompatible and hexaploid, which makes traditional genetic analysis through cross-breeding difficult (Drewlow et al., 1973). Chrysanthemums could be sampled for parents that would make good breeding pairs because of their genetic diversity.

Marker-assisted selection has received considerable attention in recent years as an important tool for the improvement of major-gene disease and insect pest resistance in crop plants (see review by Melchinger, 1990). RFLP and RAPD have been used to identify and locate useful genes. To find specific genes, it is necessary to have a technique sensitive enough to differentiate closely related plants. In order to identify genomic regions linked to the gene of interest, it is necessary to differentiate pairs of backcross-derived nearly isogenic lines (NILs) (Young et al., 1988; Tanksley et al., 1995). The basic objective is to identify

markers located in the linkage block that surround the gene of interest (Melchinger, 1990).

The cultivars of chrysanthemum included in this study belong to six different series. Each cultivar in a series is a sport or somatic mutant that arose from another cultivar within that series. A working hypothesis would be that only a few AFLP's will be identified within a series comparison, but a greater number would be expected in between series comparisons. In order to patent cultivars and perform marker, mapping and breeding studies, a method sensitive enough to differentiate cultivars within series would be helpful. Chrysanthemum cultivars are especially suited for this type of research because they are propagated asexually via cuttings. Therefore, all plants in a cultivar are considered to be clones and genetically identical. Consequently, genetic differences should not exist between individual plants that might interfere with the identification of cultivars. This is an advantage over sexually reproduced plants, especially obligate outcrossers such as chrysanthemum. The information generated by the various fingerprinting techniques can serve many practical purposes including patent protection, phylogenic analysis, breeding studies and marker-facilitated selection. In this case, many individuals must be examined to realize the potential range of genetic variability in the population. The first step is to evaluate the capability of DAF to assess genetic variability of closely related cultivars of chrysanthemum and to develop markers that would be useful in identifying cultivars and series of chrysanthemum.

Materials and Methods

Plant material

Rooted cuttings of 21 chrysanthemum cultivars (Table 1) were obtained from Yoder Brothers, Inc. (Barberton, Ohio). All of the cultivars were a non-hardy pot type chrysanthemum. Cultivars in the Anne, Boaldi, Charm and Pomona series have a decorative type flower, whereas the Davis and Blush series cultivars have a daisy type flower. The plants were grown in the laboratory to prevent insect infestation, disease and to limit carbohydrate accumulation that could possibly interfere with DNA extraction. The plants were maintained vegetatively under fluorescent and incandescent lights ($\approx$ 75 $\mu mol \cdot sec^{-1}\ m^{-2}$) using a 16 hour light: 8 hour dark cycle. Plants were pinched twice a month, watered once a week and fertigated once a week with Peters® 20-10-20 (300 ppm). In addition, terminal cuttings were made and rooted twice a year to maintain the vigorously growing plants.

Experimental design

Amplified DNA products from each cultivar of a series were separated electrophoretically on an individual gel along with one cultivar from two other series used as outliers. DNA was extracted from three individual plants of three cultivars to determine if the DNA profile was identical between plants of the same cultivar. Additionally, DNA from all the cultivars in a series were bulked together using DNA extracted from each cultivar mixed together in equal amounts or alternatively, plant leaves from each cultivar were mixed together in equal weights and then DNA was extracted. The bulking methods of combining DNA of a series were compared using several primers and run on a gel for between series comparison. A total of twenty-five primers were screened; eleven primers were chosen to fingerprint the

Table 1. Chrysanthemum Series and Cultivars

Series	Cultivar
Anne	Bright Golden Anne
	Cream Yellow Princess Anne
	Peacock
Blush	Blush
	Coral Blush
	White Blush
Boaldi	Boaldi
	Yellow Boaldi
Charm	Charm
	Coral Charm
	Dark Bronze Charm
	Dark Charm
	Salmon Charm
Davis	Davis
	Coral Davis
	Light Davis
	Regal Davis
Pomona	Pomona
	Cherry Pomona
	Coral Pomona
	Dark Pomona

21 cultivars. Amplifications from the fourteen additional primers were not included in the interpretation of this study because the most of those primers did not support amplification with the chrysanthemum DNA and a few primers produced products that could not be visualized without modifying the electrophoresis technique. For each of the eleven primers, at least three different gels were run for each cultivar comparison. Each of the amplifications used a DNA template from a separate extraction to demonstrate that consistent profiles could be obtained.

Tissue collection

Young, not yet fully expanded light green leaves were selected for DNA extraction because they have a greater percentage of DNA and do not contain as many secondary plant products (e.g. phenols) as older leaves that can make DNA extraction more difficult and less efficient. Three to six leaves, weighing approximately 50 mg, were used for each extraction. All tissue was collected after the dark period to reduce interference of polysaccharides with DNA recovery. Leaves were periodically collected and placed in individual whirl-pacs and stored in a -70°C freezer for future use.

DNA extraction

The DNA was extracted using the method of Yoon et al. (1991) as modified by Trigiano et al. (1995). Approximately 50 mg of chrysanthemum leaves were placed in a pre-chilled mortar. Leaves were frozen with liquid nitrogen and ground to a powder with a pestle. One-hundred milligrams of polyvinyl-polypyrrolidone was added to the leaf powder then ground again with a pestle under liquid nitrogen. One ml of Yoon et al. (1991) extraction buffer [50 mM Tris-HCl, pH 7.2; 50 mM EDTA; 3% sodium dodecyl sulfate; and 1% 2-mercaptoethanol] was added to the powder and allowed to thaw at room temperature. The extraction buffer lysed the cell membranes and released the DNA. The resultant slurry was

divided equally between two sterile 1.5 ml Eppendorf tubes placed in a 65°C water bath for one hour and the contents mixed by inversion at 20 minute intervals. The remaining volume in the Eppendorf tube was filled to capacity with chloroform, inverted several times and centrifuged for 15 minutes. The supernatant was then transferred to a freshly autoclaved 1.5 ml Eppendorf tube. This procedure separated the DNA from large particles of unwanted plant material and was repeated several times until the fluid was clear. Next, 35 µl of 4°C, 3M sodium acetate was added for every 200 µl of extract, then centrifuged for 5 minutes. The supernatant was collected and placed in a fresh sterile tube and 500 µl of -20°C absolute ethanol was added for every 200 µl of sample to precipitate the DNA. The tube was inverted gently several times and placed in a -20°C freezer for a minimum of 30 minutes. The sample was centrifuged at 14,000 X g for 10 minutes and the supernatant discarded. The remaining pellet was rinsed with 4°C 70% ethanol and centrifuged for 5 minutes. The alcohol was discarded and the residual removed by blotting the Eppendorf tube on kim-wipes. After the pellet was dried, it was redissolved in 100 µl of room temperature TE buffer [10 mM Tris-HCl and 0.1 mM EDTA, pH 7.2]. A 65°C water bath was sometimes used to facilitate dissolving the DNA. One hundred and fifty µl of chloroform was added to the extract and the contents vortexed gently and centrifuged for 10 minutes. The supernatant was transferred to a sterile 1.5 ml Eppendorf tube and 100 µl of TE buffer and 100 mg of CsCl added and dissolved. The extract was then centrifuged for 5 minutes. The pellet was discarded and the supernatant was transferred to a sterile 1.5 ml eppendorf tube. An additional 300 µl of TE buffer and 1.0 ml of cold absolute ethanol was added. The tube was then stored at -20°C for a minimum of 30 minutes and then centrifuged for 15 minutes. Then, the supernatant was discarded and the pellet rinsed with 4°C 70% ethanol and centrifuged for an additional 5 minutes. The ethanol was discarded and the pellet air-dried. Finally, 35 µl of autoclaved distilled water was added, the pellet redissolved and stored at 4° C overnight.

DNA Quantification

The extracted DNA was quantified using a micro assay technique on a TKO 100 Dedicated Mini Fluorometer. A working dye solution was made with 100 ml 1X TNE working buffer [10 mM Tris, 1 mM EDTA, 2M NaCl, pH 7.4] and 10 µl of Hoechst 33258 stock [10 mg Hoechst 33258, 10 ml H_2O]. The fluorometer was calibrated with standard of 100 ng/µl calf thymus DNA. The chrysanthemum DNA extraction was quantified by pipetting 2 µl of the extract into 2 ml of working dye solution and the amount of DNA (ng/µl) determined on the fluorometer (Hoefer Scientific Instruments, San Francisco, CA). After fluorometric analyses, DNA stocks for amplification were diluted with autoclaved nanopure water to yield a final concentration of 0.5 ng/µl.

The extraction procedure consistently yielded between 1.75-8.75 ng DNA per 50 mg of leaf tissue. The Yoon et al., (1991) method was developed for DNA extraction from fungi. Chrysanthemum DNA yields were 5 to 10 times higher than fungi yields using this method. The modified Yoon extraction (Trigiano et al., 1995) is considerably less complicated than traditional plant DNA extractions, for example Dellaporta et al., (1983), nevertheless the Yoon procedure provided ample DNA suitable for DNA Amplification Fingerprinting.

