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TRANSPOSON COPY NUMBER IN SELF- AND CROSS-FERTILIZING TAXA OF AMSINCKIA (BORAGINACEAE)

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June 1997.

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science.

PREFACE

This thesis carries a credit weight of 39 credits, from a total of 45 credits required for the Master's degree. Graduate credits are a measure of the time assigned to a given task in the graduate program. They are based on the consideration that a term of full-time graduate work is equivalent to 12 to 16 credits, depending on the intensity of the program.

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ABSTRACT

Copy number of a copia-like retrotransposon was compared in self- and cross-fertilizing taxa of the annual plant genus, Amsinckia. It was hypothesized that variation in copy number between populations with contrasting mating systems could be used to interpret the relative importance of factors influencing copy number (purging of element-induced mutations, ectopic exchange, and the level of heterozygosity). Populations of Amsinckia spectabilis var. microcarpa and A. furcata, two outcrossing taxa, and their self-fertilizing relatives, A. spectabilis var. spectabilis and A. vernicosa were studied. Probes for Southern hybridisation were generated for each respective taxa through the amplification of a conserved region of the retrotransposon sequence. There were no observable differences in the numbers or patterns of hybridised bands between related self- and cross-fertilizing taxa. The retrotransposon family studied may have been inactive before divergence of Amsinckia taxa, or the factors influencing copy number and genome location may be expressed in such a way as to yield no observable differences in copy number between species with contrasting mating systems.

RÉSUMÉ

Le nombre de copies du rétrotransposon «copia-like» a été comparé chez des individus de populations autofécondées et à fécondation croisée du genre Amsinckia. L'hypothèse de départ était que les différences entre les deux modes de reproduction peuvent être utilisées pour interpréter l'importance relative des facteurs qui influencent l'accumulation des rétrotransposons «copia-like» (e.g., l'élimination de mutations causées par la transposition, l'échange ectopique, et le niveau d'hétérozygosité des individus qui échangent les transposons). Nous avons étudié deux taxa qui utilisent la fécondation croisée, Amsinckia spectabilis var. microcarpa et A. furcata, ainsi que leurs taxa autofécondées alliées, A. spectabilis var. spectabilis et A. vernicosa. Les sondes utilisées pour l'hybridation de type Southern proviennent d'une région bien conservée codant pour l'enzyme transcriptase inverse du rétrotransposon «copia-like». Aucune différence n'a été observée dans le nombre ou dans l'emplacement des bandes hybridées entre les taxa autofécondés et celles à fécondation croisée. La famille de rétrotransposons de cette étude aurait pu être inactivée avant la divergence des systèmes de reproduction d'Amsinckia. Alternativement, il est possible que les forces qui influencent le nombre de copies de rétrotransposons produisent des différences imperceptibles entre ces espèces.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. D.J. Schoen for his many thoughts and actions, and the rest of my supervisory committee, Dr. C. Waddell and Dr. G.A. Bell, and to Dr. T.E. Bureau and T. Batallion for their advice on theoretical aspects of the work presented here. I really appreciated N.O. Tremblay, A.M. L'Heureux, A. Babwah, N.M. Hamel and N. Elrouby for helping me confront "common" lab problems, and thanks also to R.B. Scott, H.R. Kleb, H.M. Gray, G. Stirling, J. Brinkman, Z.M. Wang for their individual input. I would also like to thank R. Lamarche, the Phytotron group and the Photo Lab staff as well as the Technical staff of Qiagen and Boehringer Mannheim.

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INTRODUCTION

Transposable elements and their characteristics

Transposable elements, also known as transposons, are segments of DNA that have the capacity for self-replication and reinsertion into the genome (Werren et al., 1988; Konieczny et al., 1991; Hickey, 1992; Cummings, 1994). There are two main classes of such elements, defined principally by whether the mechanism of transposition involves a DNA or an RNA intermediate.

The first class are the retrotransposons (Cummings, 1994; McDonald, 1995). These elements do not excise from their genomic positions. Instead, an RNA transcript is converted into a DNA copy through the action of a reverse transcriptase encoded in the element itself, and this DNA copy is integrated elsewhere in the genome (Cummings, 1994; McDonald, 1995).

Retrotransposons can be further classified on the basis of their molecular structures. Some elements, such as the gypsy and copia retrotransposons, are characterized by long terminal repeats (LTRs) of DNA (hundreds of base pairs in length) flanking both ends of the element (Bingham and Zachar, 1989; Purugganan, 1993; Cummings, 1994; Zeyl and Bell, 1996). The other type of retrotransposons are the non-LTR retrotransposons, such as SINES (Short Interspersed Elements) and Drosophila P-elements (Cummings, 1994; Zeyl and Bell, 1996).

Retrotransposons, the most common class of eukaryotic transposable elements, have recently been shown to occupy a large proportion of many plant genomes (Kumar, 1996). Active retrotransposons have been observed to transpose when the host plant is caused, for example, by wounding, pathogen attack or tissue culture (Hirochika et al., 1996; Matyunina et al., 1996; Pearce et al., 1996a; Wessler, 1996). The reverse transcriptases of such elements do not have a proofreading mechanism for DNA synthesis, so retrotransposon sequences tend to accumulate mutations at higher rates than those of other genes, resulting in the formation of retrotransposon families (Flavell et al., 1992a; Voytas et al., 1992). Active retrotransposon families may undergo periods of elevated mutation and recombination, leading to the inactivation of the affected lineage. The expression of the copia-like retrotransposon has been attributed to both element and host regulatory variation (Matyunina et al., 1996). As retrotransposons can also remain quiescent in the genome for extended periods of time, different element lineages may have evolved at different rates (Voytas et al., 1992).

The second major class of transposons consist of elements that transpose via a DNA intermediate (Purugganan, 1993; Cummings, 1994; McDonald, 1995). These are the inverted repeat (IR) elements, named after the short segments (11-200 base pairs) of inverted repeat sequences located at each end of the element (Purugganan, 1993; Cummings, 1994; Young et al., 1994). Such transposons are transferred from one chromosomal location to another upon physical contact between donor and recipient genome segments, through the action of an element-encoded transposase (Hickey, 1993; Cummings, 1994; McDonald, 1995). Transposition of these types of elements tends to occur during DNA replication, thus allowing the untransposed sister

chromatid to be used as a template for restoring a copy of the transposed element in its recently excised position (Zeyl and Bell, 1996). There are usually short direct repeats of chromosomal DNA flanking the transposon; a result of the staggered cuts made by the transposase during the insertion of the element (Hickey, 1993; Purugganan, 1993; Cummings, 1994; Young et al, 1994; McDonald, 1995).

It has been suggested that transposable elements and their remnants make up the majority of interspersed repetitive DNA in eukaryotes (Werren et al., 1988; Charlesworth et al, 1994; Bureau and Wessler, 1994). The larger genome sizes of flowering plants (i.e., compared to that of other higher eukaryotes) is thought to be due to the larger number of transposable elements in their genomes (Bureau and Wessler, 1994). This viewpoint is supported by the discovery that some plant transposon families occur in high copy number (Bureau and Wessler, 1994).

Factors regulating transposon copy number

Transposons have typically been treated either as sequences that regulate a particular function within the host cell, or alternatively, as "selfish DNA" that "infects" host genomes solely because it is able to (Doolittle and Sapienza, 1980; Orgel and Crick, 1980; Werren et al., 1988; Hickey, 1993). The latter interpretation has gained considerable support in recent years (Charlesworth et al., 1994; Zeyl and Bell, 1996).

Transposons are expected to remain in the host genome until they are excised by splicing that

is induced either by the host or by the transposon itself (Charlesworth et al., 1994; Cummings, 1994; Matyunina et al., 1996). Since transposable elements should be maintained in the host population by virtue of their ability to replicate within the host genome and be passed on to individuals in the population during mating, mutant elements with increased rates of transposition may often be selectively favoured among transposons (Charlesworth and Langley, 1986; Zeyl and Bell, 1996). Yet elements with high rates of transposition may not ultimately be the most evolutionarily successful elements. Transposons may cause mutations when new copies reintegrate into a gene or near a modifying factor for a gene (Werren et al., 1988; Bell, 1993; Charlesworth et al, 1994; Francis et al, 1995; Bureau and Wessler, 1994; Bureau et al, 1996). Assuming that most mutations caused by transposable element insertion are deleterious to the host, and in view of the possibly unregulated increase in genome size caused by proliferation of such elements, there is likely to be counterselection against highly active transposons (Charlesworth et al., 1994). Transposon copy number might then best be viewed as reflecting the balance between the propensity of such elements to increase in number, and counteracting selection brought about by the deleterious effects of such elements on host fitness.

Selection against transposons is expected to vary with the location of insertion. For example, transposons are often found in genomic regions where recombination is infrequent. This has been interpreted as evidence that recombination between mismatched regions of DNA lead to chromosomal aberrations; as may occur when similar transposons are located at very different loci. Unequal exchange between transposons may have acted in the past as a selective force to restrict transposon increase (Werren et al., 1988; Charlesworth et al., 1994). There is also

expected to be less selection against transposon accumulation in non-coding regions of chromosomes or when mutations caused by transposon insertion are recessive (Werren et al., 1988; Charlesworth et al., 1994).

Transposon copy number in self- and cross-fertilizing organisms

Mutation accumulation and mating system. The mating system of a population may play an important role in determining copy number of transposable elements (Francis et al., 1995). In species with high levels of inbreeding, such as self-fertilizing plants, recessive lethal and semi-lethal mutations may be quickly eliminated because they are selected against in a homozygous state soon after their occurrence (Charlesworth and Langley, 1986). To the extent that transposon insertion causes such mutations, inbreeding will facilitate selection against transposons and high transposition rates, and thereby contribute to restricting their copy number. Outcrossing populations may, however, contain such mutations in a heterozygous form.

Ectopic exchange. Chromosomal pairing and meiotic exchange between elements at non-homologous sites (ectopic exchange) can also cause reductions in host fitness; i.e., through chromosomal deletions and duplications. Such effects are expected to be dominant in their expression. Ectopic exchange should oppose transposable element increase by reducing the fertility of individuals with high copy numbers of transposable elements (Langley et al., 1988; Montgomery et al., 1991). Ectopic exchange is expected to be more prevalent in outcrossers due to their higher levels of heterozygosity. In inbreeders, most non-lethal element insertions would

quickly become homozygous, resulting in lower frequencies of recombination between element sequences located at different chromosomal positions (i.e., reduced likelihood of ectopic exchange). There is direct evidence for the occurrence of ectopic exchange between element sequences in Drosophila, and in humans (Charlesworth et al., 1994). Studies in Drosophila have confirmed that ectopic exchange occurs more frequently in chromosomal heterozygotes than in chromosomal homozygotes (Montgomery et al., 1991; Francis et al., 1995).

Transfer of elements through the mating process. Outbreeding may facilitate the spread of transposable elements in populations through their transmission to the offspring of unrelated individuals (Werren et al., 1988). In outcrossing populations, genetic parasites are expected to infect new lines of descent through gamete fusion, and so high rates of transposition and replication may be less detrimental to the fate of very active transposons over the long term, as their descendants would be able to continually infect other lineages (Bell, 1993; Hickey, 1993). Charlesworth and Langley (1986) showed that the conditions for the spread of elements with reduced rates of transposition are easier to satisfy in host populations that have low rates of genetic recombination. In other words, there may be stronger selection against transpositionally-active elements in selfers as the lines containing such elements may accumulate a high copy number of transposons, many of which will cause a mutation in the function of the gene it inserted into. These mutations would be homozygous due to the selfing nature of the mating system and therefore the line may be more prone to extinction, thereby removing such elements from the population.

Another theoretical effect of the mating system of the host on transmission of transposons concerns exposure to the elements accompanying mating, or in other words, the likelihood of an element being transferred during the sexual process. In general, outcrossing should lead to greater "exposure" of a parent's progeny to infection by transposons than selfing (Charlesworth et al., 1994).

While it is possible to postulate the different effects of the mating system on transposon dynamics, when comparing copy numbers in selfers and outcrossers that share common relatives (as in the present study), it may be more difficult to predict the effect of the transmission process on copy number, as the taxa compared may have harboured the element(s) before their divergence. At the start of the investigation we had no evidence to suggest that the elements for which probes were developed were either active or inactive. It seems likely that in the case of the taxonomically-widespread copia family of elements investigated in the present study, the common ancestors of each pair of related selfers and outcrossers included in the analysis would have contained the elements.

Inferences about factors determining transposon dynamics in self- and cross-fertilizing populations

The factors mentioned above influence transposon copy number differently according to the host mating system (Table 1). Copy number is limited mainly through homozygous deleterious mutations in selfers, and by ectopic exchange in outcrossers. It seems reasonable, therefore, that a comparison of copy number in related selfing and outcrossing taxa may help to reveal their relative importance. For example, if it is found that selfing taxa, on average, contain more transposons per individual than related outcrossing taxa, this would suggest that ectopic exchange may be a more important mechanism in limiting copy number, compared with selection against transposon-induced mutations caused by element insertion (which is expected to be the main force limiting transposon number in selfers). Alternatively, if outcrossing species contain a higher average number of transposons per individual than related selfing species, this would suggest that selection against deleterious mutation may be the more important mechanism of containing copy number.

Mating system variation in the genus Amsinckia

The genus Amsinckia is comprised of species that are self-fertilizing or predominantly cross-fertilizing (Johnston and Schoen, 1995, 1996). Often this is the sole attribute that differentiates one related species from another (Ray and Chisaki, 1957). It has been suggested that the homostylous selfing species originated from heterostylous ancestors (Ray and Chisaki,

1957). Molecular phylogenetic analysis of the taxa supports this view (Schoen et al., in press).

