THESIS

CHARACTERIZATION OF *ALSTROEMERIA* SPECIES AND CULTIVARS USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS

Submitted by

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY DERIC D. PICTON ENTITLED CHARACTERIZATION OF *ALSTROEMERIA* SPECIES AND CULTIVARS USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

CHARACTERIZATION OF *ALSTROEMERIA* SPECIES AND CULTIVARS USING RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS

The characterization of *Alstroemeria* has not been effective through the use of conventional morphological markers. This is mainly due to the confused status of many of the species. Breeding of *Alstroemeria* has involved interspecific crosses as well as the use of mutagens and chromosome doubling for the creation of cultivars. Through the use of molecular techniques, in particular random amplified polymorphic DNA (RAPD) analysis, it is possible to uniquely characterize the species and cultivars. Fifteen species and hybrids along with 25 cultivars were examined using RAPD analysis. Four primers were eventually used for the analysis. All amplification products were seperated using a 5% polyacrylamide gel and stained with silver nitrate for maximum differentiation of the fragments. The four primers yielded 73 amplification products which were polymorphic. When analysed using cluster analysis, all species and cultivars were uniquely characterized and putative parentages of many of the cultivars were determined.

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Chapter 1

INTRODUCTION

The genus *Alstroemeria* has become an important cut flower in the last 15 years in the United States and in Europe. Its requirement for lower temperatures for flowering and growth compared to many other greenhouse flowers make it less costly to produce in high cost energy areas. Long vase life, up to 4 weeks, also make *Alstroemeria* popular as a cut flower. Many of the cultivars grown in the United States were developed and are owned by European firms based mainly in the Netherlands and England. For economic reasons, the complete parentage of the cultivars has never been fully revealed by these firms. In some cases one of the reputed parents has been named but rarely are two or more of the ancestors known. Since the parentage of the commonly grown cultivars is not known, the development of cultivars by U.S. breeders has been somewhat less successful than in Europe.

There are reportedly over 60 species of *Alstroemeria* native to South America, generally from Peru, Argentina and Brazil. There are some species known to be native to Mexico and Central America as well. There is, however, some doubt as to the actual existence of some of the reported species. In some cases there are

two or more names for the same species and in other cases the species has no botanical standing in modern taxonomy texts. Since identification of *Alstroemeria* by classical taxonomic means is confused, other methods of identification may be useful.

American growers are required to pay lease fees for the European plants as well as a per stem royalty. This makes the flowers expensive for the consumer despite the lower inputs needed for growing the plant as compared to other popular cut flowers.

Identification of the species used in the development of the modern cultivars of *Alstroemeria* would greatly assist American breeders in developing competitive cultivars. Most European cultivars are the result of hybridization and polyploidization of various species. Many cultivars were also developed through mutagenesis using X-ray bombardment. This may have resulted in the alteration of the DNA either through large rearrangements of the genome or through point mutations.

In this study, Random Amplified Polymorphic DNA (RAPD) analysis was used to fingerprint the cultivars and provide information about possible breeding materials. This approach allowed for the identification of polymorphisms within the genome of the plants. By identifying unique banding patterns for each species and cultivar, it is possible to correlate the relationship of cultivar to species, provided there is an adequate representation of each species, using cluster analysis .

Large format polyacrylamide gels and silver staining were used to separate and characterize polymorphisms in each species of *Alstroemeria* studied. Analysis

of 25 commonly grown cultivars were conducted along with the species in order to compare them to RAPD patterns present in the respective species. Where a high number of shared bands was present in a cultivar and species, a parental link was proposed. In some cases more than one possible parent was identified.

The analysis of the species and the analysis of the cultivars are presented in two separate chapters and are discussed separately. A separate chapter discusses the possible linkages between the species and cultivars studied.

The results obtained from this research provide further information regarding the characterization and identification of *Alstroemeria* species and cultivars. The relationship of the RAPD banding patterns of the species allows for further support on the putative parents of the cultivars as previously indicated by the research of Stephens (1995) using isozyme and karyotype analysis.