DNA amplification

DNA was amplified using the method of Caetano-Anollés et al. (1991b). Initially, 25 octamer primers were screened. The 11 primers and their sequences that gave sound amplifications are shown in Table 2. Amplification components were added to the individual autoclaved 0.6 ml eppendorf tubes in the following order: 4 µl autoclaved nanopure water; 1 µl TTNK10 reaction buffer [20 mM Tris-HCl; 0.1% Triton X-100; 4mM $(NH_4)_2 SO_4$; 10 mM KCl]; 1 µl deoxyribonucleotides [2 mM of each dNTP]; 0.6 µl

Table 2. Primer codes and sequences

Primer code	Sequence 5' ---> 3'
8.6A	GA GCC TGT
8.6D	GT AAC GCC
8.6F	GA TGC AGG
8.6H	GA AAC GCC
8.6I	GT TAC GCC
8.6J	GT ATC GCC
8.6M	GT AAC CGC
8.7A	AA TGC AGC
8.7D	CC GAG CTG
8.7F	CG TGG TGG
8.9A	CG CGG CCA

First number denotes number of bases and the second, percentage of GC content.

TTNK10 $MgSO_4$ [25 mM]; 0.4 µl DNA Polymerase [10 units/µl, Ampli-*taq* Stoffel fragment (Perkin-Elmer/Cetus)]; 1 µl primer [30 µM (Table 2)]; 2 µl 0.5 ng/µl DNA template, for a final volume of 10 µl (Caetano-Anollés et al., 1991a). A master mix was prepared containing all the components except DNA template. The 2 µl of template and 8 µl of master mix was pipetted into each 0.6 ml eppendorf tube individually.

The mixture was vortexed briefly, centrifuged and a drop of heavy white mineral oil (Paraffin oil, Liquid Petrolatum) added to prevent evaporation of the mixture in the thermocycler. The Twin Block System, Easy Cycler (Ericomp Inc., San Diego, Ca) was programmed in a two step cycle, without an extension phase, 10 seconds at 96°C and 10 seconds at 30°C. Thirty-five cycles, each cycle requiring about 5.5 minutes, were completed for each amplification. Amplified DNA was pipetted from under the oil making sure that no oil remained with the solution and placed in a fresh 0.6 ml eppendorf tube. The amplified products were diluted, if necessary, with 10 µl of sterile nanopure water and stored at 4°C.

DNA electrophoresis

The amplified products were separated electrophoretically on 5% polyacrylamide gels backed on GelBond PAG polyester film (FMC, Rockland, Maine) using a Mini-Protean apparatus. The gel was prepared by mixing 4.2 g of urea (Biorad ®), 2 ml of 5X running buffer [60.6 g trizma base 0.5 M, 25.7 g Boric acid (0.42M), and 1.86 g $EDTANa_2 \cdot H_2O$ diluted to 1 liter with nanopure water]; 1.2 ml of 38% acrylamide and 2% PDA, and enough water to bring the meniscus of the solution to the 10 ml mark in a 20 ml beaker. A stir bar was added and the mixture placed on a magnetic stir plate. When the urea was dissolved, 15 µl of TEMED and 150 µl of a 10% ammonium persulfate solution were

added to polymerize the acrylamide. After an additional 10 seconds of mixing, the solution was loaded into a syringe and filtered through a 0.45 μm acrodisc to remove particulates. The acrylamide solution was then delivered to the Mini-Protean apparatus and 10 well Teflon combs put in place.

The gel apparati were mounted onto the electrode core and placed in a buffer tank after the acrylamide had polymerized (approximately 25-30 minutes). The tank and apparatus reservoir was filled with 1X running buffer, the combs removed and the wells cleaned with 1X running buffer with a 1cc tuberculin syringe. The gels were pre-run at 120 V for five minutes while the DNA samples were mixed.

Each individual amplified DNA sample (3 μl) was mixed with 3 μl of loading buffer [6g urea, 4 mg xylene cyanol FF in 5 ml nanopure water] in a clean 96 well titer plate. The biomarker® (1:9) [molecular base pairs: 1000, 700, 525, 500, 300, 200, 100 and 50] was also mixed in the same fashion on the plate. Next, the wells in the gel were cleaned again with 1X running buffer. The samples were loaded with flat ended pipette tips from the 96 well titer plate into the individual wells. Each DNA sample, in conjunction with the well they were loaded into was carefully documented. Amplification products were separated by electrophoresis at 120 volts for approximately 90 minutes or until the tracking dye reached the level of the bottom electrode. The gels were then carefully removed from the apparatus under deionized water.

Silver staining

DNA amplification fragments were visualized using a fast and sensitive silver stain (Bassam et al., 1991) as modified by Trigiano (unpublished). This stain detects about 1 pg DNA/mm^2 band cross section. The gels were placed in individual containers on a rotary

shaker for the entire staining process. First, gels were fixed in 7.5% acetic acid for 10 minutes and then the gel was rinsed with nanopure water three times for two minutes each. Then, the gels were then soaked in the silver stain [1g/l silver nitrate, 3.45 ml/l 16% EM grade formaldehyde] for 30 minutes, and then rinsed quickly in nanopure water. The stain was developed using 4°C 30 g/l AC grade sodium carbonate, 2.5 ml/l 16% EM grade formaldehyde, 20 mg/l sodium thiosulfate. The developer was removed after the bands had developed, but before the edges of the gel started to discolor. The development was halted by rinsing the gels in 4°C 7.5% acetic acid and after 5 minutes rinsed in nanopure water until the acetic acid odor could no longer be detected.

Data analyses

Amplification products (bands) from single primers, representing all isolates, were examined on a light box. Bands of 700 bp or less were scored as either present (1) or absent (0) (See Appendix for raw data). At least two gels were compared to assure the profiles were consistent and to ensure a minimum of artifactual data.

All Statistic Analyses were performed using NTSYS-pc Numerical Taxonomy and Multivariate Analysis System for PC, version 1.70 created by F. James Rohlf, Exeter Software, Serauket, N.Y. For Principle Coordinate Analyses, Dice similarity coefficients were calculated and then aligned using the double center option with distances squared. Eigenvectors were calculated from the transformed matrix using the square root (lambda) scaling option. The results were graphic displayed without normalization of scales. Genetic similarity matrices (Dice coefficients) were computed and cluster analysis performed using the UPGMA option set for a maximum of 25 tied trees. Dice coefficients were arbitrarily chosen from a group of similarity coefficients available in the NTSYS program.

The Dice coefficients were calculated using the following equation:

$$2a/(2a+b+c)$$

where: a=bands present in both taxa; b and c = band present in one taxum and not the other and vice versa; lack of bands are not considered.

Results and Discussion

The DAF technique produced highly consistent and relatively complex DNA fingerprints compared to other fingerprinting techniques. Reproducibility of results with all template/primer combinations was verified. Each amplification profile was repeated with the same primer from two to six independent DNA isolations during 2 years of this study. Despite the variability in staining intensity and crossectional area of some amplification products in some of the repeated profiles, the overall profile patterns remained consistent between independent DNA isolations and amplifications for all template/primer combinations. Experiments have shown that mobility and detectability of a band generated by the DAF method is highly reproducible in this study as well as others (Trigiano et al., 1995; Gresshoff and MacKenzie, 1994).

Clonal fidelity within a series was determined for several cultivars of chrysanthemum. DNA was extracted and amplified from three different plants of each of the cultivars Salmon Charm, Light Davis and Pomona. Polymorphic DNA sequences between individual plants of the same cultivar were not detected by any of the eleven primers (Figure 1). The individual plants of each cultivar are asexually propagated by cuttings and therefore, DAF profiles should not have contained polymorphisms between plants that should be genetically identical or clonal. These results (no polymorphisms) support the

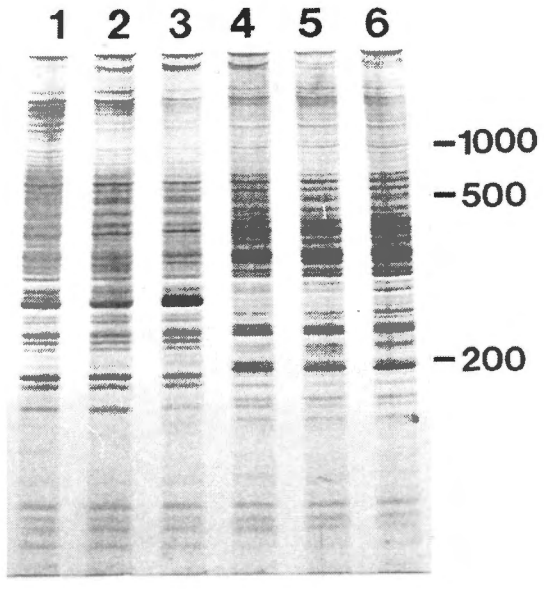

Figure 1. Clonal DAF profiles generated with primer 8.6A (GA GCC TGT) for three individual plants of Salmon Charm (lanes 1-3) and three individual plants of Light Davis (lanes 4-6). Polymorphisms were not detected for vegetatively propagated or clonal cultivars.

decision to represent each individual cultivar in the series with a few plants and alleviates the need to bulk DNA to detect variability within cultivars as was necessary for genetically heterogenic Petunia cultivars (Cerny and Starman, 1995).

Eleven octamer primers were used to amplify genomic DNA from twenty-one chrysanthemums cultivars. The number of bands produced for each primer/template (DNA) varied from 13 to 41 with a mean of 28 bands. Over 250 bands for each series were scored at or below 700 base pairs and many additional bands of higher molecular weight were not considered because they were too difficult to read with consistency using the electrophoresis conditions of these studies. Polymorphic DNA was found that could identify or discriminate between discrete cultivars within series. There were 13 AFLPs for Anne, 7 for Charm, 11 for Davis and 15 for Pomona (Table 3). Between series comparisons with bulked DNA yielded 113 AFLPs. Some primers, such as CC GAG CTG, detected AFLPs between series, but did not amplify any polymorphic DNA regions within series (Figures 2 and 3). In contrast, primers GA TGC AGG and GT ATC GCC yielded AFLPs between cultivars within series of chrysanthemum in all series tested.