Two pairs of taxa were studied in this investigation: (1) Amsinckia spectabilis var. microcarpa (outcrossing) and its relative A. spectabilis var. spectabilis (selfing); and (2) A. furcata (outcrossing) and its relative A. vernicosa (selfing). The related selfing and outcrossing taxa possess the same diploid number of chromosomes; 2n=10 for the two varieties of A. spectabilis, and 2n=14 for A. furcata and A. vernicosa. Restriction enzyme analysis of the chloroplast genome supports the hypothesis that the species within each pair are closely related (Schoen et al., in press).

The copia group of retrotransposons

The copia family of retrotransposons was chosen for analysis. These elements have a nearly ubiquitous distribution in higher plants, and published sequences for consensus primers that can be used in PCR amplification of a conserved region of these elements (the pol gene) are available (Voytas et al., 1992). Although there is a large diversity of nucleotide sequences of the copia group retrotransposons (Flavell et al., 1992a, 1992b), the amino acid sequence of the reverse transcriptase has been conserved sufficiently for accurate recognition of this class of retrotransposons (Voytas et al., 1992). A survey of the copia-like retrotransposon from 31 plants (Flavell et al., 1992b) revealed that more than half of the copia group retrotransposon fragments carried either stop codons or frameshifts in the isolated fragment of the reverse transcriptase reading frame. Given the small percentage element sequences isolated with intact open reading

frames, it is likely many of these copia-like retrotransposons are presently transpositionally inactive (Flavell et al., 1992b). This does not rule out the likelihood that such elements were active in the past, and have had their population dynamics and distributions influenced by the host mating system.

Objectives of this thesis

The objectives of this thesis project were to: (1) develop probes for copia-like retrotransposons derived from populations of Amsinckia with contrasting mating systems; and (2) use these probes to compare the copy number of these sequences in selfing and outcrossing populations. Such a comparison has the potential to help suggest which factor(s) are the most influential in determining transposon copy number.

MATERIALS AND METHODS

Plant populations and culture conditions

A total of four taxa from Amsinckia, two self-fertilizing taxa and their nearest cross-fertilizing taxa, were used in this study. Amsinckia spectabilis var. spectabilis and Amsinckia vernicosa produce homostylous flowers and use a self-pollinating mating system. Amsinckia spectabilis var. microcarpa and Amsinckia furcata produce heterostylous flowers and are partially outcrossing. Each population was given an identification number at the time of seed collection. These numbers are used below in referring to the population (Table 2).

Seeds from different populations of the Amsinckia taxa used in this investigation were obtained in 1988 and 1991 during field work in southern California (see Johnston and Schoen, 1995, 1996). Two populations were sampled for each taxon studied. Approximately 30 seeds per population were selected from each seed collection. Seeds were nicked to improve water uptake and placed in small petri dishes lined with moistened Whatman#1 filter paper. The first week, seeds were incubated in complete darkness at 4° C. After this period, the seeds were transferred to room temperature, away from direct sunlight. Upon germination, the young seedlings were planted in a soil-sand-vermiculite mixture and cultured in growth chambers. They received a 12 hr photoperiod with temperatures of 22° C (day)/14° C (night). The plants were watered from the bottom of the pots. A 10% solution of 20:20:20 N:P:K fertilizer was applied once a week. Leaf tissue was harvested once each plant had produced at least 1 g (fresh weight) of leaf tissue.

Genomic DNA extraction for restriction digests and Southern hybridisations

Genomic DNA was obtained following a procedure modified from Doyle and Doyle (1987). To begin, leaf tissue (1 g per plant) was frozen in liquid nitrogen and ground in a mortar at -20° C. The ground tissue was transferred to a tube containing 7 ml of CTAB solution (2%CTAB, 100 mM Tris-HCl (pH 8), 1.4 M NaCl, 20mM EDTA) at 60° C, to which 14 µl ß-mercaptoethanol was subsequently added. The mixture was incubated for 30 min at 60° C. Purification of the DNA was achieved with phenol/chloroform extractions. An equal volume of 25:24:1 phenol/chloroform/isoamyl alcohol was added to the tubes, and the solution was mixed gently. The resulting emulsion was centrifuged at 4° C for 1 min in a clinical centrifuge at high speed. The aqueous layer was removed to a clean tube, and the extraction was repeated twice. One or two further extractions were performed using 24:1 chloroform/isoamyl alcohol to remove residual phenol. Half a volume of 5 M NaCl was added to the aqueous layer (transferred to a new tube) to remove polysaccharides from the product. Precipitation of DNA was performed by adding 2/3 volumes of cold isopropanol to this aqueous layer. After a 12-16 hr incubation at -20° C, the solutions were spun at 4° C for 5 min in a clinical centrifuge at high speed. The pellets were rinsed in -20° C 95% alcohol (removed after spinning at 4° C, 5 min at high speed), twice with -20° C 70% alcohol (spun at 4° C for 5 min). The pellet was allowed to air dry, and was resuspended in 1 ml sterile TE buffer (10 mM Tris, pH 8, 1 mM EDTA) at 37° C for 1 hr.

DNA extractions for PCR amplification

One hundred mg of leaf tissue was frozen in liquid nitrogen and then ground with a cold autoclaved pestle in 1.5 ml microcentrifuge tubes. A 0.5 ml aliquot of 60° C CTAB solution (see above) was added and the tube was incubated for 60 min at 60° C. One half ml of 24:1 chloroform/isoamyl was added. The contents of the tube were gently shaken into an emulsion and spun at 14,000 rpm for 6 min. The top phase was transferred to a new tube to which 0.5 ml cold isopropanol was added. The mixture was incubated at 4° C for 12-16 hrs, then spun at 14,000 rpm for 15 min. The pellet was washed with 0.5 ml wash buffer (70% w/v ethanol, 10mM NaAc), air dried, and resuspended in 0.1 ml ddH_2O at 37° C.

PCR amplification of probe sequences

The probes used to test for the presence of transposons in Amsinckia populations were produced via PCR amplification, using a portion of the highly conserved reverse transcriptase gene from the copia-like retrotransposon as primer sequences (Flavell et al., 1992b; Voytas et al., 1992). PCR amplifications were performed in 25 µl reactions with 0.01 µl (~10 µg) DNA, 10 mM each dNTP, 32.26 pmol of each primer, 5 units of Taq polymerase, and the buffer supplied by the manufacturer (Boehringer Mannheim). Temperature cycling was as follows: 94° C for 1 min, 55° C for 1 min, 72° C for 2 min (40 cycles). Oligonucleotide sequences for PCR primers were:

5' primer: GGAATTCGAYGTNAARACNGCNTTYYT;

3' primer: GGGATCCAYRTCRTCNACRTANARNA

where N = A + C + G + T, R = A + G, and Y = T + C. Each sample was replicated six times. The reaction products from each replicate were pooled and separated on a 0.7% agarose gel in 1X TBE buffer at 60 V-100 V. Products of the appropriate size (200-300 Kb) were isolated from the gel using the Qiagen Gel Extraction kit, and reamplified, again with six replications, using the above conditions for PCR, with an additional tailing period of 7 min at 72° C. The products were separated on a 0.7% gel in 1X TBE buffer. Bands of approximate size (300bp) were excised from the gel, purified and cloned into EcoRI-digested BluescriptKS+ vectors in E. coli strain DH5α.

Cloning and sequencing of probes

In a 10 µl reaction, 1 µl of BluescriptKS+ vector was restriction-digested with 10 units of EcoRI at 37° C for 1 hr. T-tailing was performed in a 25 µl reaction with 9 µl of the aforementioned mixture, 1 µl of 5 mM dTTP, 6.5 µl sterile dH_2O, 5 units Taq polymerase, and 2.5 µl 10X reaction buffer (Boehringer Mannheim). The reaction was held at 72° C for 2 hrs. After the PCR amplification, the appropriate-sized vector was gel-isolated and purified using the Qiagen Gel Extraction kit. The ligation reaction of the vector to the PCR sequence was performed using the Boehringer Mannheim Rapid Ligation kit.

DH5α E. coli cells were cultured for 16-20 hrs at 37° C in a shaker-incubator. Following

incubation, 500 ml of fresh LB media (10 g bactotryptone, 5 g bacto-yeast extract, 10 g NaCl, pH 7.0 in 1 L) was inoculated with 3 ml of the E. coli culture, and this was incubated until the OD_{550} reading was 0.3 - 0.5 units (about 2 hrs). The culture was spun at 4000 rpm for 10 min, the supernatant was removed, and the pellet was resuspended in 10ml of 50 mM $CaCl_2$ and incubated for 30 min on ice. The cells were then spun down once again at 4000 rpm for 10 min and the pellet was resuspended in 1-2 ml $CaCl_2$.

Heat shock transformation was performed using 200 µl of the prepared cells, and 10 µl of the ligation reaction. This mixture was set on ice for 30 min, then at 42° C for 2 min, and then back to ice for 2 min, after which 800 µl of SOC medium (1 M glucose, 250 mM KCl, 2M $MgCl_2$, 20 g bactotryptone, 5 g bacto-yeast extract, 0.5 g NaCl in 1 L) was added. The reaction was incubated at 37° C for 1 hr. After incubation, the volume of the reaction was reduced to approximately 100 µl, and the cells were aliquotted onto three LB plates and incubated 16-20 hrs at 37° C.

Transformed cells were grown on LB media in the presence of ampicillin (100 mg/L), X-gal (40 mg/L) and IPTG (4 mg/L). Blue colonies were considered to be potential carriers of the PCR product. These were picked, cultured, and the plasmid extracted using the Qiagen Qiaprep kit. Five µg of the isolated plasmid was sent for sequencing, either to the Queen's University Sequencing Facility in Kingston, Ontario, or to Sheldon Biotechnology at the Royal Victoria Hospital in Montreal, Quebec. Returned sequences were compared with those already recorded in the NCBI Genebank using the BLASTX algorithm (Altschul et al., 1990). Sequences with

homology to the copia-like retrotransposon reverse transcriptase gene were used as probes for the experiment.

The plasmids containing the probe sequences were linearized and used as templates to reamplify the actual sequence from the vector, using the same protocol as that for the initial amplification of the sequence from the genomic DNA (6 replications, temperature cycling as stated before). Seventeen µg of DNA were labelled with DIG-dUTP for each probe using the Boehringer Mannheim Random Primer Labelling kit.

Characterization of probes

Integrity of the transposon sequences. The amplified sequences were examined for open-reading frames using WebTrans (Sharp and Li, 1987). As the copia-like retrotransposon contains a single open-reading frame from the gag to the pol genes (Varmus and Brown, 1989), sequences from active retrotransposons should contain an open-reading frames throughout their length.

Transposon homology within and between populations. Sequence homology of the probes extracted from within the same and between the different populations of Amsinckia used in this study was analyzed using the ALIGN computer program (Gotoh, 1982; Myers and Miller, 1988). After alignment, the sequences were compared by eye in a pairwise fashion with respect to their overall similarity of base composition. The complete set of Amsinckia pol gene sequences from all of the species studied were aligned using the CLUSTALW program (Higgins et al., 1992;

Thompson et al., 1994) and cladistic analyses were performed using the exhaustive search algorithm (with bases as unordered characters) in PAUP version 3.1.1. (Swofford, 1993). Midpoint rooting was used in tree construction as no outgroup was available for rooting the phylogeny.

Restriction sites in the probe sequences. The sequences were checked for the possibility that they contained the restriction sites for the same restriction enzymes used to digest Amsinckia genomic DNA using the TACG program (Mangalem and Irvine, 1996). This information was deemed necessary for interpretation of the probing results (see below). If the probe sequence contained one or more sites for restriction enzymes used to digest the plant genomic DNA prior to probing, the bands observed when the probe was used in the Southern hybridisations would each contain a portion of the probe instead of a complete sequence. This could lead to an overestimation of copy number.

Southern transfers

Restriction enzymes that reliably cut the genomic DNA of the related outcrossing and selfing species used in this study were determined by testing several samples of the DNA with various restriction enzymes. Of the twelve restriction enzymes tested, only two completely digested the DNA of all four species reliably (e.g., without problems of partial digestion). Since only limited amounts of genomic DNA were available, all of the DNA for one individual sample was used to make up 1 mg for each individual digest. Each sample was divided into three volumes and

digested in a total volume of 200 ml, using 20-30 units of restriction enzyme. After 1 hr, an additional 5 units of enzyme was added to the sample and the reaction was allowed to continue for 12-16 hrs. After digestion, the three reaction products from a single digestion were pooled and reduced to a final volume of 50 µl. Half a µl of (10 mg/ml) RNase A was added, and the reaction mixture was incubated at 37° C for 1hr. This product was separated on a 1% agarose gel (without ethidium bromide) in 1X tris-borate EDTA (TBE) buffer at 15 V for 30-36 hrs. Following this, the DNA was transferred from the gel to a nitrocellulose membrane according to the protocol of a Southern transfer as described by Sambrook et al. (1989). The principal steps were as follows: gel washed in denaturing solution 30 min; gel placed in neutralizing solution and nitro-cellulose membrane placed in 10X SSC solution, 20 min; 10X SSC used as the transfer buffer during the 16-20 hr transfer reaction; nitro-cellulose membrane washed 15 min in 2X SSC for the stop reaction, air dried, exposed to UV-light to link DNA to membrane.