Chapter 2

LITERATURE REVIEW

The species *Alstroemeria* is a native of South America, in particular the Andean highlands of Peru and Chile as well as the Pampas of Brazil and Argentina. The first specimens of *Alstroemeria* were introduced into Europe in 1714 by Father Fueillée, a representative of the Spanish church. Fueillée then gave seeds of *Alstroemeria pelegrina* to the monk Alstromer who passed them onto fellow monk and naturalist Carolus Linnaeus. Linneaus then described and illustrated the plant and gave the genus the name *Alstroemeria* in honor of Alstromer. Due to its reported use in the gardens of the Incan aristocracy, *Alstroemeria* is also known as Peruvian Lily or Lily of the Incas. *Alstroemeria pelegrina* was first introduced to Kew gardens in England in 1756 (Stephens, 1995).

There are currently more than 60 species of *Alstroemeria* recorded, some of which have little or no standing in modern botanical texts (Bailey and Bailey, 1976). The species grow in climates that range from dry costal sand dunes, deserts, river valleys, and scrub up to the snowline of the Andean mountains. The genus has a desired average growing temperature of 15° Celsius and will enter a dormant period if the temperature rises above 26° Celsius. The plants are also drought tolerant but

will enter dormancy if they receive less than 20 mm of water per week regularly (Keil, 1987).

Alstroemeria belongs to its own family, the *ALSTROEMERIACEAE*, in which there are four genera, *Alstroemeria, Bomarea, Leontochir* and *Schickendantzia.* Only species of *Alstroemeria* and *Bomarea* are grown for commercial flower production. The *ALSTROEMERIACEAE* is in the class Monocotyledons and order Liliales, making it a true monocot.

The genus *Alstroemeria* consists of herbaceous perennials that have a white fleshy rhizome from which arise the stems and a fibrous root system. Some of the roots are modified to store water and nutrients. The aerial portion of the stem contains no axillary buds above the first two nodes of the shoot which are located underground or along the soil surface. These buds allow for the continued development of the main rhizome and for the lateral branching of the rhizome. Each new aerial shoot will arise from the first node of the previous shoot. The shoot may be vegetative or reproductive depending on the existing environmental conditions. The stems range from six centimeters to four meters in length depending on the species and environmental conditions. The leaves are linear to lanceolate to ovoid and, in most of the species, as the leaves unfold they rotate 180° so that they are 'upside down' or resupinate. Stomata are present on the surface facing the ground with guard cells which are not 'grass type' in shape.

The reproductive stems end in umbel-like inflorescences which contain from one to many individual flowers. The flowers are often subtended by green, leaf like

bracts that range from minute to quite large in size. The flowers are usually large with the perianth being spotted and are trimerous, epigynous, bisexual and actinomorphic or lightly zygomorphic. The lower segment of the inner whorl differs in shape and coloring from the other two segments whereas the segments of the outer whorl differ in shape from the inner whorl.

There are six stamens which have narrow filaments and small oblong anthers which are attached to the base. The ovary is locular and three celled with each cell containing many ovules. The single style is filiform, the stigma being separated into three parts or lobes about halfway down with a wet stigmatic surface. The pollen has generally matured and been released prior to the stigma becoming receptive, thus promoting cross-pollination. The fruit is a dehiscent, loculicidally three-valved capsule and the seeds are globose with oily endosperm (Bailey, 1947).

Although morphology is adequate for the differentiation of species for most plants, there are some problems with this in terms of *Alstroemeria* species. Most *Alstroemeria* species evolved in isolated mountainous areas of South America and therefore have similar morphological characteristics. Thus, separation of species may require analysis techniques other than the use of morphology to differentiate the species. One of these techniques is the use of karyotype analysis. It is possible, although infrequent, for two species of the same genus to have aneuploid variation. It is also possible, and much more probable, for two species of one genus to have different ploidy levels. This has been demonstrated in potato *(Solanum)* species (Hosaka et al., 1994).

The development of the cultivars of *Alstroemeria* began over 100 years ago in England. Prior to 1960, the breeding of *Alstroemeria* involved the crossing of the various species of *Alstroemeria*. This type of breeding resulted in few hybrids due to strong incompatibilities among the species. Those hybrids that were created were usually crossed with another *Alstroemeria* species in an attempt to improve the cross. Cultivars were released around 1960.

The application of colchicine aided in their development, by allowing the chromosome numbers of the species to be doubled. This doubling of chromosome number allowed the breeders to create many of the triploid cultivars by crossing a diploid with a tetraploid cultivar. Since *Alstroemeria* is a clonally propagated species it is not necessary for the cultivars to reproduce sexually.

The application of X-rays to the cultivars has also been used to induce further mutations and create sterile lines. The development of sterile cultivars in clonally propagated species is important for the floral industry since it prevents the use of superior cultivars for breeding as well as generally increasing the longevity of the cut flower. This lack of breeding ability also tends to create a monopolistic tendency in the development of new cultivars.