Polymorphic DNA was detected in DAF profiles of the Anne series, which included the cultivars Bright Golden Anne, Cream Yellow Princess Anne and Peacock. Polymorphisms between the cultivars were produced by the primers GA GCC TGT, GT AAC GCC, GA TGC AGG (Figure 4), GT TAC GCC (Figure 5) and GT ATC GCC (Figure 6). All polymorphisms were located between 100-300 base pairs. Bright Golden Anne and Cream Yellow Princess Anne can be uniquely identified with bands at 165 and 175 bp bands produced by primer GT AAC GCC; respectively, whereas Peacock can be distinguished by bands at 160 and 115 bp products generated by primer GA TGC AGG. Specific diagnostic bands that can be used to identify cultivars of the Anne series can be found in Table 3.

Table 3: Amplified Fragment Length Polymorphisms (AFLPs) within four series of chrysanthemum

Series	Primers										
	8.6A	8.6D	8.6F	8.6H	8.6I	8.6J	8.6M	8.7A	8.7D	8.7F	8.9A
Anne	250 (110)[a] 160 (110)	180 (101) 175 (010) 165 (100)	240 (110) 160 (001) 115 (001)	---[b]	260 (100)	290 (011) 200 (010) 155 (010) 140 (011)	---	---	---	---	---
Charm	---	---	270 (01100)[c] 200 (00100) 200 (00100) 180 (00100) 145 (01100) 140 (00010)	---	---	250 (00101)	---	---	---	---	---
Davis	160 (0010)[d]	195 (1101)	210 (1010) 200 (1000)	---	---	315 (1001) 250 (1100) 245 (1011) 240 (0100) 215 (1011) 210 (1101) 130 (1101)	---	140 (0100)	---	---	---
Pomona	205 (0111)[e] 200 (0001) 140 (0110) 135 (1100) 130 (0101)	270 (1110)	215 (0010) 210 (1000) 190 (1000) 140 (1000)	---	310 (1000) 270 (1101) 210 (1101) 200 (1100)	225 (0100) 200 (0101)	---	---	---	---	---

a: first number = base pairs; second number: 1 = present, 0 = absent; Order of cultivars in Anne: Bright Golden Anne, Cream Yellow Princess Anne and Peacock
b: no polymorphisms
c: first number = base pairs; second number: 1 = present, 0 = absent; Order of cultivars in Charm: Charm, Coral Charm, Dark Bronze Charm, Dark Charm and Salmon Charm
d: first number = base pairs; second number: 1 = present, 0 = absent; Order of cultivars in Davis: Coral Davis, Davis, Light Davis and Regal Davis
e: first number = base pairs; second number: 1 = present, 0 = absent; Order of cultivars in Pomona: Cherry Pomona, Coral Pomona, Dark Pomona and Pomona

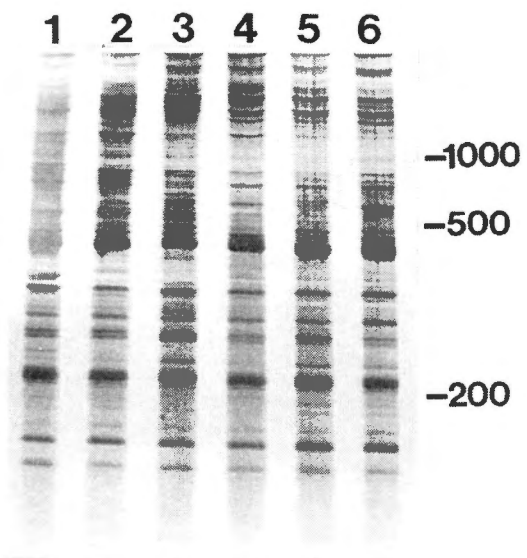

Figure 2. Polymorphic DAF profile of chrysanthemum series generated with primer 8.7D (CC GAG CTG). Lanes 1-6 in order, series Anne, Blush, Boaldi, Charm, Davis and Pomona. Numbers on right indicate base pairs.

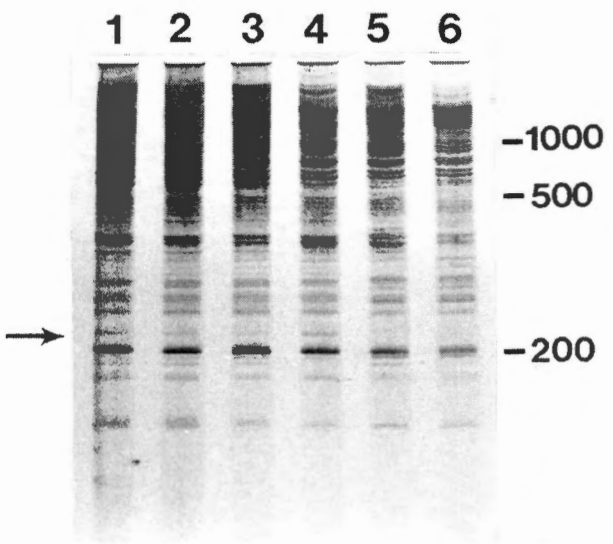

Figure 3. DAF profiles generated with primer 8.7D (CC GAG CTG) for chrysanthemum Davis series and outliers. Lanes 1-6 in order, cultivars Coral Davis, Davis, Light Davis, Regal Davis, Charm and Pomona. Arrow indicates polymorphism between series; there were no polymorphisms within the series. Numbers on right indicate base pairs.

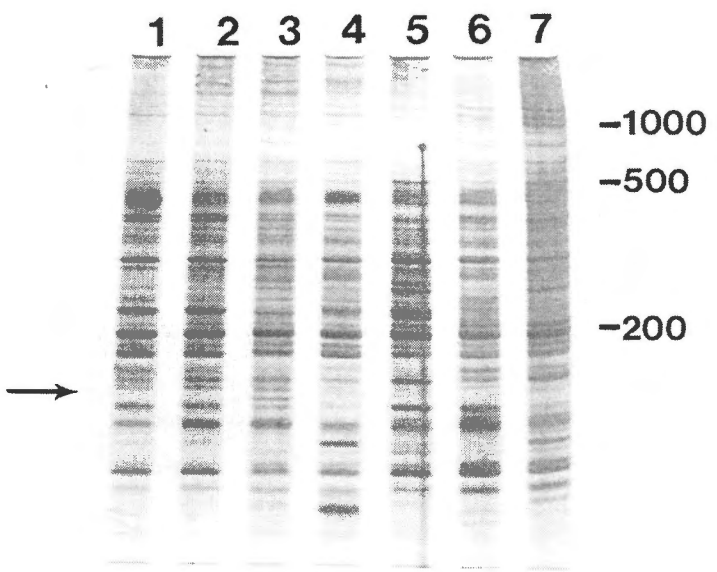

Figure 4. Polymorphic DAF profiles generated with primer 8.6 F (GA TGC AGC) for chrysanthemum Anne series and outliers. Lanes 1-7 in order, cultivars Bright Golden Anne, Cream Yellow Princess Anne, Peacock, Blush, Boaldi, Charm and Davis. Arrow indicates polymorphism. Numbers on right indicate base pairs.

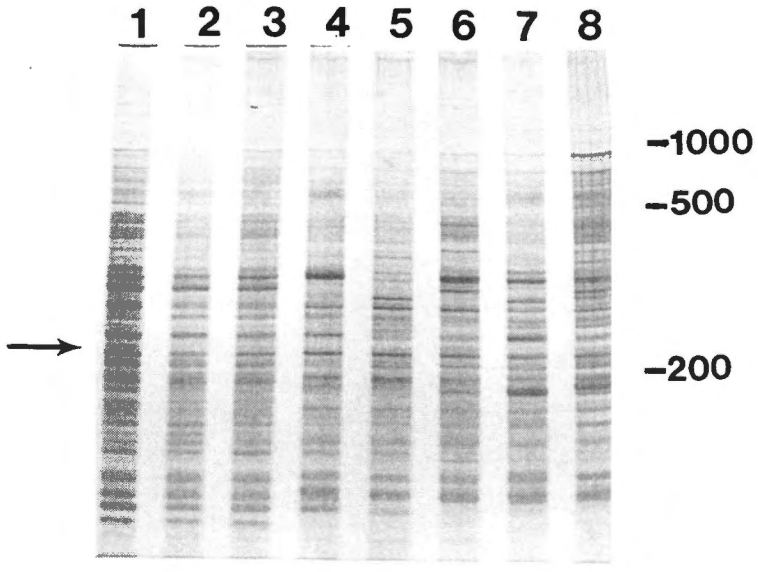

Figure 5. Polymorphic DAF profiles generated with primer 8.6l (GT TAC GCC) for chrysanthemum Anne series and outliers. Lanes 1-8 in order, cultivars Bright Golden Anne, Cream Yellow Princess Anne, Peacock, Blush, Boaldi, Charm, Davis and Pomona. Arrow indicates polymorphism. Numbers on right indicate base pairs.