Southern hybridisation and colorimetric reaction

Southern hybridisation and the colorimetric reactions for probe detection were performed according to the protocol given by Boehringer-Mannheim with their DIG-dUTP (digoxigenin-dUTP) labelling kit. Membranes were placed in a hybridisation bag with 30 ml of prehybridisation solution for 1 hr at 37° C in a shaking waterbath. The prehybridisation solution was recovered, and 15 ml of hybridisation solution containing the DIG-dUTP labelled probe was added to the contents of the hybridisation bag. The membrane was incubated overnight at 37° C in a shaking waterbath, and the hybridisation solution was recovered. Post-hybridisation

washes were performed in 2X SSC solution. For low stringency washes, the membrane was rinsed with 2X SSC for 5 min at room temperature, then twice more at 37° C for 15 min each. For high stringency washes, the temperature of the second and third washes was set to 55° C. The membrane was then washed with Buffer 1 (0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl) for 30 min. The membrane was then coated via a 30 min wash in Buffer 2 (6.25 g powdered skim milk, 100 ml Buffer 1). A short rinse with Buffer 1 for 2 min was followed by washing the membrane with 90 ml of Buffer 1 containing 30 µl anti-digoxigenin antibody for 30 min. Two rinses, 15 min each, were performed to rinse off excess antibody from the membrane. A short soak with Buffer 3 (0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 50mM MgCl, filter sterilized through 45 µm mesh) preceded the colorimetric reaction. For the colorimetric reaction, the membrane was placed inside a clean hybridisation bag with 30 ml Buffer 3, 145 µl NBT (75 mg/ml nitroblue tetrazolium salt, 70 % dimethylformamide, 30 % ddH_2O) and 135 µl X-phosphate (50 mg/ml 5-bromo-4-chloro-3-indoyl phosphate toludinium salt, 100% dimethyl formamide). The reaction was allowed to continue overnight. Once the bands from the colorimetric reaction were sufficiently developed, the membrane was placed in stop solution (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) for 15 min after which it was dried and photographed. Since the gels used in the Southern transfer and the membranes after the colorimetric reaction were both photographed with a 1 cm ruler with 1 mm markings, the size of the bands could be determined by comparing measurements between the two pictures.

RESULTS

Probe sequences

At the time of seed collection, individual populations of Amsinckia were given identification numbers. These numbers were used in the experiments to label the probes and the membranes. Table 3 provides a summary of the identification numbers and probe sequences for each probe.

Probe production. PCR amplification of the genomic DNA from each species except one produced ample quantities of fragments of the desired size. From these species, three non-identical sequences with sequence homology to the reverse transcriptase gene fragment were used as probes. In the fourth species, only one sequence was found to be homologous to the reverse transcriptase gene. Sequence homology between sequences is presented in Table 3.

Figure 1 shows the results of the cladistic analyses of the sequences. Two most parsimonious trees were found using PAUP, both of length 491 steps, and with consistency indices of 0.60. In one tree, pol gene sequences from A. spectabilis were in a separate clade from those of A. furcata and A. vernicosa, whereas in the other tree sequences from all species fall together in several clades.

Sequence homology between the probes ranged from 0.468 to 0.789. Homology within the A. spectabilis var. spectabilis, A. spectabilis var. microcarpa and A. furcata populations were 0.726 (0.051), 0.646 (0.077) and 0.681 (0.028) respectively (standard deviation shown in brackets). Homology between related species of different mating types were as follows; between A.

spectabilis var. spectabilis and A. spectabilis var. microcarpa; 0.722 (0.028) and A. furcata and A. vernicosa; 0.694 (0.076). There was no significant difference (t-test α=0.05) in sequence homology with regard to sequences within a species, or between related and unrelated species.

Restriction sites. Two restriction enzymes were used separately to digest the plant genomic DNA. HaeIII recognizes a four base-pair sequence, GG/CC, and HindIII recognizes a six base pair sequence, A/AGCTT. All of the ten probes did not possess restriction sites for the enzyme, HindIII. The probe 8A, however, contains one restriction site for the enzyme HaeIII, but all of the other probe sequences did not possess this site.

Southern Hybridisations

Figures 2 through 5 illustrate results from the hybridisation reactions using plant genomic DNA from the two populations from A. spectabilis var. spectabilis and A. spectabilis var. microcarpa, digested with HaeIII. Figures 6 through 9 illustrate the hybridisation reactions for plant genomic DNA from the two populations of A. spectabilis var. spectabilis and A. spectabilis var. microcarpa, digested with HindIII. Figures 10 through 13 illustrate the hybridisation reactions for plant genomic DNA from the two populations from A. furcata and A. vernicosa digested with HaeIII. Figures 14 through 17 illustrate the hybridisation reactions for plant genomic DNA from the two populations of A. furcata and A. vernicosa digested with HindIII. Gels showing the digestion patterns of the genomic DNA are given in Appendix A. The size of the bands were

determined using the size marker from the gel as a standard curve. Sizes of the hybridised bands are shown in Tables 5-8.

Three to four bands were found in each hybridisation. The banding patterns observed when using plant DNA from populations within the same species were very similar to one another in size and band number. When the banding pattern between related plant species of opposing mating types were compared, hybridisations containing DNA digested with HaeIII showed similar banding patterns. The banding patterns of membranes containing DNA digested with HindIII from related species with opposing mating types also exhibited similar banding patterns.

Hybridisation at high stringency (Figs. 18-21, Table 6) produced fewer bands than observed at low stringency, and both the number and size of the bands that were hybridised at high stringency differed between mating types of related species, but in general, there were few bands in each case.

Cross hybridisation (Figs. 22-25, Table 7) gave results very similar to those produced from low stringency hybridisations, though a fragment of a particular size would not often hybridise to the foreign probe. This supports the idea that the sequences used as probes are the copia-like retrotransposon and not relics from the PCR amplification, as all of the probes hybridised to the same sequences on all membranes. Membranes containing digested DNA from A. vernicosa, the species from which only one probe was amplified, did not hybridise well to its own probe, but did hybridise well to foreign probes.

Multiple alignment of the ten probes with one another is shown in Table 8. The ten pol gene sequences are very similar to one another. Comparison of the amino acid sequences to those from Voytas (1992) and Flavell (1992a) (not shown) were also very similar, thereby establishing the fact that these probes are representative of the pol gene of the copia-like retrotransposon.

DISCUSSION

Relationships among the retrotransposon sequences

The sequences amplified from the Amsinckia populations studied show homology to copia retroelements discovered in the genomes of a large number of plant species, particularly at the amino acid sequence level (Voytas et al., 1992). This supports the assumption that the probes developed in this investigation can be used to assess retroelement copy number in Southern hybridizations of Amsinckia genomic DNA digests.

At the nucleotide level, there is considerable variation among the sequences obtained from different populations. The phylogenetic relationships among sequences in one of the two most parsimonious trees (Fig.1) parallels that of the species (and population) phylogeny obtained by earlier analysis of chloroplast DNA restriction site variation (Schoen et al., in press), suggesting that the elements are transmitted vertically. There is less support for this interpretation in the second tree, in which element sequences extracted from the same population are found together in several clades (Fig. 1). VanderWiel et al. (1993) also found incomplete congruence between

the phylogeny of copia retrotransposon sequences and that of wild cotton relatives from which the sequences were obtained. They pointed out that such incongruence need not stem from horizontal element transmission, but that sampling error, varying degrees of orthology and paralogy, as well as differential lineage loss and proliferation of elements, could obscure phylogenetic relationships based on element divergence expected under vertical transmission alone.

Copy number of retrotransposon elements and population mating system

Factors influencing the population dynamics of transposable elements have been discussed by a number of researchers (e.g., Charlesworth and Charlesworth, 1983; Werren et al., 1988; Hickey, 1993; Charlesworth et al., 1994; McDonald, 1995). While the mating system of the population has been implicated as one of several factors influencing transposon abundance, there have been few comparative investigations that have allowed this hypothesis to be explored. One notable exception, a comparison of transposon abundance in accessions of the tomato genus Lycopersicon by Young et al. (1994), revealed a higher copy number in the red- as opposed to green-fruited species. The former are largely (but not exclusively) self-compatible, whereas the latter are largely (but not exclusively) self-incompatible. This contrast in transposon abundance was interpreted as evidence that ectopic exchange (infrequent in selfers) is a factor important in inhibiting increases in copy numbers of transposons (Charlesworth and Charlesworth, 1995). The interpretation is further supported by the low number of retroelements found in the genome of the selfing species Arabadopsis thaliana (Konieczny et al., 1991), together with higher copy numbers in outcrossing and partially outcrossing species such as Zea mays (Voytas et al., 1992).

But Francis et al. (1995) argue that even among the tomato relatives they surveyed, other factors such as phylogeny, may better account for transposon abundance patterns.

Results from the present study show no clear pattern of transposon abundance in relation to plant mating system. Elements in the retrotransposon family studied in this investigation were found in low copy number throughout all the genomes assayed, and the number and the size of the fragments carrying such elements did not vary in any systematic way between populations with contrasting mating systems. The lack of a contrast in element copy number between populations with contrasting mating system, and the similarities in the hybridisation reactions among species has several possible explanations.

First, the elements probed (and their relatives) may be inactive; i.e., no longer capable of transposition. This interpretation is supported by the finding of similar banding patterns among individuals within all populations, and especially the similarity of banding patterns of individuals from populations belonging to pairs of related species. In other words, if the elements were inactive before divergence of the taxa compared, then all individuals who share a common ancestor (and in whose genome the retrotransposon became inactive) would be expected to carry retrotransposons at the same location in their genomes (as suggested by the Southern hybridization results). Retrotransposons do not encode a proof-reading mechanism in their reverse transcriptase (VanderWiel et al., 1993). Errors are introduced at a frequency of over six orders of magnitude greater than those of normal transcription rates (VanderWiel et al., 1993; Flavell et al., 1992b), thus many modifications to the retrotransposon sequence can occur.

Mutations incorporated into integrated retrotransposons could render the retrotransposon inactive and create new subfamilies of retrotransposons. On the other hand, evidence against this hypothesis comes from the similarities between the amino acid sequences of these probes and those of active elements studied by Flavell et al. (1992b) and Voytas et al. (1992), and the finding of open reading frames spanning the element sections sequenced.

A second explanation for the similarity among species in banding pattern and number is the existence of specific and conserved insertion sites in the genomes of the species studied. While there is no known specific insertion sequence for copia-like retrotransposons (Varmus and Brown, 1989), genome locations for copia-like retrotransposons have been found to differ among species, with some species having concentrations in centromeric locations and others in telomeric regions of heterochromatin (Kumar, 1996; Pearce et al., 1996b). Varmus and Brown (1989) suggest that the insertion site (chromosomal location) for the copia-like retrotransposon is specific, even though the actual sequence at the insertion sequence may show heterogeneity. Studies of a copia-like retrotransposon in barley (Hordeum vulgare L.) by Suoniemi et al. (1996) also indicate a possible specific target site for insertion in that species. If, in fact, there is a specific, conserved repeat region of retrotransposon incorporation in the Amsinckia genome, the determination of retrotransposon copy number by counting up fragments hybridising to retrotransposon probes, as done in this study, may be inadequate for estimating transposon copy number since the banding patterns observed would be similar, even among plants with differing numbers of elements. Instead, a method for quantifying the number of elements in each band would be needed. The latter were, however, beyond the scope of the present investigation.

Technical problems and the estimation of element copy number

Several technical challenges were encountered during this investigation that may have interfered with the accurate assessment of retroelement copy number in the different populations of Amsinckia studied. These are discussed individually in the paragraphs below.

First, given the diversity of sequences observed for this retrotransposon family, the entire complement of the copia-like retrotransposons may not have been be fully revealed by use of a few probe sequences in each population. Since a greater number of sequences were recognised by probing at lower stringency, it is possible that there are additional (i.e., more divergent) copia retroelement sequences present in the Amsinckia populations studied that were not revealed by the Southern hybridizations. This could interfere with accurate enumeration of copy number.

Second, the retrotransposon copy number estimated for plants in each population was determined under the assumption that each of the fragments that the probe hybridised to correspond with the presence of a single element in the retrotransposon family, or that the fragments did not differ greatly in numbers of element copies present. This assumption could not be investigated given the time constraints of the investigation. If it is not correct, it may obscure possible differences in copy number between populations.

Third, in Amsinckia vernicosa, it was difficult to obtain probes corresponding to the pol gene (few PCR products of the size expected for the conserved region of the pol gene (approximately 250 to 300 bp) were amplified). Similar problems were reported by Flavell et al. (1992a, 1992b)

and Voytas et al. (1992). The use of only a single probe derived for examining copy number in this species could lead to underestimation of the number of copia elements present in the genome if it was more distantly related to the overall diversity of the element family compared with other probes used in this investigation. However, comparisons among the sequences of the probes reveal that the single A. vernicosa probe used is no less similar in homology than are the other probes with each other.

Finally, there is the assumption of each band representing a single copy of the retrotransposon. This was untrue in the case of one of the ten probes (from A. furcata) which was found to contain a site for HaeIII, also used in the plant genomic DNA digests. This could have artificially increased the number of bands observed when the HaeIII digests of A. furcata and A. vernicosa were hybridised with their respective probes.

Conclusions and suggestions for future work

The copia element family was selected as the focal group of study because it is well studied, abundant throughout the plant kingdom, and because of the availability of published primer sequences that facilitated cloning (Voytas et al., 1992). It was, therefore, possible to proceed with the investigation with assurance that clones for transposons could be made available. But the work described was initiated on the assumption that elements cloned represent sequences from active transposable elements, or at least that elements could be cloned that may be presently inactive but that had been active for some of the evolutionary history following divergence of

related selfing and outcrossing populations a common ancestor--i.e., elements whose distribution and abundance could potentially be influenced by the mating system. In retrospect, this task may have proved more difficult than anticipated at the start, as there rests some uncertainty as to whether the elements studied were active or inactive. Future tests of the hypothesis that the mating system can influence copy number may best be carried out after recognising and cloning elements observed to actively transpose (e.g., following crosses), and by using techniques that permit the number of the elements to be more accurately assessed.