Chromosome analysis allows for the confirmation of ploidy level. Chromosome analysis of *Alstroemeria* was first published in 1882 by Strasberger when he recorded the basic chromosome number as x=8. These findings have subsequently been confirmed by various sources.

During meiotic cell division, the chromosomes pair into homologous units. These homologous pairs are not necessarily a pairing of exact chromosomes but of similar chromosomes. Thus, it is possible for the pair to consist of chromosomes that contain significant differences. These differences may not be directly visible but can be shown by staining the chromosomes with certain dyes. One of the limitations of chromosome staining is that only non-homologous chromosomes can be differentiated.

The preferred dye used for staining is Giemsa, as it binds to heterochromatin (Tamarin, 1991). Heterochromatin is a region that remains coiled during the interphase portion of cell division. These areas vary among species and can be different enough to allow for the differentiation of the species. Since these heterochromatic areas may vary with each species they can be used for the development of parental heritages if the parents used in the crosses are sufficiently diverse. Hybrids within species and between highly related species are usually not distinguishable with Giemsa staining due to the lack of variability of the heterochromatin banding patterns. It is also difficult to differentiate cultivars which were developed using mutation breeding which usually affects areas other than those stained by Giemsa. Since not all differences may be detected with whole chromosome analysis, it is important that the DNA or the products of DNA, the enzymes, be analyzed.

Isozyme analysis allows for the identification of differences in individual enzymes (Gardiner et al., 1996). Since the enzyme is a product of the DNA it is possible for any differences in the DNA to show up in the enzyme when run on an

electrophoretic gel. These differences cause the enzymes to migrate to different positions with in the gel. Enzymes usually vary from species to species but can also vary within a species, which can lead to problems with identification of an enzymatic pattern for a species. Since the enzymes in a plant are not normally coded from a single allele of a gene but from multiple alleles and undergo various processes in their assembly, there are usually multiple enzymes, isozymes, with similar function in a plant. This variability can lead to problems in identifying the parents of a hybrid if the enzyme patterns are very complex or if the species crossed are very closely related. This problem can be reduced if the DNA of a plant is directly analyzed using one of the various PCR based techniques.

The polymerase chain reaction (PCR) is a technique that allows for the in vitro amplification of DNA sequences by the simultaneous extension of primers of complementary strands of DNA. The PCR is used to amplify a segment of DNA that lies between two regions of known sequence. The original protocols for PCR used the Klenow fragment of *E. coli* DNA polymerase I to catalyze the extension of the annealed primers (Mullis et al., 1986; Mullis and Faloona, 1987; Saiki et al, 1988). The major problem with the use of the Klenow fragment is that it is inactivated at the temperature needed to denature the DNA (Eckert and Kunkel, 1993). Thus it is necessary to replace the enzyme for each round of DNA fragment synthesis. The use of the Klenow fragment is also only useful for amplification of fragments of around 200 base pairs. The PCR was not widely used until the development of a heat stable DNA polymerase from the bacterium *Thermus aquaticus.* This

polymerase, known as Taq polymerase, has the ability to withstand temperatures up to 95°C without denaturing. The use of Taq polymerase also allows for the amplification of much larger DNA fragments. The exact size of the fragment depends on the type of primer used. PCR is a major advance in the analysis of DNA and RNA as it has simplified existing technologies and enabled the rapid development of new techniques for nucleic acid analysis.

The DNA polymerase carries out the synthesis of a complementary strand of DNA in the 5' to 3' direction using a single strand of DNA from the double stranded DNA helix. Two oligonucleotides or short DNA segments are used for the priming of the DNA fragment. These primers have a known sequence and range from five to thirty-five bases in length. The primers act as the starting and ending points for the synthesis of nucleic acid segments. For this to work the primers are complementary to sequences on opposite strands of the target DNA. For DNA synthesis to occur it is necessary for the original DNA template to be single stranded, which under most circumstances it is not. Separating the DNA strands can be achieved by the heating of the double-stranded DNA molecule to around 94°C. This heating causes the hydrogen bonds holding the single strands of the DNA to break, thus creating single-stranded DNA molecules. The primers are then positioned on the template, or original DNA in such a manner as to allow for the extension of the primers toward each other. Therefore, the first primer directs the synthesis of a strand of DNA which can then be primed by the second primer. The result of this process is the creation of a segment of DNA with a specific length flanked by the two primers.