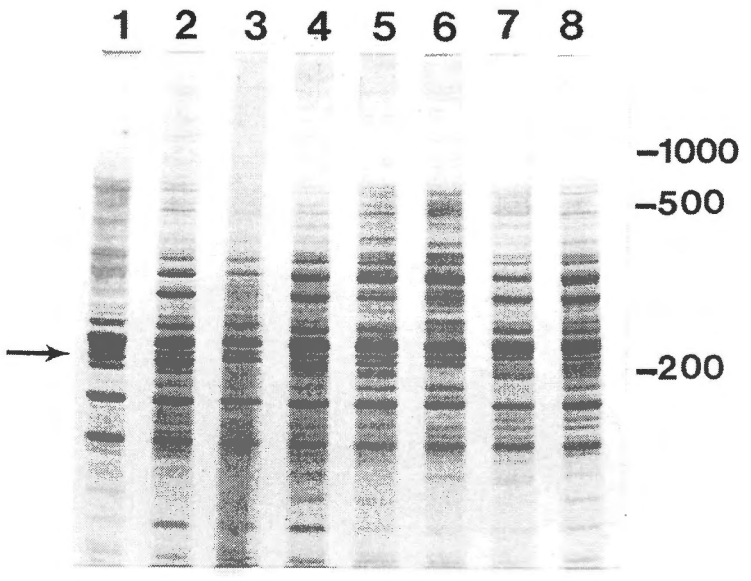

Figure 6. Polymorphic DAF profiles generated with primer 8.6J (GT ATC GCC) for chrysanthemum Anne series and outliers. Lanes 1-8 in order, cultivars Bright Golden Anne, Cream Yellow Princess Anne, Peacock, Blush, Boaldi, Charm, Davis and Pomona. Arrow indicates polymorphism. Numbers on right indicate base pairs.

Polymorphic DNA was detected in DAF profiles of the Charm series, which included the cultivars Charm, Coral Charm, Dark Bronze Charm, Dark Charm and Salmon Charm. Polymorphisms between the cultivars were produced by the primers GA TGC AGG (Figure 7) and GT ATC GCC. A total of 270 loci were scored for the Charm series, all polymorphisms were located between 100-300 base pairs. Dark Bronze Charm and Dark Charm can be uniquely identified within the series at 200, 180 and 140 bp the bands were produced by primer GA TGC AGG. Diagnostic bands for distinguishing cultivars are listed in Table 3.

Polymorphic DNA was detected in DAF profiles of the Davis series, which included the cultivars Davis, Coral Davis, Light Davis and Regal Davis. Polymorphisms between the cultivars were produced by the primers GA GCC TGT, GT AAC GCC (Figure 8), GA TGC AGG (Figure 9), GT ATC GCC (Figure 10) and AA TGC AGC. A total of 278 loci were scored for the Davis series, all polymorphisms were located between 100-320 base pairs. Light Davis can be uniquely identified within the series at 160 bp with a band produced by GA GCC TGT; respectively, whereas Coral Davis can be distinguished by the 200 bp product generated by primer GA TGC AGG. Additional, diagnostic bands for distinguishing cultivars are listed in Table 3.

Polymorphic DNA was detected in DAF profiles of the Pomona series, which included the cultivars Pomona, Cherry Pomona, Coral Pomona and Dark Pomona. Polymorphisms between the cultivars were produced by the primers GA GCC TGT, GT AAC GCC, GA TGC AGG (Figure 11), GT TAC GCC and GT ATC GCC. A total of 283 loci were scored for the Pomona series, all polymorphisms were located between 100-320 base pairs. Dark Pomona and Cherry Pomona can be uniquely identified within the series at 215 and 190 bp the bands were produced by primer GA TGC AGG; respectively, whereas

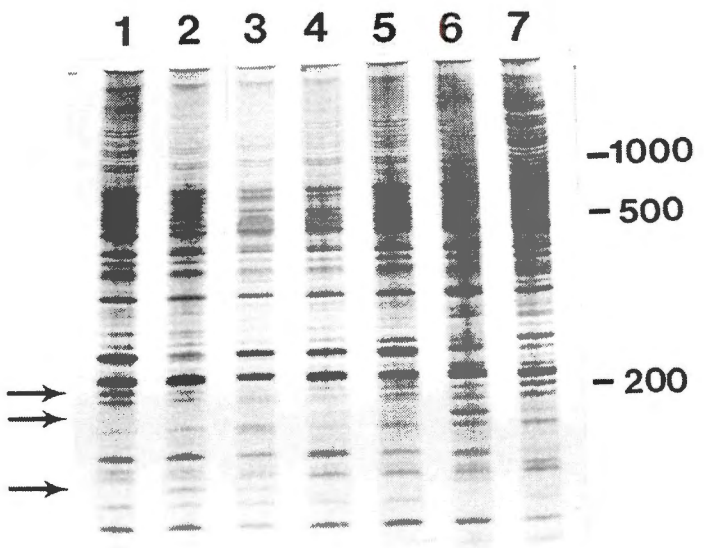

Figure 7. Polymorphic DAF profiles generated with primer 8.6F (GA TGC AGG) for chrysanthemum Charm series and outliers. Lanes 1-7 in order, cultivars Charm, Coral Charm, Dark Bronze Charm, Dark Charm, Salmon Charm, Davis and Pomona. Arrow indicates polymorphism. Numbers on right indicate base pairs.

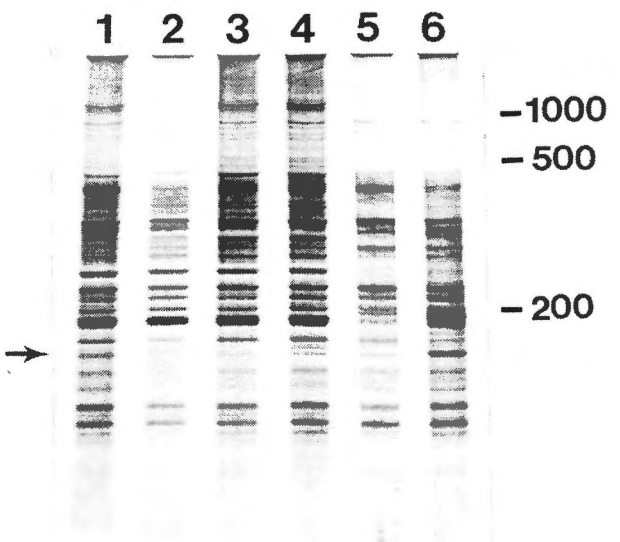

Figure 8. Polymorphic DAF profiles generated with primer 8.6D (GT AAC GCC) for chrysanthemum Davis series and outliers. Lanes 1-6 in order, cultivars Coral Davis, Davis, Light Davis, Regal Davis, Charm and Pomona. Arrow indicates polymorphism. Numbers on right indicate base pairs.

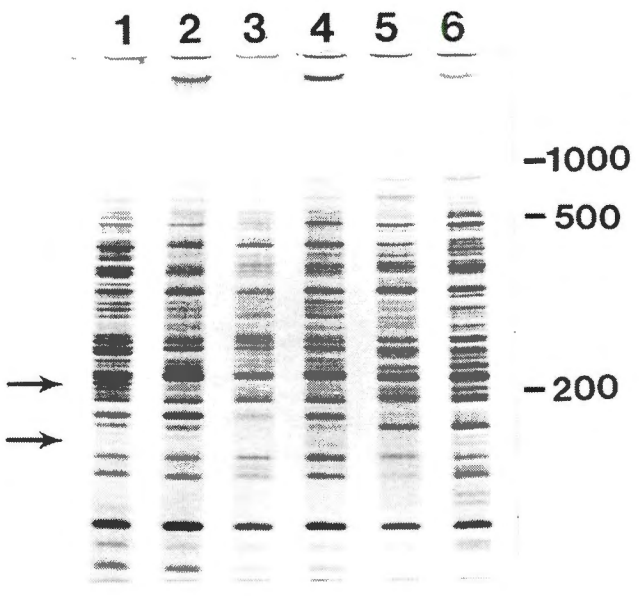

Figure 9. Polymorphic DAF profiles generated with primer 8.6F (GA TGC AGG) for chrysanthemum Davis series and outliers. Lanes 1-6 in order, cultivars Coral Davis, Davis, Light Davis, Regal Davis, Charm and Pomona. Arrow indicates polymorphism. Numbers on right indicate base pairs.

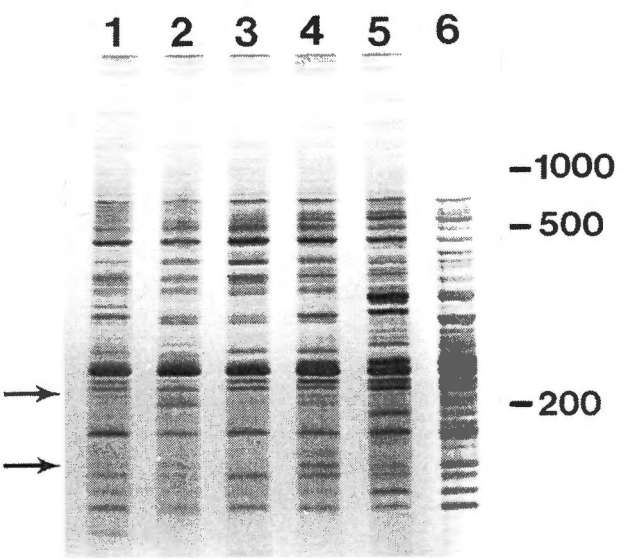

Figure 10. Polymorphic DAF profiles generated with primer 8.6J (GT ATC GCC) for chrysanthemum Davis series and outliers. Lanes 1-6 in order, cultivars Coral Davis, Davis, Light Davis, Regal Davis, Charm and Pomona. Arrow indicates polymorphism. Numbers on right indicate base pairs.