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Internet sites

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ALIGN from Indiana University :

http://iubio.bio.indiana.edu/soft/molbio/align

BLASTX from National Centre for Biotechnology Information - GenBank :

http://www.ncbi.nlm.nih.gov/BLAST

CLUSTALW from Biology Workbench:

http://biology.ncsa.uiuc.edu

TACG from Biology Workbench:

http://biology.ncsa.uiuc.edu

WebTrans from Virtual Genome Centre:

http://alces.med.umn.edu/cuse.html

Figures 1 to 25

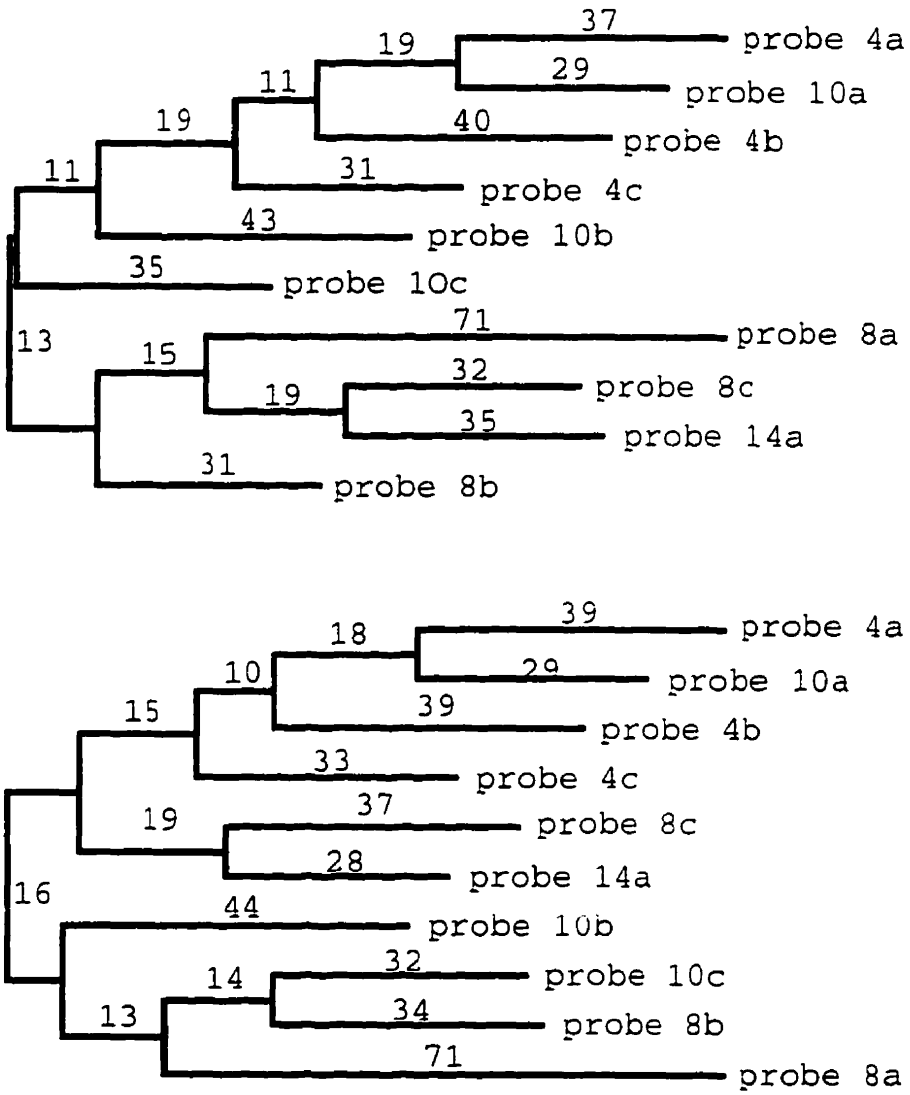


Figure 1.

The two most parsimonious trees for probes derived from conserved regions of the pol gene in four populations of Amsinckia (See Table 2 for details of species origin and base sequence). Numbers along branches indicate base changes.

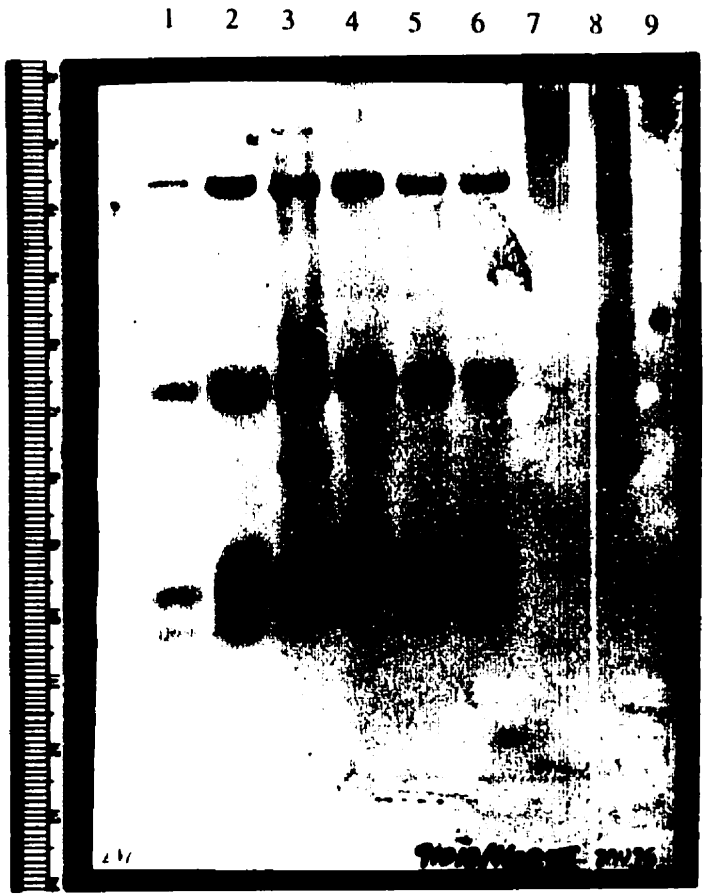


Figure 2.

DNA from Amsinckia spectabilis var. spectabilis digested with HaeIII, hybridised to probe 10A.
lanes 1-8: A. spectabilis var. spectabilis DNA
lane 9: 1 kb marker

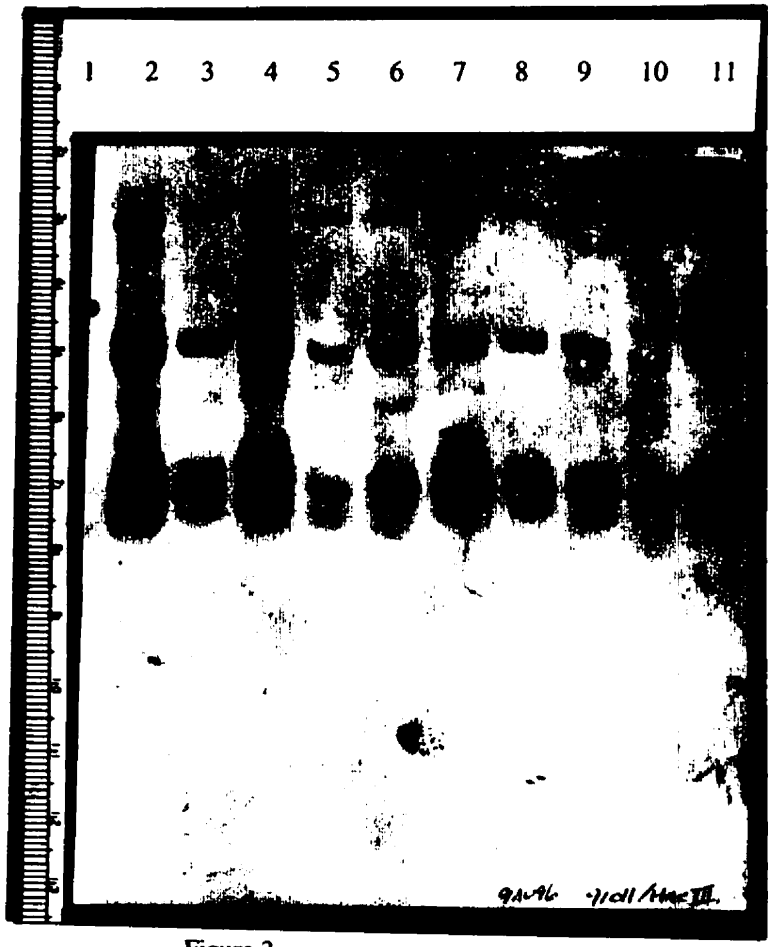


Figure 3.

DNA from Amsinckia spectabilis var. spectabilis digested with HaeIII, hybridised to probe 10C.
lane 1: 1 kb marker
lanes 2-11: A. spectabilis var. spectabilis DNA

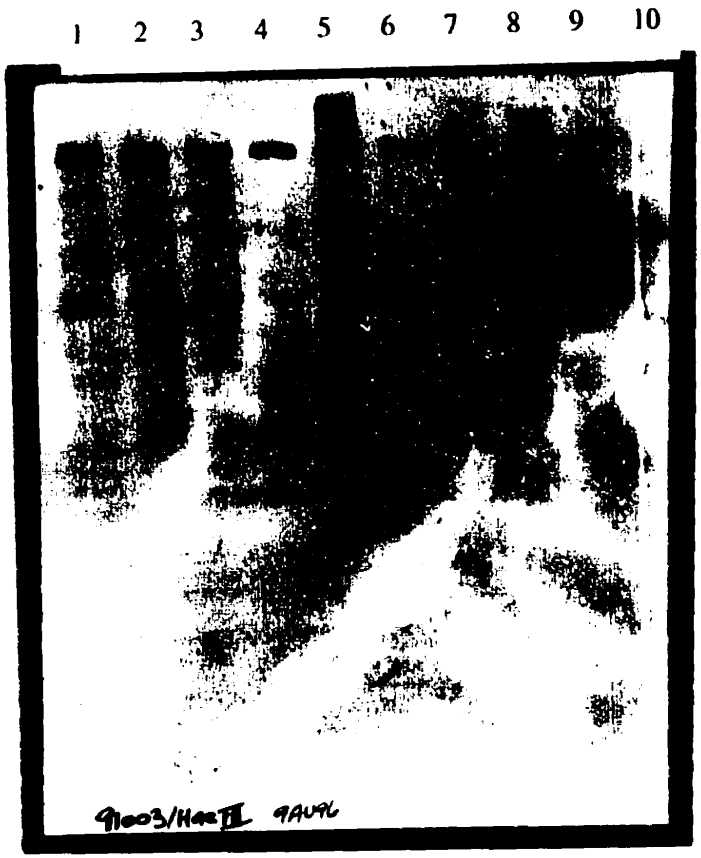


Figure 4.

DNA from Amsinckia spectabilis var. microcarpa digested with HaeIII, hybridised to probe 4A.
lanes 1-9: A. spectabilis var. microcarpa DNA
lane 10: 1 kb marker

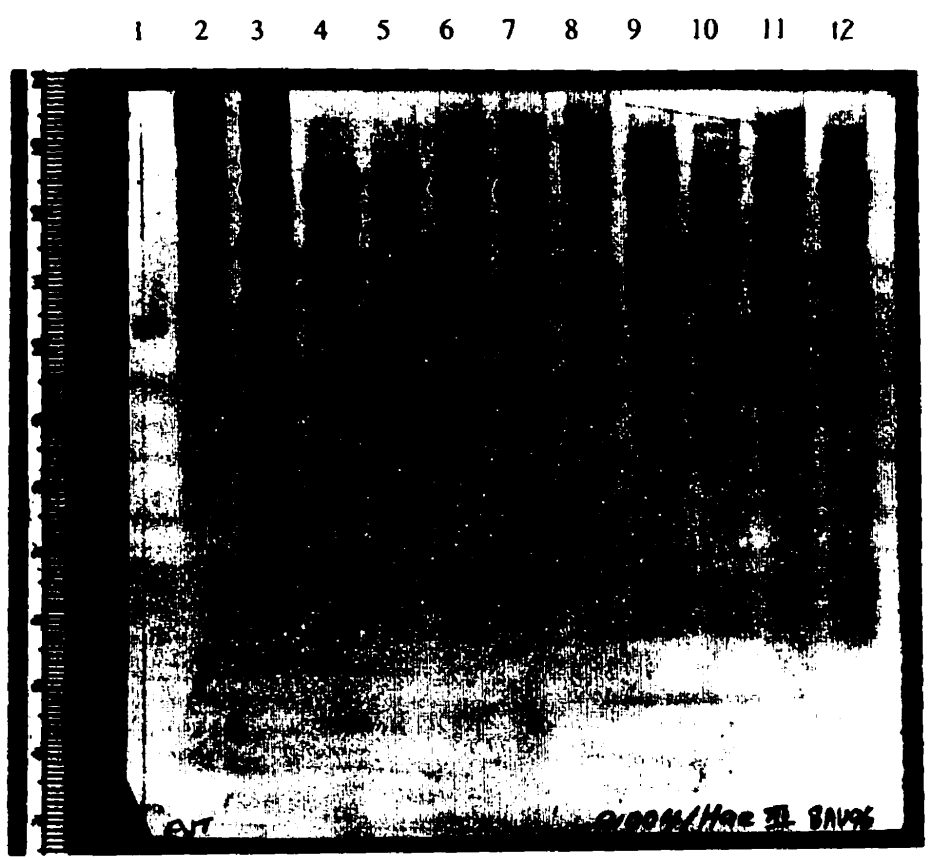


Figure 5.