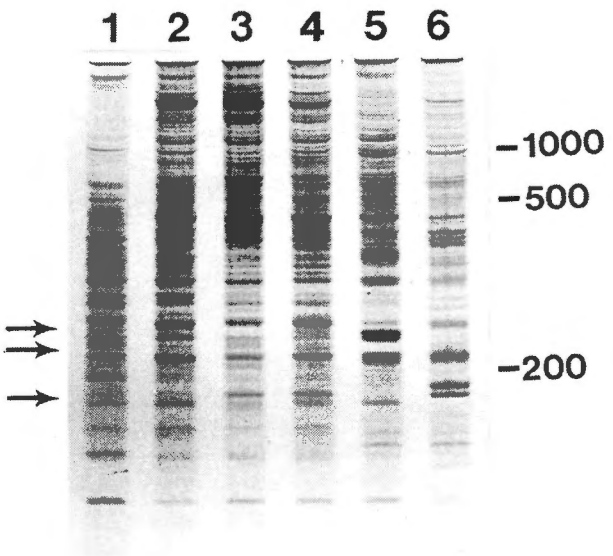

Figure 11. Polymorphic DAF profiles generated with primer 8.6F (GA TGC AGG) for chrysanthemum Pomona series and outliers. Lanes 1-6 in order, cultivars Cherry Pomona, Coral Pomona, Dark Pomona, Pomona, Charm and Davis. Arrow indicates polymorphism. Numbers on right indicate base pairs.

Pomona can be distinguished by 200 bp product generated by GA GCC TGT. Additional diagnostic bands for distinguishing cultivars are listed in Table 3.

The application of DAF to determine cultivar identity would be of value to horticultural breeders. For example, primer GA TGC AGG could be used to identify cultivars in the Anne, Charm, Davis and Pomona series (Table 3). If the primers that are known to give highly polymorphic band patterns are selected, only a few primers are necessary to distinguish cultivars within a series. The method DAF is sensitive enough to detect differences within series of chrysanthemums. In contrast, RAPD analysis of similar series of chrysanthemums was unable to distinguish individual cultivars that were derived vegetatively. With RAPD analysis only one primer out of 27 tested gave slightly different patterns for the 13 mutant cultivars (cultivars within series) tested. The polymorphic bands produced were very faint. An average of seven bands were produced per isolate (Wolff and Peters Van Rijn, 1993b). Using the DAF technique an average of 12 polymophic bands were seen between cultivars within a series from a total of approximately 280 bands scored. The RAPD method produced approximately 190 bands, but yielded no conclusive polymorphisms within series.

The members of series originate from each other by somatic mutations, and furthermore, a vast majority of horticulturally important chrysanthemum cultivars are derived vegetatively, since breeding is exceedingly difficult (Dowrick and El-Bayoumi, 1966). Consequently, there seems to be need for a more sensitive or discriminating technique that can differentiate closely related or vegetatively propagated chrysanthemum cultivars. DAF would seem to be the method of choice among MAAP technologies to identify closely related individuals within series of chrysanthemum. Furthermore, because cultivars have the majority of their genome in common (conserved) with the cultivars in their series, it may be possible to

locate genes of interest by comparing DNA fingerprints between cultivars within series. For example, the cultivars studied appeared to have the same phenotype within series except for flower color. Therefore, by comparing fingerprints between cultivars within a series, a polymorphic marker may be linked to a gene or genes regulating color. However, it is not known whether the differences in phenotype arise due to point mutations or inversions, deletions or even loss of chromosomes (Wolff and Peters Van Rijn, 1993b). Polymorphic markers may be used to find a gene of interest, but the marker probably would not represent the specific gene. However, the search for markers linked to a specific trait may be confounded since chrysanthemums are hexaploid and one or more genes on the six copies may influence any gene trait.

Genetic distances or similarities within and between series were calculated using Dice coefficients. Results indicated genetic distance between cultivars within series differed from only 0.004 to 0.035. This indicates the genomes of these cultivars are highly conserved, as would be expected from series that arose through a few somatic mutations. Conversely, genetic distances calculated from cultivars from the other series (0.074-0.123)(Table 4, 5 and 6) demonstrated at least a double to thirty-fold greater level of genomic variability.

Genetic distances between series from bulk analyses calculated using Dice coefficients ranged from 0.090 to 0.112 (Table 7). Individual differences between several series are lessened since all of the heterogeneity of the series (AFLPs between individual cultivars within series) is obliterated by the bulk profile. In contrast, within series comparison included outliers to illustrate the genetic distance between series as compared to different cultivars within series. This comparison does not accurately show differences between series because the individual differences of the outlier cultivar is not taken into account.

Table 4. Similarity (Dice coefficients) matrix among chrysanthemum cultivars of the Charm series and Davis and Pomona cultivars.

Cultivars	1	2	3	4	5	6	7
1 Charm	1.000						
2 Coral Charm	0.993	1.000					
3 Dark Bronze Charm	0.985	0.991	1.000				
4 Dark Charm	0.996	0.993	0.985	1.000			
5 Salmon Charm	0.996	0.993	0.989	0.996	1.000		
6 Davis	0.884	0.886	0.891	0.888	0.884	1.000	
7 Pomona	0.926	0.933	0.934	0.931	0.926	0.886	1.000

Table 5. Similarity (Dice coefficients) matrix among chrysanthemum cultivars of the Davis series and Charm and Pomona cultivars.

Cultivars	1	2	3	4	5	6
1 Coral Davis	1.000					
2 Davis	0.985	1.000				
3 Light Davis	0.985	0.979	1.000			
4 Regal Davis	0.992	0.985	0.985	1.000		
5 Charm	0.894	0.891	0.887	0.893	1.000	
6 Pomona	0.881	0.878	0.873	0.880	0.936	1.000

Table 6. Similarity (Dice coefficients) matrix among chrysanthemum cultivars of the Pomona series and Charm and Davis cultivars.

Cultivars	1	2	3	4	5	6
1 Cherry Pomona	1.000					
2 Coral Pomona	0.980	1.000				
3 Dark Pomona	0.977	0.981	1.000			
4 Pomona	0.975	0.988	0.981	1.000		
5 Charm	0.912	0.920	0.913	0.911	1.000	
6 Davis	0.877	0.885	0.881	0.880	0.886	1.000

Table 7. Similarity (Dice coefficients) matrix for bulk analysis of six series of chrysanthemum.

Series	1	2	3	4	5	6
1 Anne	1.000					
2 Blush	0.913	1.000				
3 Boaldi	0.910	0.892	1.000			
4 Charm	0.901	0.889	0.894	1.000		
5 Davis	0.888	0.910	0.904	0.897	1.000	
6 Pomona	0.898	0.879	0.891	0.928	0.895	1.000

However, according to the Similarity (Dice coefficients) matrix, the outliers between series seem to facilitate an accurate assessment of genetic distance between series.

The DAF banding patterns obtained from bulking of individual cultivars within a series supported the original hypothesis that there would be many polymorphisms between the six series. In the future, bulking may prove useful in the identification and characterization of chrysanthemum series that would be of help to nurseries in breeding and patent protection. Bulking the DNA of several cultivars of the same series generates products that are unique for that series and these markers may be useful for breeding for following series' traits through cultivar development. Bulk assays are valuable for studying populations instead of sampling several different individuals within the population. There were no differences in bulking patterns from DNA that was extracted from each cultivar individually or if leaf tissue from each cultivar was combined and extracted. In the study of bulked DNA, polymorphic loci were detected in every banding profile generated by all of the primers tested (Figure 12-16) (see Appendix for DAF generated data).

One goal of this investigation was to measure genetic variation within and between series of chrysanthemum cultivars. Data analysis estimates genetic distances between subjects with greater confidence when the data sets are sufficiently large and include at least as many informative characters as there are taxa in the study (Stewart, 1993). As the sample set gets larger the distribution of markers approaches a relatively uniform distribution over the entire genome, and a relatively accurate assessment of the genetic difference or similarity between two genotypes can be achieved (Skroch, 1992). There were approximately 30 scorable bands per primer and over 250 total bands. This did not include any bands located above the 700 base pair. Examples of RAPD analysis of genomic DNA of other chrysanthemums, demonstrated that typically seven products were produced per decamer

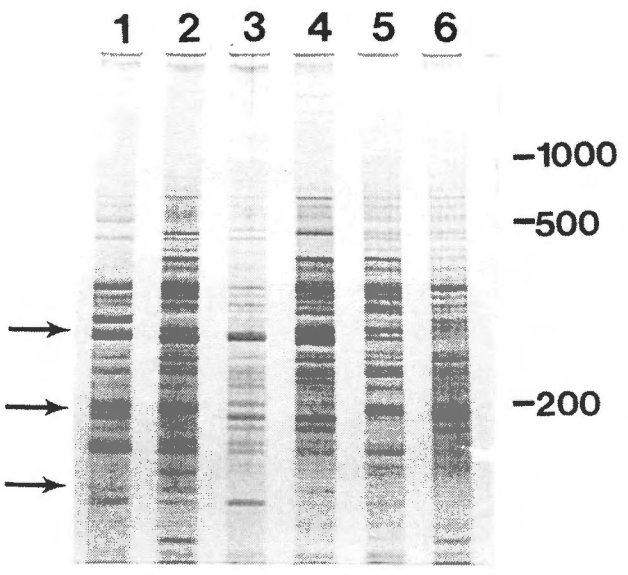

Figure 12. Polymorphic DAF profiles generated with primer 8.6A (GA GCC TGT) of chrysanthemum series. Lanes 1-6 in order, series Anne, Blush, Boaldi, Charm, Davis and Pomona. Arrow indicates polymorphism. Numbers on right indicate base pairs.

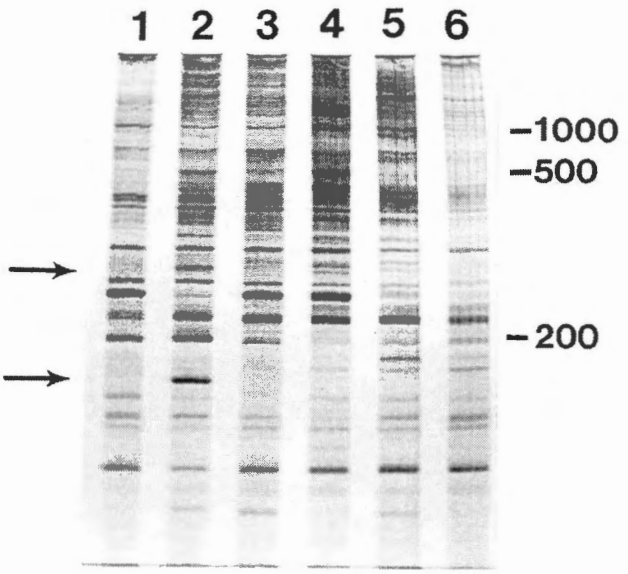

Figure 13. Polymorphic DAF profiles generated with primer 8.6F (GA TGC AGG) of chrysanthemum series. Lanes 1-6 in order, series Anne, Blush, Boaldi, Charm, Davis and Pomona. Arrow indicates polymorphism. Numbers on right indicate base pairs.

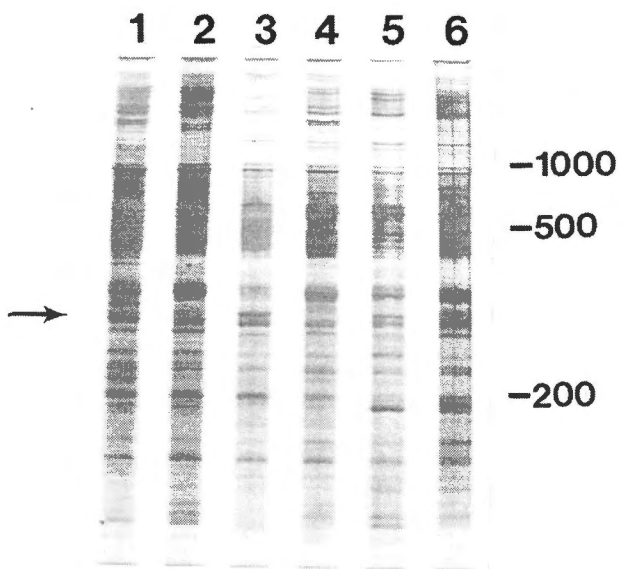

Figure 14. Polymorphic DAF profiles generated with primer 8.6I (GT TAC GCC) of chrysanthemum series. Lanes 1-6 in order, series Anne, Blush, Boaldi, Charm, Davis and Pomona. Arrow indicates polymorphism. Numbers on right indicate base pairs.

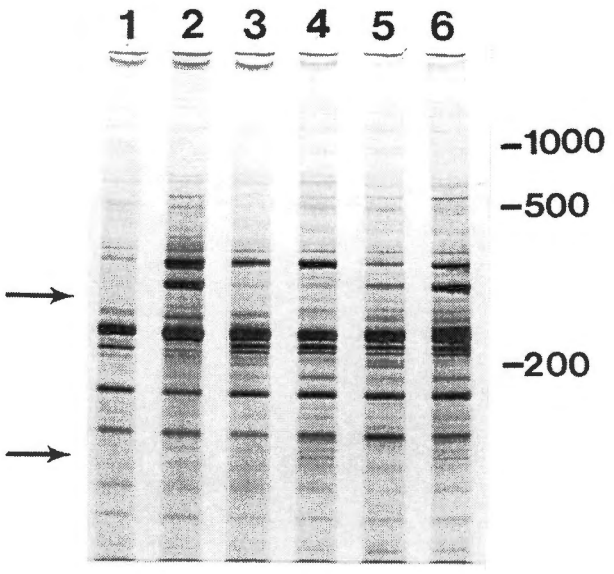

Figure 15. Polymorphic DAF profiles generated with primer 8.6J (GT ATC GCC) of chrysanthemum series. Lanes 1-6 in order, series Anne, Blush, Boaldi, Charm, Davis and Pomona. Arrow indicates polymorphism. Numbers on right indicate base pairs.

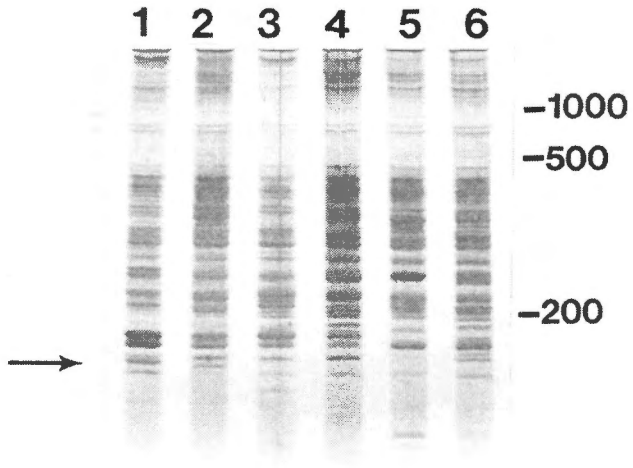

Figure 16. Polymorphic DAF profiles generated with primer 8.7A (AA TGC AGC) of chrysanthemum series. Lanes 1-6 in order, series Anne, Blush, Boaldi, Charm, Davis and Pomona. Arrow indicates polymorphism. Numbers on right indicate base pairs.

times the number of primers in our study to obtain a similar data set containing more than 250 characters or loci. Therefore, DAF technology offers a potentially more efficient method of determining genetic variation.

Phenotypic traits have been the major taxonomic characteristics used to separate chrysanthemum cultivars (Cummings, 1964; Dowrick and El-Bayoumi, 1965; Smith, 1975). There are phenotypic differences within and between the series of chrysanthemum cultivars. Within each of the series studied the only phenotypic difference seems to be flower color. Therefore, cultivars within series should be very closely related in contrast to between series comparison. Genetic analysis for similarity (Dice coefficients) indicated that the hypothesis was correct (Figure 17-19). In contrast, there are many phenotypic differences between series including flower and leaf shape. Preliminary studies showed that a series that had the same flower shape were more closely related than those of different flower types. Specifically, flowers that had decorative shape, Charm and Pomona, were more closely related than Davis that had daisy type flowers. However, when DNA bulk DAF profiles were analyzed for Anne, Blush, Boaldi, Charm, Davis and Pomona, they did not group according to flower shape. Pomona and Charm were still clustered together, but Davis appeared to be more closely related to Anne, a decorative. Furthermore, Blush, a daisy, and Boaldi, a decorative, appeared to be closely related (Figure 20-21). According to phenotype no explanation could be given for the clustering seen between series.

The DAF technique utilizes primers, polyacrylamide gel electrophoresis, and silver staining in order to produce, separate and detect a complex array of DNA products. The DAF technique is capable of generating an informative fingerprint for each genotype examined. Despite the fact that additional bands present in a silver stained DAF gel may be more

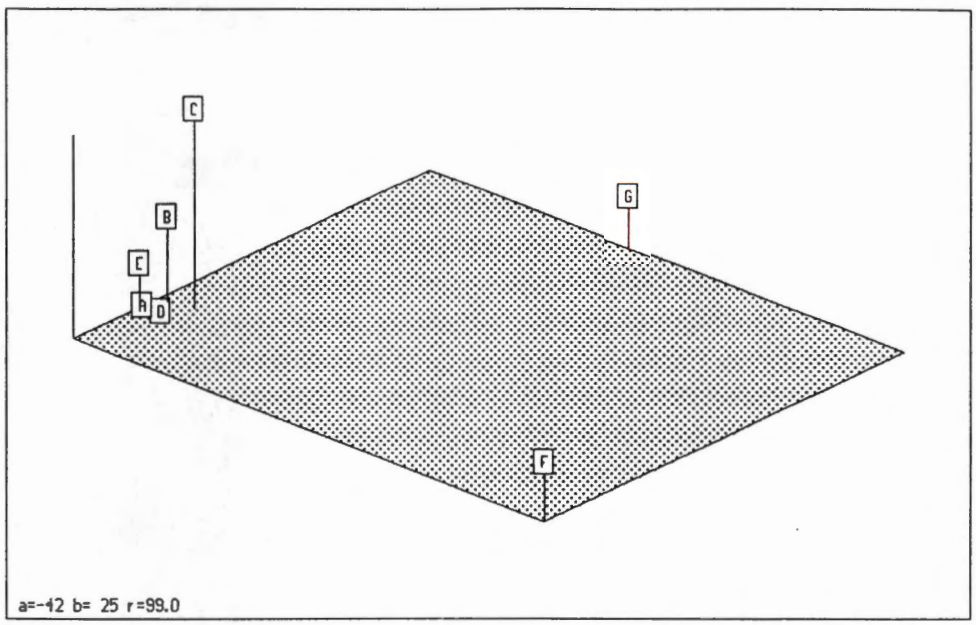

Figure 17. Principal Coordinate Analysis for Charm cultivars, Davis and Pomona. A. Charm B. Coral Charm C. Dark Bronze Charm D. Dark Charm E. Salmon Charm F. Davis G. Pomona