DNA from Amsinckia spectabilis var. microcarpa digested with HaeIII, hybridised to probe 4A.
lane 1: 1 kb marker
lanes 2-12: A. spectabilis var. microcarpa DNA

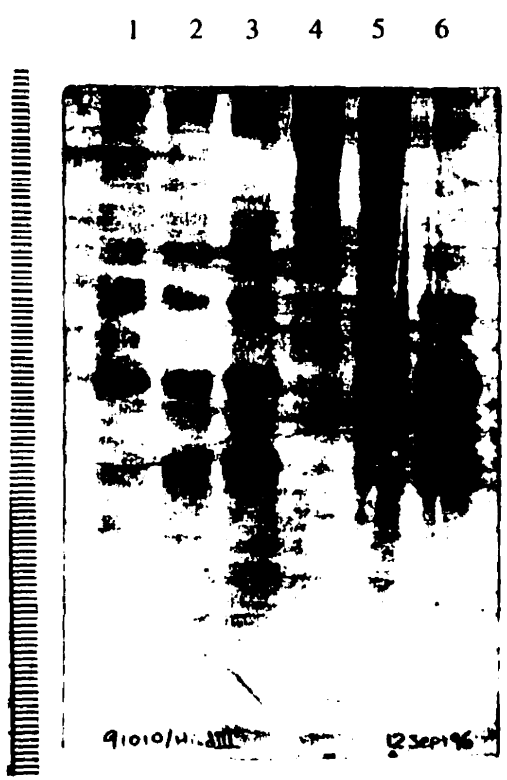


Figure 6.

DNA from Amsinckia spectabilis var. spectabilis digested with HindIII, hybridised to probe 10A.
lanes 1-6: A. spectabilis var. spectabilis DNA

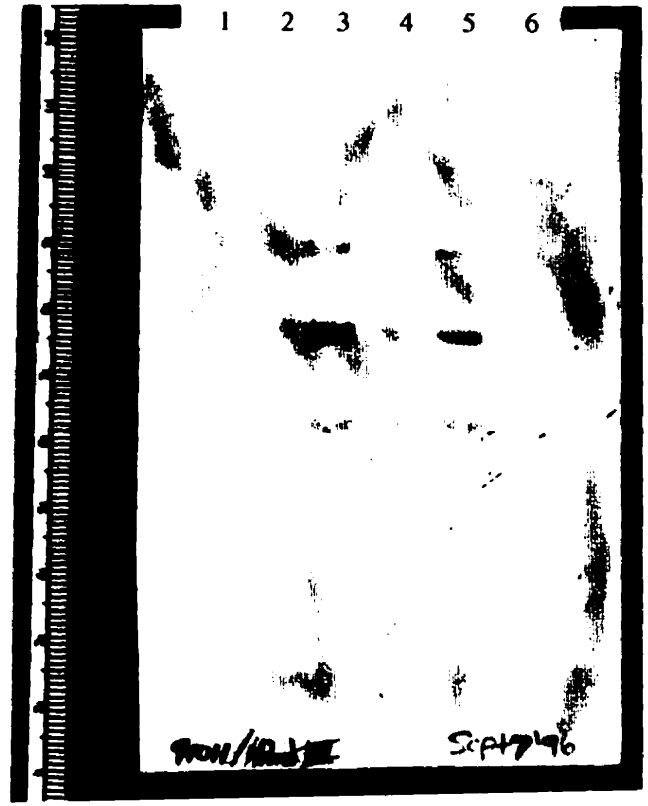


Figure 7.

DNA from Amsinckia spectabilis var. spectabilis digested with HindIII, hybridised to probe 10A.
lanes 1-6: A. spectabilis var. spectabilis DNA

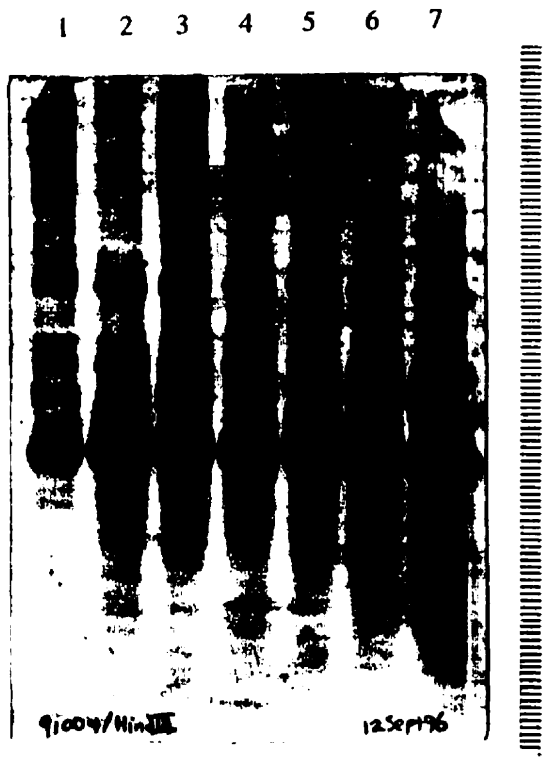


Figure 8.

DNA from Amsinckia spectabilis var. microcarpa digested with HindIII, hybridised to probe 4A.
lanes 1-7: A. spectabilis var. microcarpa DNA

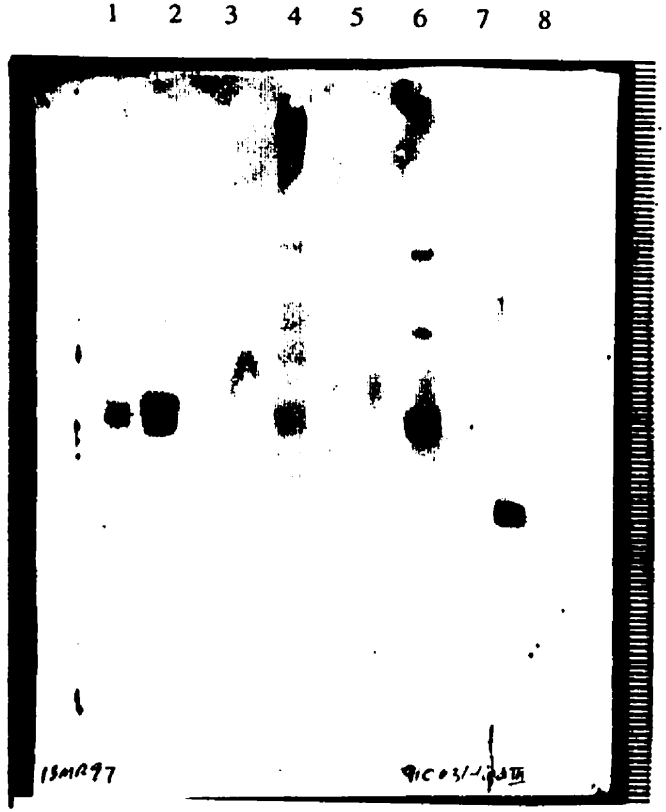


Figure 9.

DNA from Amsinckia spectabilis var. microcarpa digested with HindIII, hybridised to probe 4A.
lanes 1-7: A. spectabilis var. microcarpa DNA
lane 8: 1 kb marker

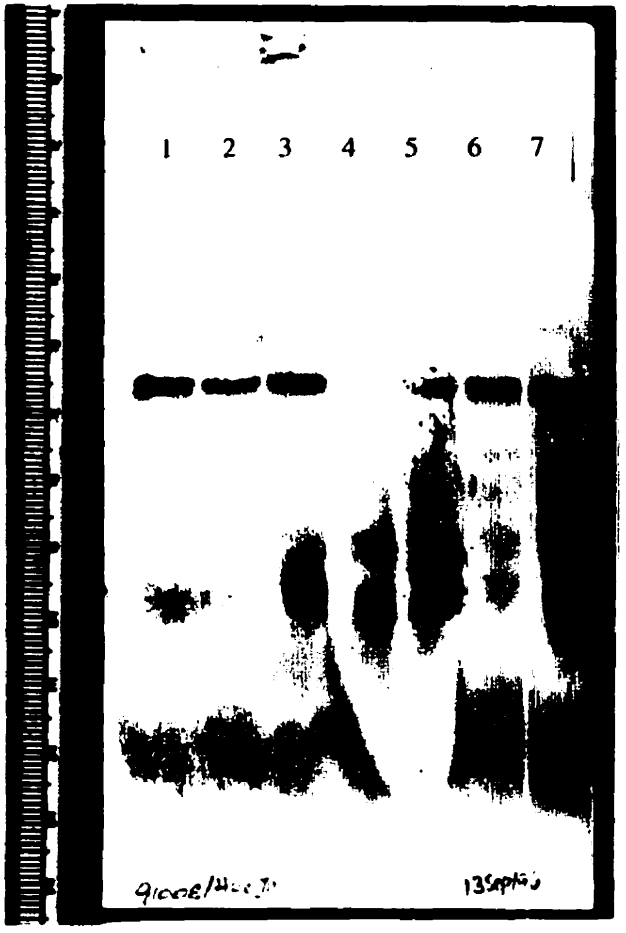


Figure 10.

DNA from Amsinckia furcata digested with HaeIII, hybridised to probe 8A.
lanes 1-7: A. furcata DNA

Figure 11.

DNA from Amsinckia furcata digested with HaeIII, hybridised to probe 8B.
lanes 1-7: A. furcata DNA

Figure 12.

DNA from Amsinckia vernicosa digested with HaeIII, hybridised to probe 14A.
lanes 1-7: A. vernicosa DNA

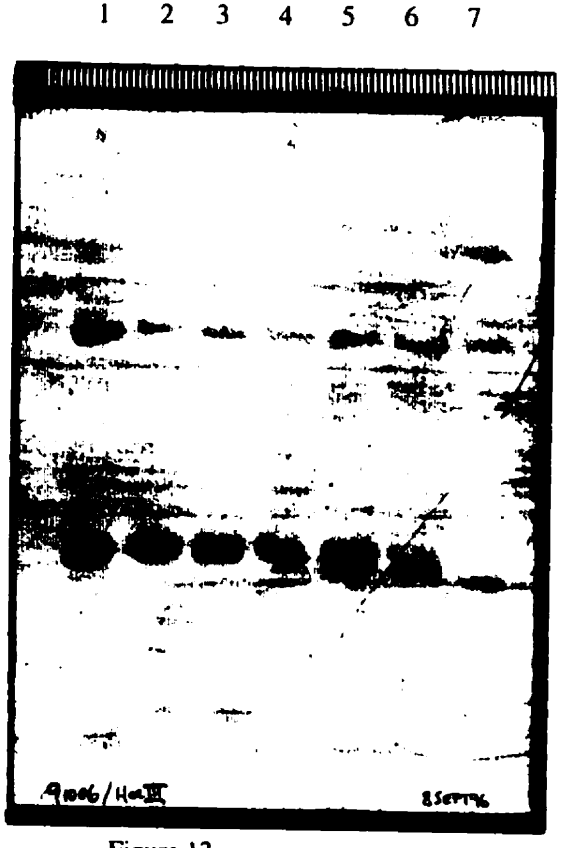


Figure 13.

DNA from Amsinckia vernicosa digested with HaeIII, hybridised to probe 14A.
lanes 1-7: A. vernicosa DNA

Figure 14.

DNA from Amsinckia furcata digested with HindIII, hybridised to probe 8C.
lanes 1-6: A. furcata DNA

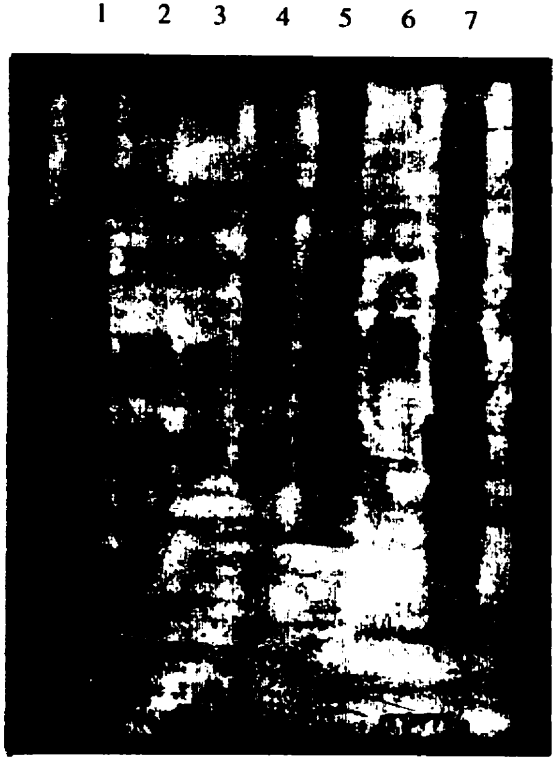


Figure 15.

DNA from Amsinckia furcata digested with HindIII, hybridised to probe 8C.
lanes 1-7: A. furcata DNA

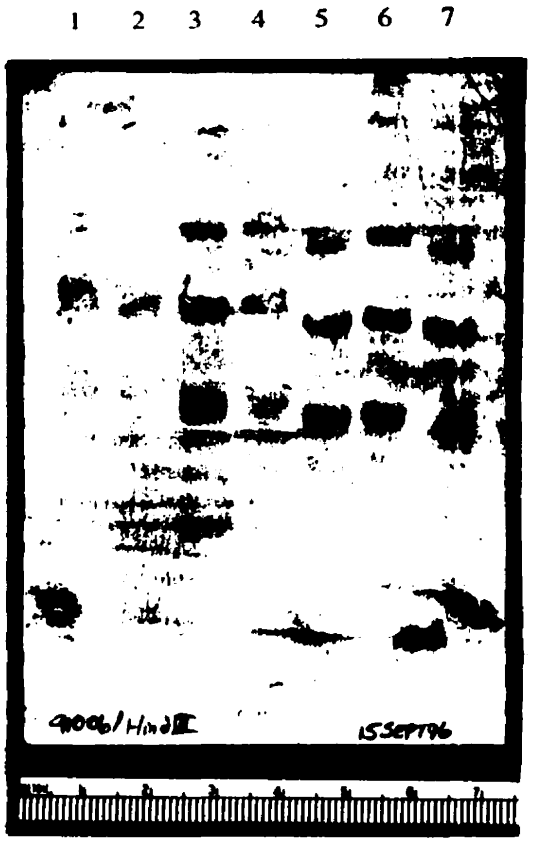


Figure 16.

DNA from Amsinckia vernicosa digested with HindIII, hybridised to probe 14A.
lanes 1-7: A. vernicosa DNA

Figure 17.

DNA from Amsinckia vernicosa digested with HindIII, hybridised to probe 14A.
lanes 1-9: A. vernicosa DNA

Figure 18.

DNA from Amsinckia spectabilis var. spectabilis digested with HindIII, hybridised to the probe 10B at high stringency.
lanes 1-6: A. spectabilis var. spectabilis DNA

Figure 19.

DNA from Amsinckia spectabilis var. microcarpa digested with HindIII, hybridised to the probe 4C at high stringency.
lanes 1-7: A. spectabilis var. microcarpa DNA

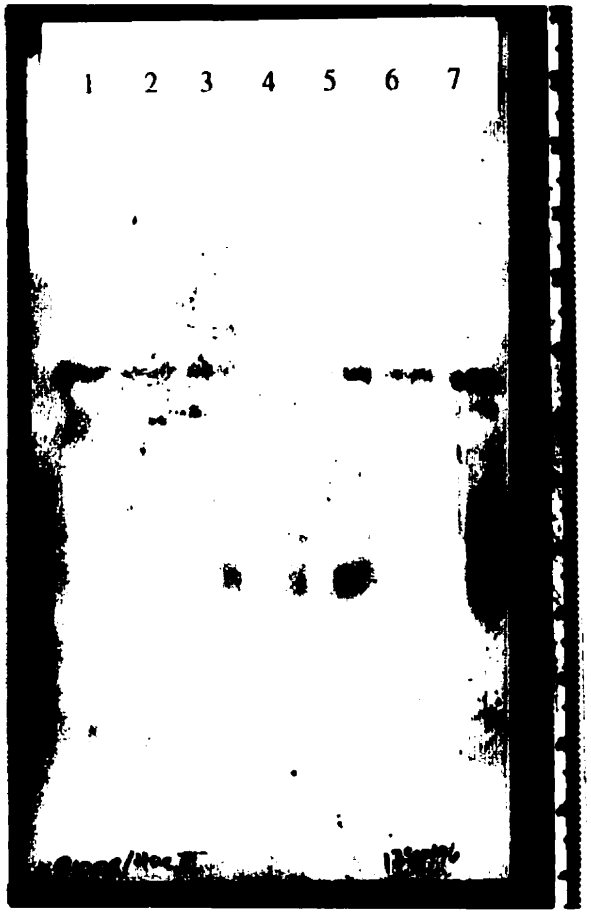


Figure 20.

DNA from Amsinckia furcata digested with HaeIII, hybridised to the probe 8C at high stringency.
lanes 1-7: A. furcata DNA

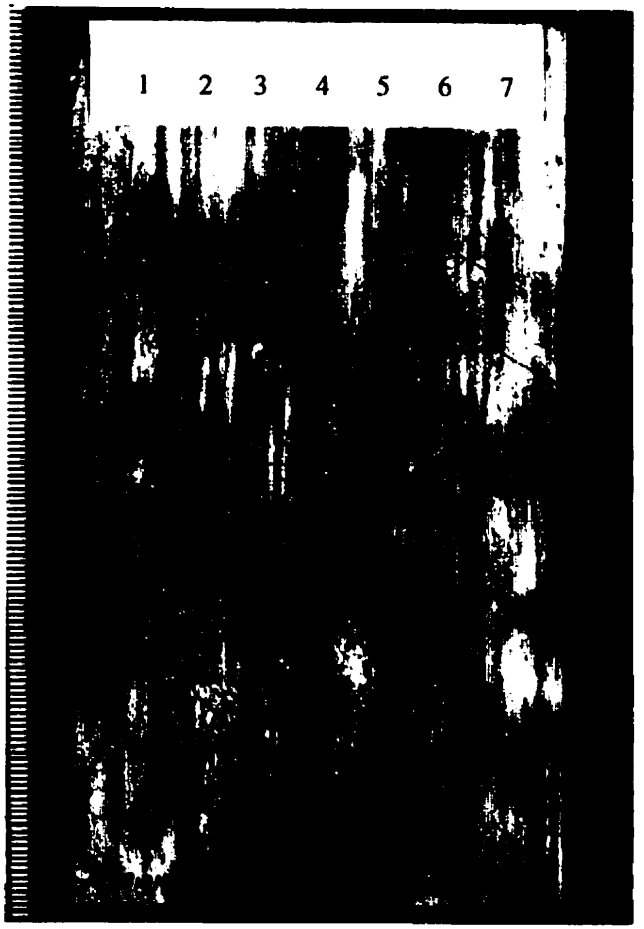


Figure 21.

DNA from Amsinckia vernicosa digested with HaeIII, hybridised to the probe 14A at high stringency.
lanes 1-7: A. vernicosa DNA

Figure 22.

DNA from Amsinckia spectabilis var. spectabilis digested with HindIII, hybridised to the probe to the probe 8A, from Amsinckia furcata.
lanes 1-6: A. spectabilis var. spectabilis DNA

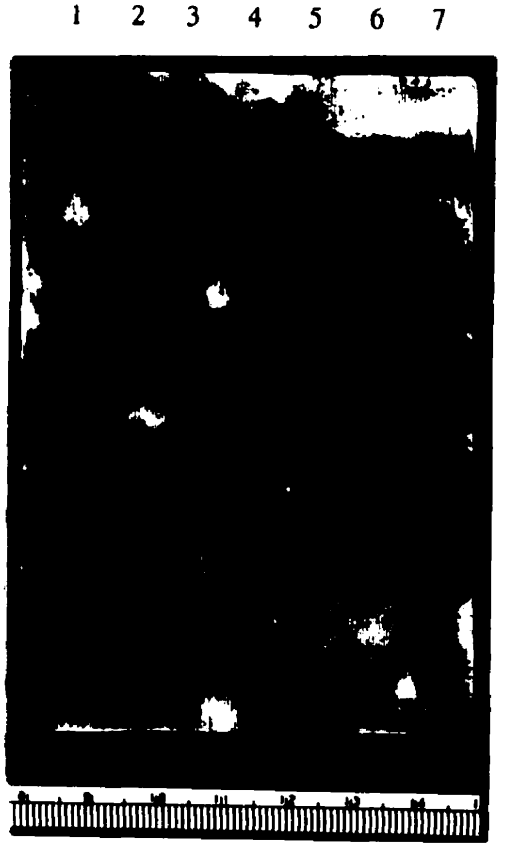


Figure 23.

DNA from Amsinckia spectabilis var. microcarpa digested with HindIII, hybridised to the probe 8A, from Amsinckia vernicosa.
lanes 1-7: A. spectabilis var. microcarpa DNA

Figure 24.

DNA from Amsinckia furcata digested with HindIII, hybridised to the probe 10B, from Amsinckia spectabilis var. spectabilis.
lanes 1-7: A. furcata DNA

Figure 25.

DNA from Amsinckia vernicosa digested with HindIII, hybridised to the probe 4A, from Amsinckia spectabilis var. microcarpa.
lanes 1-7: A. vernicosa DNA

Tables 1-9

Table 1 Selection of transposon copy number in selfing and outcrossing plant populations

Selective Factor	Mating system of the population	
	Self-fertilising	Cross-fertilising
Mutations caused by transposon insertion in/near functional genes	Strong selection against transposons and high rates of transposition due to homozygosity of mutations.	Weaker selection against transposons and high rates of transposition (due to the heterozygosity of the majority of transposon-induced mutations).
Ectopic exchange	Weak selection against transposons and high rates of transposition, as most elements should be present in homologous chromosomal positions.	Strong selection against transposons and high rates of transposition, as many elements should be present in non-homologous chromosomal positions.
Transmission of transposons between individuals	Strong selection against high rates of transposition, as lineages containing transpositionally-active elements will be more prone to extinction.	Weaker selection against high rates of transposition, as there are few discrete lineages and transpositionally-active lineages will not be confined to single lines.

Table 2 Species and populations of Amsinckia studied

species	population number	mating system
A. spectabilis var. spectabilis	91010	self-pollinating (homostylous)
A. spectabilis var. spectabilis	91011	self-pollinating (homostylous)
A. spectabilis var. microcarpa	91004	cross-pollinating (heterostylous)
A. spectabilis var. microcarpa	91003	cross-pollinating (heterostylous)
A. vernicosa	91014	self-pollinating (homostylous)
A. vernicosa	91006	self-pollinating (homostylous)
A. furcata	91008	cross-pollinating (heterostylous)
A. furcata	91007	cross-pollinating (heterostylous)

Table 3 Probes isolated from Amsinckia taxa for determining copy number of copia retroelements

Probe	Isolated from	Base sequence *
4A	A. spectabilis var. microcarpa (population 91004)	GCATGGTGATTTAGAAGAGGACATCCATATGGAAC AACCCGACGGCTTTTGTGTGAAAGGATAG**GAGGAG TATGTGTGTAAACTGCTAAAGTCCCTCTACGGCT TAAGCAAGCACCTCGACAATGGTACAGAAAATTT GATCAATTTATTTGATCATGGATTCAATAAAACA AAGATAGACTCTTGTGTATTCGTCAAATGGCTGG AAGAGGATAACTTCATCATATTG**
4B	A. spectabilis var. microcarpa (population 91004)	**GCATGGGGATCTTGAAGAGGACATACACATGGA ACAACCTGATGGCTTCTATGTCAAGGGGAAAGA ACATCTGGTTTGCAAGCTACAAAAATCATTGTAT GGACTCAAACAAGCCCCTCGTCAGTGGTATAAG AAATTCGATCAATTCATGAGTGACATGATCAGCT** AGACTTAGATGAACCCTGAGTCTCATGTAGAGAAT GATGCAGMGATCATCTACTACTACT
4C	A. spectabilis var. microcarpa (population 91004)	**ACATGAAGACTTAGAAGAGAACATTCATATGGA ACAACCAGATGGATTTATTGCTGAAGGGAAGGA ACATCTTGTTTGCAAGCTGCAAAATCCTTGTATG GTCTTAAACAAGCACCACGTCAGTGTAATTAAGA AATTTGAGCAATTAATGACAGAACAAGAGTTCAA GAGAACTAACGTTGATCACTGTGTTTTCTTGAAA AACTATGATGATGGGAGCTTCATTATTCTAC**

* open reading frame shown in boldface type

Table 3 (cont'd) Probes isolated from Amsinckia taxa for determining copy number of copia retroelements

Probe	Isolated from	Base sequence *
10A	A. spectabilis var. spectabilis (population 91010)	**ACATGGAGACCTGGAAGAAGACATTCATATGGA GCAACCTGATGGTTTTTATGTTAAAGGAAAGAAG AATTTTGTGTGTCAAACTACAACAATCCCTATAT GGACTCAAGCAAACTACATGACAGTGGTATAAG AAATTCGATCAGTTTATGGGGGAGCATGGCTTC AACAAAACTCAAA**TAGATGCTTGTGTGTTCATTAA GAGACTGAAATGTGACAACTTCATTTTATTACTACT CTACGC
10B	A. spectabilis var. spectabilis (population 91010)	**GCATGGTGAGCTTGAAGAAAATATCTTTATGGAA CAACCACTGGGTTTTGTGGAAGAAGGGAAGGAA GAATGGGTCTGTAAATTGAACAGGAGCTTGTAT GGACTAAAACAAGCACCAAGGCAATGGTACAAA AGATTTGATGCCTTCATGCTTGACCAAGAATTCA AGAAAACACTTTCAGACCACTGTGTATTCATCAA AAGATTTGACTCAGGTGATTTCTTGATACTCC**
10C	A. spectabilis var. spectabilis (population 91010)	**GCATGGTGAGTTAGATGAGGAAATATACATGGA ACAACCATTAGGATTCATTAAGGAAGGTGAGGA AGAGCTTGTGTGTAAATTAACTAAGAGTTTATAC GGCTTGAAACAAGCTCCTCGACAATGGTATAGG AAGTTTGATTCGTTTATGCTTAAACATGATTACA AAAGAACACTAGTTGATCACTGTGTTTTTGTTAA AAGATTTGGGCAAAACGATTTCATTATCCTAC**

* open reading frame shown in boldface type

Table 3 (cont'd) Probes isolated from Amsinckia taxa for determining copy number of copia retroelements