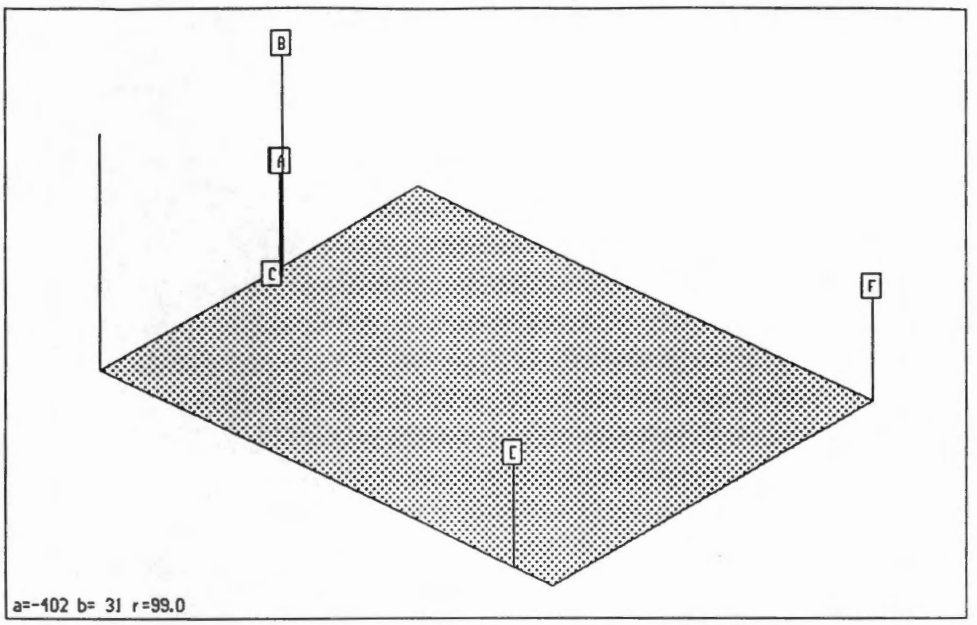

Figure 18. Principal Coordinate Analysis for Davis cultivars, Charm and Pomona. A. Coral Davis B. Davis C. Light Davis D. Regal Davis E. Charm F. Pomona

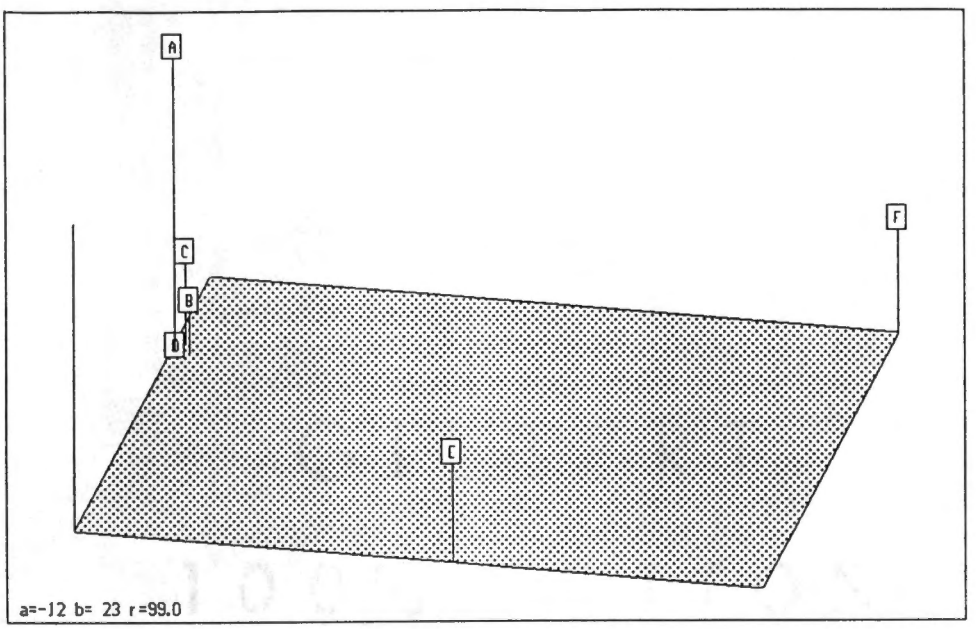

Figure 19. Principal Coordinate Analysis for Pomona cultivars, Charm and Davis. A. Cherry Pomona B Coral Pomona C. Dark Pomona D. Pomona E. Charm F. Davis

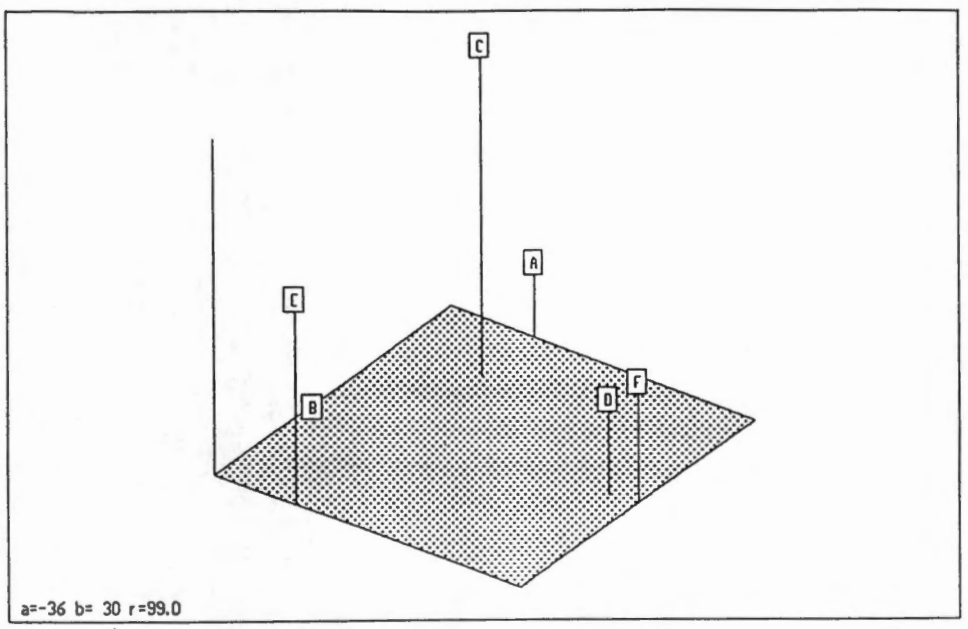

Figure 20. Principal Coordinate Analysis for bulked cultivars of a series.
A. Anne B. Blush C. Boaldi D. Charm E. Davis F. Pomona

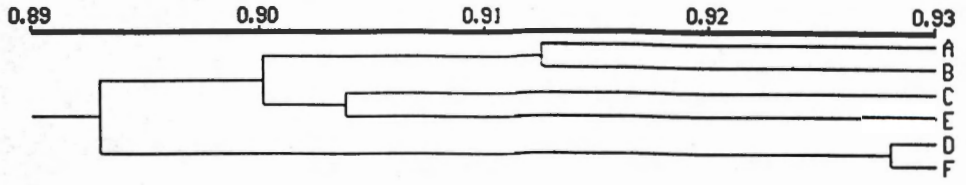

Figure 21. Genetic Similarity computed using Dice coefficients and Cluster Analysis (UPGMA) for bulk series. A. Anne B. Blush C. Boaldi D. Charm E. Davis F. Pomona

difficult to interpret in genetic studies, they are more informative for establishing the unambiguous identity of an accession (Eskew et al., 1993). Other advantages are that the plastic backed polyacrylamide gels may be stored indefinitely after they are dried, and that the bands can be dissected out, re-amplified, and used as hybridization probes (Caetano-Anollés et al., 1993b).

The levels of variability of DAF fragments in chrysanthemum individuals as well as bulked series may be suitable for cultivar identification, breeding programs, mapping studies, and marker analysis. Although specific traits of series cannot be assigned to individual loci, bulk profiles and within series comparison serve to identify potentially unique amplified products that may be associated with some horticultural characteristics. Furthermore, using the DAF technique it was possible to separate cultivars derived asexually (i.e. sports or somatic mutation) and individual AFLPs could be used in cultivar identification and patent protection. In future experiments many cultivars will be screened using the most productive primers for the purpose of exploring marker facilitated selection, breeding studies and phylogenic origins. The results shown in these preliminary experiments demonstrate the utility of DAF for separating closely related cultivars and series of chrysanthemum.

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APPENDIX

DAF GENERATED DATA

Banding patterns generated for eleven different octamer primers were scored as present (1) or absent (0) for each of six series and cultivars within chrysanthemum series. The numbers on left side of data correspond to the chrysanthemum series or cultivar.

Bulk series: 1 Anne 2 Blush 3 Boaldi 4 Charm 5 Davis 6 Pomona. Charm cultivars and outliers: 1 Charm 2 Coral Charm 3 Dark Bronze Charm 4 Dark Charm 5 Salmon Charm 6 Davis 7 Pomona. Davis Cultivars and outliers: 1 Coral Davis 2 Davis 3 Light Davis 4 Regal Davis 5 Charm 6 Pomona. Pomona cultivars and outliers: 1 Cherry Pomona 2 Coral Pomona 3 Dark Pomona 4 Pomona 5 Charm 6 Davis.