Probe	Isolated from	Base sequence *
8A	A. furcata (population 91008)	GCACGGGGACTTACATGAAGAGGTATACATGCGAAT **ACCACAAGGTTACGATAAAGGGGGAGGAACAAAAGT GTGTANGCTAAGGAAGTCGCTATATGGGTTGAAGCA GGCCTCAAGAAATTGGTATCAAAAATCTACATCAGCC CTTTTGGAACTCAGTTACAAGCAATCCGCTGCAGACC ACTCGCTTCTTATATACAAGGAANGAAGCACCTTTGT** CACCGCAC
8B	A. furcata (population 91008)	GCATAGGGATTTAGATGAAAACATCTACATGAAACAG **CCTTTAGGGTTTATAGAAGAGGGAAAAGAAGAGTTA GTTTGCAAGTTGAACAAGAGTTTGTATGGCTTGCAAC AAGCCTCTGGGCAATGGTATCGAAAGTTTGATTCATT CATGTTGAAGCACGATTATAAAAGGACCTTAGTGGAT CATTGTGTATTCATTAAGAAATTTGAACAATCTGATT** TTATCATACTTC
8C	A. furcata (population 91008)	ACATGGAGATTTGGATGAGGAGATTTATATGGAGCA **ACCAGAAGGTTTTATTGTTAAGGGAAAAGAGTCGTT AGTTTGCAGATTGCAGAAAAGTCTCTATGGGTTGAA GCAAGCTCCCAGGCAATGGTATCTTAAGTTTGATGG ATTCATGGTGAGTCAAGGCTTTGTAAGAGCTGCTATT GATCACTGTGTGTATCTCAAAAGACTTGAAAATGAAA** GTTTCATTATCTTGT
14A	A. vernicosa (population 91014)	ACATGGCGAGTTGGACGAGGAGATTTACATGGAACA **ACCTGATGGATTTGTCGTTAGAGGCAAGGAATCTAT GGTTTGCAAGTTACAGAAAAGCCTTTATGGTTTGAAA CAGGCTCCAAGGCAGTGGTACTTGAAGTTTGAAGGA TTTATGATCAATCAAGGTTTCTGCAAAATCATTATTG AACATTGTGTGTTTATGAAGACATTTGAAGATAGAAG** TTTCATTATATTGC

* open reading frame shown in boldface type

Table 4. Comparison of sequence homology for probes isolated within and between species of Amsinckia

	Probes from A. spectabilis*					A. furcata and A. vernicosa*			
	4B	**4C**	**10A**	**10B**	**10C**	**8A**	**8B**	**8C**	**14A**
4A	.687o	.708	.733	.715	.730	.626	.656	.756	.670
4B	-	.784	.789	.696	.700	.604	.736	.727	.727
4C	-	-	.725	.708	.704	.537	.468	.738	.730
10A	-	-	-	.674	.657	.621	.682	.691	.708
10B	-	-	-	-	.712	.573	.712	.670	.682
10C	-	-	-	-	-	.639	.747	.708	.708
8A	-	-	-	-	-	-	.568	.648	.621
8B	-	-	-	-	-	-	-	.721	.687
8C	-	-	-	-	-	-	-	-	.773

* Probe origins given in Table 2.
o Numbers of homologous nucleotides over the total number of nucleotides found in the shorter probe sequence.

Table 5 Fragment sizes of bands which hybridised in A. spectabilis taxa

Species	Genomic DNA digest (Population/restriction enzyme)	Copia-retrotransposon probe	Fragment sizes (kb)
A. spectabilis var. spectabilis	91010/HaeIII	10A	3.94, 1.21 0.86
A. spectabilis var. spectabilis	91011/HaeIII	10C	4.52, 1.20, 0.65, 0.58
A. spectabilis var. microcarpa	91003/HaeIII	4A	4.52, 1.11, 0.64
A. spectabilis var. microcarpa	91004/HaeIII	4A	5.91, 5.08, 3.94, 1.16, 0.90
A. spectabilis var. spectabilis	91010/HindIII	10A	6.36, 5.15, 3.58, 2.42
A. spectabilis var. spectabilis	91011/HindIII	10A	5.00, 3.37, 2.21
A. spectabilis var. microcarpa	91004/HindIII	4A	5.47, 4.30, 3.81, 3.27, 2.40
A. spectabilis var. microcarpa	91003/HindIII	4A	5.86, 4.10, 2.73

Table 6 Fragment sizes of bands which hybridised in A. vernicosa and A. furcata

Species	Genomic DNA digest (Population/restriction enzyme)	Copia retrotransposon probe	Fragment sizes (kb)
A. furcata	91008/HaeIII	8A	4.44, 2.00, 1.65
A. furcata	91007/HaeIII	8B	4.84, 3.64, 2.22
A. vernicosa	91014/HaeIII	14A	4.3, 2.9, 1.7
A. vernicosa	91006/HaeIII	14A	3.64, 1.32
A. furcata	91008/HindIII	8C	4.07, 2.80, 1.80
A. furcata	91007/HindIII	8C	4.82, 3.83, 2.60
A. vernicosa	91006/HindIII	14A	5.44, 3.52, 2.50
A. vernicosa	91014/HindIII	14A	5.80, 4.07, 2.70

Table 7 Fragment sizes of bands which hybridised using high stringency hybridisation reactions

Species	Genomic DNA digest (Population/restriction enzyme)	Copia retrotransposon probe	Fragment sizes (kb)
A. spectabilis var. spectabilis	91010/HindIII	10B	5.00, 3.58, 2.42
A. spectabilis var. microcarpa	91004/HindIII	4C	5.15, 3.69
A. furcata	91008/HaeIII	8C	1.8, 0.64
A. vernicosa	91014/HaeIII	14A	1.62, 0.58

Table 8 Fragment sizes of bands which hybridised to membranes containing foreign DNA

Species	Genomic DNA digest (Population/restriction enzyme)	Copia retrotransposon probe	Fragment sizes (kb)
A. spectabilis var. spectabilis	91010/HindIII	8A	5.00, 3.48, 2.35
A. spectabilis var. microcarpa	91004/HindIII	8A	5.15, 3.48
A. furcata	91007/HindIII	10B	4.37, 2.77, 2.01, 1.60
A. vernicosa	91006/HindIII	4A	6.45, 5.58, 4.28, 2.98

Table 9 Alignment of probe sequences for A. spectabilis var microcarpa (91004), A. spectabilis var spectabilis (91010), A. furcata (91008) and A. vernicosa (91014).

```
Probe: sequence*
                1         2         3         4         5
91004a:GCATGGTGATTTAGAAGAGGACATCCATATGGAACAACCCGACGGCTTTTGTG
91004b:GCATGGGGATCTTGAAGAGGACATACACATGGAACAACCTGATGGCTTCTATG
91004c:ACATGAAGACTTAGAAGAGAACATTCATATGGAACAACCAGATGGATTTATTG
91010a:ACATGGAGACCTGGAAGAAGACATTCATATGGAGCAACCTGATGGTTTTTATG
91010b:GCATGGTGAGCTTGAAGAAAATATCTTTATGGAACAACCACTGGGTTTTGTGG
91010c:GCATGGTGAGTTAGATGAGGAAATATACATGGAACAACCATTAGGATTCATTA
91008a:GCACGGGGACTTACATGAAGAGGTATACATGCGAATACCACAAGGTT---ACG
91008b:GCATAGGGATTTAGATGAAAACATCTACATGAAACAGCCTTTAGGGTTTATAG
91008c:ACATGGAGATTTGGATGAGGAGATTTATATGGAGCAACCAGAAGGTTTTATTG
91014a:ACATGGCGAGTTGGACGAGGAGATTTACATGGAACAACCTGATGGATTTGTCG
             6         7         8         9         0
91004a:TGAAAGGATAGGAGGAGTATGTGTGTAAACTGCTAAAGTCCCTCTACGGCTT-
91004b:TCAAGGGGAAAGAACATCTGGTTTGCAAGCTACAAAAATCATTGTATGGACTC
91004c:CTGAAGGGAAGGAACATCTTGTTTGCAAGCTGCAAAA-TCCTTGTATGGTCTT
91010a:TTAAAGGAAAGAAGAATTTTGTGTGCAAACTACAACAATCCCTATATGGACTC
91010b:AAGAAGGGAAGGAAGAATGGGTCTGTAAATTGAACAGGAGCTTGTATGGACTA
91010c:AGGAAGGTGAGGAAGAGCTTGTGTGTAAATTAACTAAGAGTTTATACGGCTTG
91008a:ATAAAGGGGGAGGAACAAAAGTGTGTANGCTAAGGAAGTCGCTATATGGGTTG
91008b:AAGAGGGAAAAGAAGAGTTAGTTTGCAAGTTGAACAAGAGTTTGTATGGCTTG
91008c:TTAAGGGAAAAGAGTCGTTAGTTTGCAGATTGCAGAAAAGTCTCTATGGGTTG
91014a:TTAGAGGCAAGGAATCTATGGTTTGCAAGTTACAGAAAAGCCTTTATGGTTTG
          1         2         3         4         5
91004a:AAGCAAGCACCTCGACAATGGTAC-AGAAAATTTGATCAATTTAT-TTG-ATC
91004b:AAACAAGCCCCTCGTCAGTGGTAT-AAGAAATTCGATCAATTCATGAGTGA-C
91004c:AAACAAGCACCACGTCAGTGTAATTAAGAAATTTGAGCAATTAATGACAGAAC
91010a:AAGCAAACTACATGACAGTGGTAT-AAGAAATTCGATCAGTTTATGGGGGAGC
91010b:AAACAAGCACCAAGGCAATGGTAC-AAAAGATTTGATGCCTTCATGCTTGACC
91010c:AAACAAGCTCCTCGACAATGGTAT-AGGAAGTTTGATTCGTTTATGCTTAAAC
91008a:AAGCAGGCCTCAAGAAATTGGTAT-CAAAAATCTACATCAGCCCTTTTGGAAC
91008b:CAACAAGCCTCTGGGCAATGGTAT-CGAAAGTTTGATTCATTCATGTTGAAGC
91008c:AAGCAAGCTCCCAGGCAATGGTAT-CTTAAGTTTGATGGATTCATGGTGAGTC
91014a:AAACAGGCTCCAAGGCAGTGGTAC-TTGAAGTTTGAAGGATTTATGATCAATC
```

*open reading frames marked in boldface

Table 9 (cont'd) Alignment of probe sequences for A. spectabilis var microcarpa (91004), A. spectabilis var spectabilis (91010), A. furcata (91008) and A. vernicosa (91014)

```
Probe: sequence *
       6          7          8          9          0          1
91004a:ATGGATTCAATAAAACAAAGATAGACTCTTGTGTATTCGTCAA---AATGGCT
91004b:ATGA--TCAGCTAGACTTA-GATGAACCCTGAGTCTCATGTAGAG-AATGAT-
91004c:AAGAGTTCAAGAGAACTAACGTTGATCACTGTGTTTTCTTG----AAAAACTA
91010a:ATGGCTTCAACAAAACTCAAATAGATGCTTGTGTGTTCATT----AAGAGACT
91010b:AAGAATTCAAGAAAACACTTTCAGACCACTGTGTATTCATC-----AAAGATT
91010c:ATGATTACAAAAGAACACTAGTTGATCACTGTGTTTTTGTT----AAAAGATT
91008a:TCAGTTACAAGCAATCCGCTGCAGACCACTCGCTTCTTATATAC-AAGGAAN-
91008b:ACGATTATAAAAGGACCTTAGTGGATCATTGTGTATTCATT----AAGAAATT
91008c:AAGGCTTTGTAAGAGCTGCTATTGATCACTGTGTGTATCTC----AAAAGACT
91014a:AAGGTTTCTGCAAAATCATTATTGAACATTGTGTGTTTATG----AAGACATT
               2          3          4
91004a:-----------GGAAGAGGATAACTTCATCATATTG
91004b:-----GCAGNGATCATCTACTACTACT---------
91004c:TGATGATGGGAGCTTCATTATTC-------------
91010a:GAAATGTGACAACTTCATTTTATTACTACTCTACGC
91010b:TGACTCAGGTGATTTCTTGATACTCC----------
91010c:TGGGC-AAAACGATTTCATTATCCTAC---------
91008a:-----GAAGCACCTTTGTCACCGCAC----------
91008b:TGA-----ACAATCTGATTTTATCA-----------
91008c:TGAAAATGAAAGTTTCATTATCTTGT----------
91014a:TGAAGATAGAAGTTTCATTATATTGC----------
```

* open reading frames are marked in boldface type

APPENDIX

Figures A1-A8

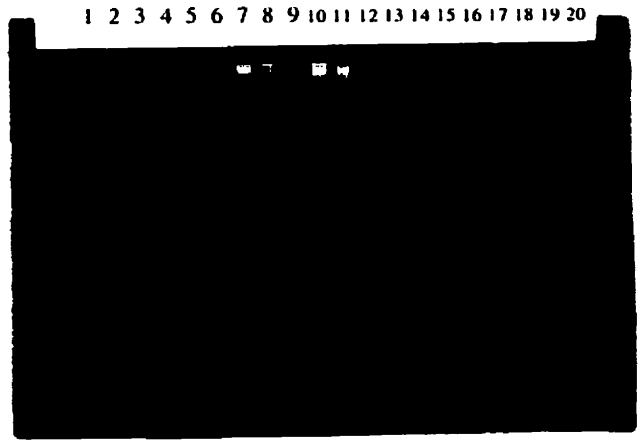


Figure A1.

HaeIII-digested DNA from Amsinckia spectabilis var. spectabilis and Amsinckia spectabilis var. microcarpa. lanes 1-8: A. spectabilis var. spectabilis (91010)
lane 9: 1 kb marker
lane 10-20: A. spectabilis var. microcarpa (91004)

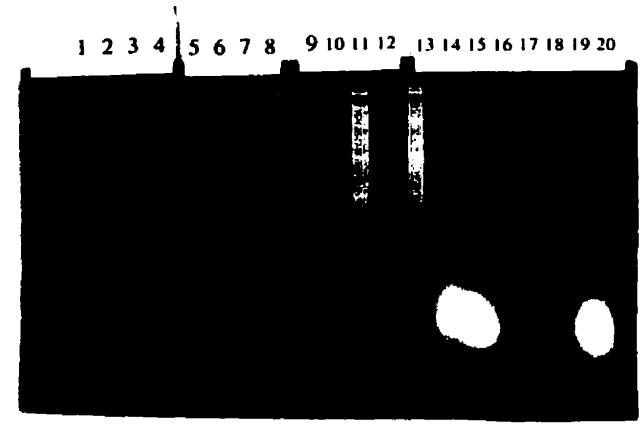


Figure A2.

HaeIII-digested DNA from Amsinckia spectabilis var. spectabilis and Amsinckia spectabilis var. microcarpa.
lanes 1-9: A. spectabilis var. microcarpa (91003)
lane 10: 1 kb marker
lanes 11-20: A. spectabilis var. spectabilis (91011)

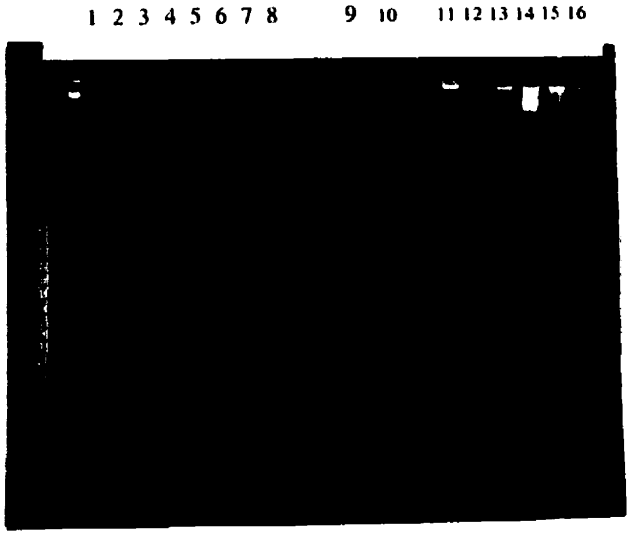


Figure A3.

HindIII-digested DNA from Amsinckia spectabilis var. spectabilis and Amsinckia spectabilis var. microcarpa.
lanes 1-8: A. spectabilis var. microcarpa (91004)
lanes 9, 10: 1 kb marker
lanes 11-16: A. spectabilis var. spectabilis (91010)

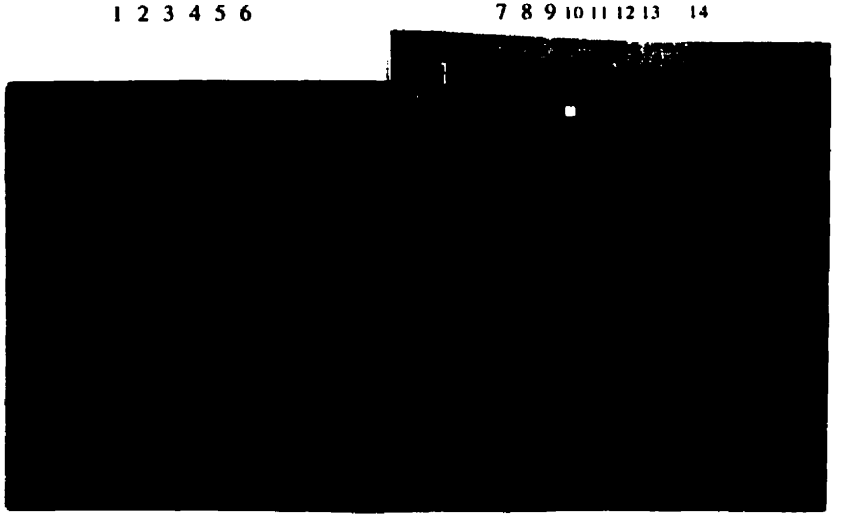


Figure A4.

HindIII-digested DNA from Amsinckia spectabilis var. spectabilis and Amsinckia spectabilis var. microcarpa.
lanes 1-6: A. spectabilis var. spectabilis (91011)
lanes 7-13: A. spectabilis var. microcarpa (91003)
lane 14: 1 kb marker

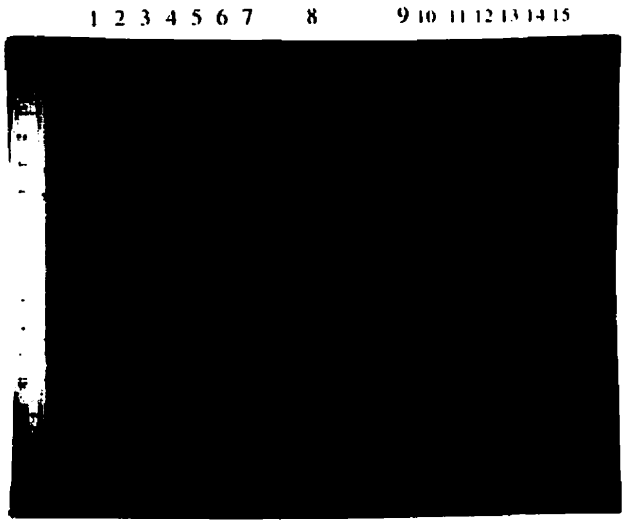


Figure A5.

HaeIII-digested DNA from Amsinckia furcata and Amsinckia vernicosa.
lanes 1-7: A. furcata (91007)
lane 8: 1 kb marker
lanes 9-15: A. vernicosa (91006)

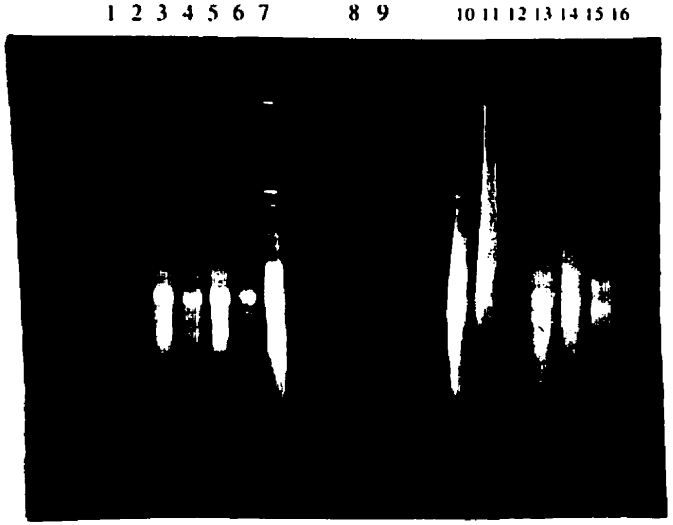


Figure A6.

HaeIII-digested DNA from Amsinckia furcata and Amsinckia vernicosa.
lanes 1-7: A. furcata (91008)
lanes 8, 9: 1 kb marker
lanes 10-16: A. vernicosa (91014)

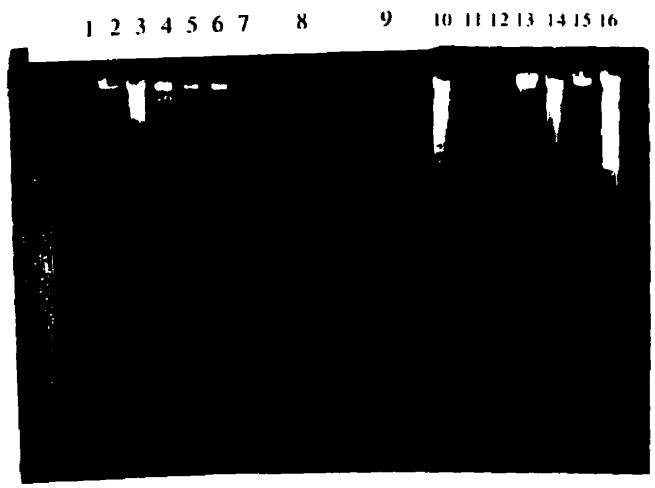


Figure A7.

HindIII-digested DNA from Amsinckia vernicosa and Amsinckia furcata.
lanes 1-7: A. vernicosa (91006)
lanes 8, 9: 1 kb ladder
lanes 10-16: A. furcata (91007)

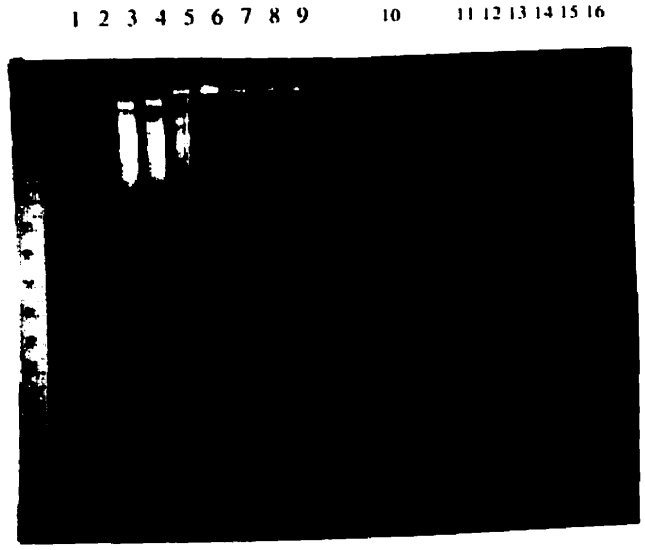
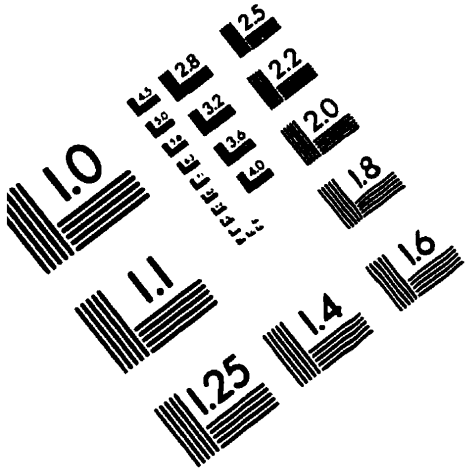
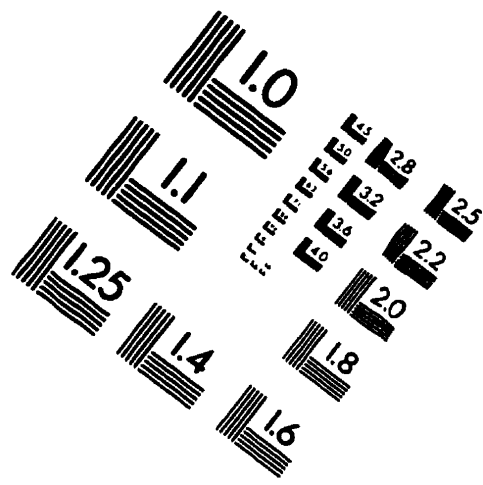


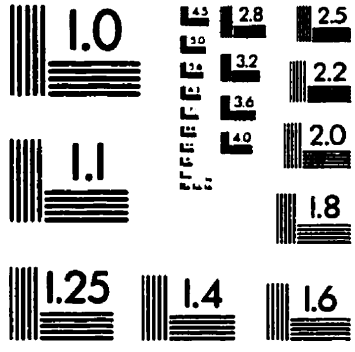
Figure A8.

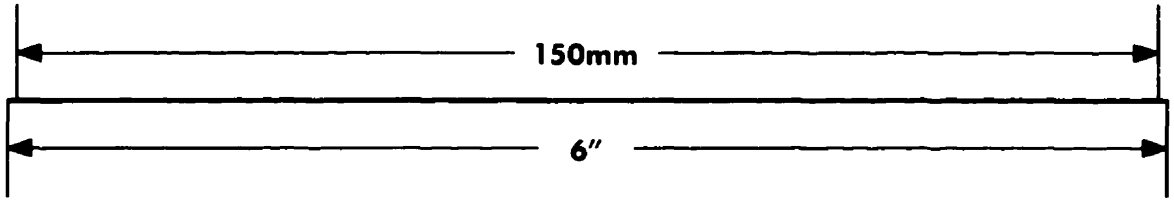
HindIII-digested DNA from Amsinckia vernicosa and Amsinckia furcata.
lanes 1-9: A. vernicosa (91014)
lane 10: 1 kb ladder
lanes 11-16: A. furcata (91008)

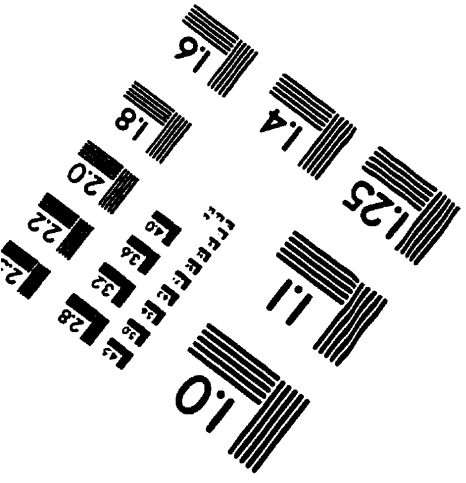
IMAGE EVALUATION
TEST TARGET (QA-3)

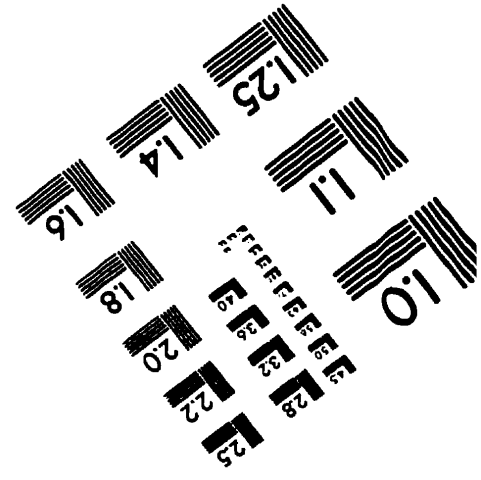












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