BULK

Series	8.6A
1	11111111011110110111011110110010110101111
2	11110111111111111111111110111110111111111
3	11111111011110111111111111011011011001111
4	11110111011010110111011111101011010101111
5	11110111110000111110111111011111110101111
6	11111111111100110101111111011011010111111

	8.6D
1	11111011101101111101011111011111011
2	11111011111011011101011111011111011
3	11111011111101111111010111111110111
4	11111011111001011111100110111110011
5	11111011111011111101101110111110111
6	11111111111001011111111111011111111

	8.6F	8.6H
1	11111111111111111011001110101100111	11111110110110111111111111
2	11111111111011111011100111101101111	11111110111110111111111111
3	11111111111101111011111100111110111	11111110110111111111111111
4	11111111111001111011110011111111111	01111110110110011111111111
5	11111111101101111011111000101110111	11111111111111001111111111
6	11111111111001101111110000011111111	11111110110110111111111111

	8.6I
1	11110111111110011111111111111100111101
2	11110111110111111111111111011110111101
3	11110011101111111111111011011100101111
4	11110011110110111111011111110101011101
5	11110111110110111111011101101010010 1101
6	11111011110110011111011111110101011001

	8.6J	8.6M
1	11111011111101100111111111111	11111111111111001111011
2	11111101111011111111010111 1	11111111111111111001011
3	11111000111101111111111101111	11111111111111011011111
4	11111111011111111111111111111	11111111111111101111111
5	11111110111101101111111011111	11111111111111011001011
6	11111111101111110111111111111	11111111111111001111111

	8.7A	8.7D
1	11111111111101111001011011111	11111111111011111111111110
2	11111111111111111001010111111	11101111111011111111111111
3	11111011111111111101101001111 1	11101101111111111111011111111
4	11111111111111111011010111111	11111111111011111101111110
5	11111111101110110110101111111	11111111111111111111011111111
6	11111111110111110101110011111	11111111111011111111011111111

BULK

Series	8.7F	8.9A
1	111111111101111011011111	111111111110111101011111111
2	111110110111111011010111	111111011110110011111101111
3	111111111101111011011111	111111111111111110101011110
4	111111111110111001101111	111111111111111100101111111
5	111110111110110111000111	111111011110110101011111111
6	111111110110110111011111	111111111111111110101111111

CHARM

Cultivar	8.6A	8.6D
1	10111111110110110110100011011111111	11101101111111111110111111
2	10111111110110110110100011011111111	11101101111111111110111111
3	10111111110110110110100011011111111	11101101111111111110111111
4	10111111110110110110100011011111111	11101101111111111110111111
5	10111111110110110110100011011111111	11101101111111111110111111
6	10111111110110110110100011011111111	11101101111111111110111111
7	11111111111011111011011111111111	11110111111111111110111111
8	10110111110101111110101011011111111	11111001111111111111111111

	8.6F	8.6H
1	11111111110111101001101010111100111	111011111111110011
2	11111111110110101101101010111110111	111011111111110011
3	11111111110110101101111111111110111	111011111111110011
4	11111111110110101001101010111011111	111011111111110011
5	11111111110110101001101010111100111	111011111111110011
6	11111111011110110010111111111111111	111111111111111110
7	11111111110110100101100011111111111	111011111111110000

	8.6I	8.6J
1	11111111110111110011011011	11111111110111111111111111100111100
2	11111111110111110011011011	11111111110111111111111111100111100
3	11111111110111110011011011	11111111110111111111111111110111100
4	11111111110111110011011011	11111111110111111111111111100111100
5	11111111110111110011011011	11111111110111111111111111110111100
6	11111111111111111001011111	11111111111111111111010100000101100
7	11111111110111110101111011	11111111111111111111111111101011011

	8.6M	8.7A	
1	11111111110111111	11111111011111111111101111111	1111111101111
2	11111111110111111	11111111011111111111101111111	1111111101111
3	11111111110111111	11111111011111111111101111111	1111111101111
4	11111111110111111	11111111011111111111101111111	1111111101111
5	11111111110111111	11111111011111111111101111111	1111111101111
6	11111111111111111	11111111111111111111011101110	11110111111111
7	11111111110111111	11111111111111111111111111111	1111111101111

	8.7F	8.9A
1	11111111010111	11111111001011111111111111111
2	11111111010111	11111111001011111111111111111
3	11111111010111	11111111001011111111111111111
4	11111111010111	11111111001011111111111111111
5	11111111010111	11111111001011111111111111111
6	11111100011111	11111111010111111011111111111
7	11111110110111	11111111011111111111111111111

DAVIS

Cultivar	8.6A	8.6D
1	11111111111111111101111011110101011	11111111111011011111111111
2	11111111111111111101111011110101011	11111111111011011111111111
3	11111111111111111101111011110101011	11111111111011001111111111
4	11111111111111111101111011110101011	11111111111010011111111111
5	11111111111111101101111110101111001	11111111101110011111101111
6	11111111111111110111111111111111010	11111111011100101111101111

	8.6F	8.6H
1	11111111011111110111110001110111111	11111111111101111
2	11111111011011010111110001110111111	11111111111101111
3	11111111011111010111110001110111111	11111111111101111
4	11111111011011010111110001110111111	11111111111101111
5	11111111111011111011111101111111111	11111111110110011
6	11110111011011111011111110111111111	11111111110100001

	8.6I	8.6J
1	10111111111111010101111111	11011111101011111101111110110001111
2	10111111111111010101111111	11011111100011111011101110110001111
3	10111111111111010101111111	11011111100011110101111010110000111
4	10111111111111010101111111	11011111101011110101111110110001111
5	11000110111111111010111	11111111100111111001111011101001111
6	11000110111111111111101111	11111111110111111001111101111111111

	8.6M	8.7A
1	11111011111111111111	11111110111111111111001100111
1	11111011111111111111	11111110111111111111001101111
1	11111011111111111111	11111110111111111111001100111
1	11111011111111111111	11111110111111111111001100111
1	11111111111111111111	11111111111011111111011110101
1	11111111111111111111	11111111111111111111111100101

	8.7D	8.7F	8.9A
1	11111011111111111	11111111101110111	11111111111111011111011111111
2	11111011111111111	11111111101110111	11111111111111011111011111111
3	11111011111111111	11111111101110111	11111111111111011111011111111
4	11111011111111111	11111111101110111	11111111111111011111011111111
5	11011111111111111	11111110111111111	11111111111011011111111111111
6	11011111111111111	11111111111111111	11111111111011111111111111111

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Cultivar	8.6A	8.6D
1	11111110110101111111001111010111111	11111110111101111111110
2	11111110110101111111101111111111111	11111110111101111111110
3	11111110110101111111101111100111111	11111110111101111111110
4	11111110110101111111111111001111111	11111110111101111111110
5	11111110111111111111101010110111111	11111110111100111011111
6	11111111111101111011001101101111111	11111111111111111101111

	8.6F	8.6H
1	11111111111110110101001111111011110010	11111111111010111
2	11111111111110110001000111100111110010	11111111111010111
3	11111111111110111001001111100111110010	11111111111010111
4	11111111111110110001000111100111110010	11111111111010111
5	11111111111110110000000011110111100111	11110111111101011
6	11111111111111110011110111101110111111	11110110111011010

	8.6I	8.6J
1	11111001101111111111110111	11111111111011011011010111111111111
2	11111000101101111111110111	11111111111011011111101111111111111
3	11111000101001111001110111	11111111111011011101101111111111111
4	11111000101101111101110111	11111111111011011101101111111111111
5	11111000011101111111111111	11111111111000011100110101101011111
6	11111101111011111011110111	11111111110100101101111101101010111

	8.6M	8.7A	8.7D
1	11111111110111111	11111110111101111111011111111	1111111111110101
2	11111111110111111	11111110111101111111011111111	1111111111110101
3	11111111110111111	11111110111101111111011111111	1111111111110101
4	11111111110111111	11111110111101111111011111111	1111111111110101
5	11111111110111111	11111110111110110111110111111	1111111110110101
6	11111111111111111	11111111111100111011011111111	1111111110111111

	8.7F	8.9A
1	11111011111111111	11111110111101111111111
2	11111011111111111	11111110111101111111111
3	11111011111111111	11111110111101111111111
4	11111011111111111	11111110111101111111111
5	11110011111111111	11111111111011111111111
6	11110111011111111	11111111111111110111111

VITA

Mary Catherine Scott was born in Pittsburgh, Pennsylvania on December 5, 1969. She graduated from Upper St. Clair High School in May 1988 and in September of the same year she entered Bethany College Bethany, West Virginia.

During her undergraduate study at Bethany, she was involved in many organizations including Zeta Tau Alpha women's fraternity, Field Hockey, Sigma Tau Epsilon Music Honorary Society, College Choir, American Chemical Society, Recycling Committee, Society of Physics students and Rotary. She was president of Beta Beta Beta the biology honorary society and captain of the Women's LaCrosse Club. She graduated in May 1992 with a Bachelor of Science in Biology. Immediately after she started work with the Environmental Protection Agency in Cincinnati.

She entered the University of Tennessee, Knoxville, in August 1993 where she worked as a Graduate Research Assistant. She is a member of Pi Alpha Xi academic fraternity, Southern Region of the American Society for Horticultural Science and the East Tennessee Chapter of Association for Women in Science. She placed third in the Norman Childress graduated student competition at The American Society for Horticultural Science, Southern Region Meeting in New Orleans and second in The University of Tennessee, Sigma Xi Scientific Research Society graduate student paper competition. She received a Master of Science degree in Ornamental Horticulture and Landscape Design in December, 1995.

Her immediate plans are to move to Cincinnati and pursue a career in molecular genetics at Children's Hospital Research Center. She will marry Mr. Daniel Edward Williams in May 1996.