The requirements of the polymerase reaction are simple: deoxynucleotides necessary for the synthesis of the DNA, a DNA polymerase, primer, template DNA, and a reaction buffer containing magnesium and other chemicals. Both the nucleotides and primers should be present in excess to ensure adequate priming of the entire template DNA as well as to ensure continued amplification of the DNA segments in later reactions. The PCR process performs DNA replication through the heating and cooling of the reaction mixture which contains all the components necessary for DNA replication. The heating and cooling cycles are repeated and DNA fragments will continue to be synthesized and accumulation will be exponential until one of the reaction products is exhausted or the DNA polymerase becomes non-functional. Generally 25-45 cycles of heating and cooling, or thermocycling, can be performed before DNA synthesis is affected. It is possible to automate much of the PCR procedure with the use of a thermocycler which allows for the automatic heating and cooling of the reaction mixture necessary for PCR based DNA synthesis. The standard thermocycler profile involves heating the reaction mixture to 94°C to denature or melt the template DNA, cooling the reaction to around 36°C to allow the annealing of the primer to the template DNA and then heating the reaction to 72°C to allow for the extension of the primers and thus DNA synthesis. Annealing temperature may need to be varied according to the composition of the primers used. Annealing temperature also will affect the ability of the primer to anneal to non-specific sequences in the template DNA. The higher the temperature, the more exact the priming will be (Kidd and Ruano, 1995).

For the PCR to work at its optimum the components of the reaction must be in specific ratios and will vary for each genus worked with (Kidd and Ruano, 1995). The most common DNA polymerase used is Taq polymerase isolated from *Thermus aquaticus,* a thermo-tolerant bacteria which naturally grows in the geothermal hot springs of geyser basins where water temperatures are at or just below 100°C. This ability to live at high temperatures allows a researcher to utilize the extracted proteins of the bacteria in applications where non-heat stable proteins would be destroyed. Alternative polymerases to the Taq polymerase that are heat stable include those isolated from *Thermus thermophilus* and *Bacillus stereothermophilus*, two bacteria commonly used to test the sterilizing ability of autoclaves. Taq polymerase is the most common and widely used of the thermostable polymerases available today. The activity of Taq polymerase is highest from 65° to 72° C at a pH of 8.2 to 9.0 (Taylor, 1993). The reaction buffer for the PCR normally consists of 10mM Tris (pH 8.4), 50mM KCl, 0.1% Triton X-100 with a variable concentration of $MgCl_2$ from 1.5mM to 4.5mM depending on the species studied.

The primers used in the PCR usually range from five to thirty-five bases in size depending on the specificity needed. The larger the number of bases in the primer the more specific the priming of the target DNA. With primers sixteen to thirty-five bases in length, the target sequence is considered specific or targeted. Primers greater than 20 bases are specific enough to be targeted to a specific sequence within a gene. With primers five to ten bases in size the target sequences are considered random or non-targeted in nature. Depending on the application it

may be better to have targeted primers rather than randomly targeted primers. Targeted PCR is generally used for the isolation of individual genes or gene segments while non-targeted primers are used for genomic sequencing and phylogenetic studies where the identification of polymorphisms within the genome is important.

In the design of the primer, depending on the technique used for analysis, it is necessary to keep in mind a few simple points. A primer should not be complementary to itself. If the primer does contain self complementary sequences it can lead to self-annealing or the annealing of two or more primers together, known as a primer-dimer (Kidd and Ruano, 1995). Both of these situations lead to inactivation or loss of potency of the primer. Primers should also not contain a 3' thiamine since thiamine is the least discriminating nucleotide and can lead to mismatching of the primer to a sequence. The dissociation or melting temperature of primer pairs, when used, should be roughly equal in order to allow for proper annealing to the template DNA. If the primers have significantly different annealing temperatures, a multi-temperature annealing protocol may need to be used (Kidd and Ruano, 1995). With the use of primers ten bases in size these recomendations can be set aside as the primers are usually to small for major problems to occur.

Magnesium is the next important factor that can affect the results of a PCR reaction. The magnesium ion concentration is relevant to both the yield and the specificity of the reaction. At high concentrations the magnesium ion will stabilize the double-stranded DNA molecule and prevent the complete denaturation of the molecule, thus reducing the ultimate yield (Kidd and Ruano, 1995). High

magnesium concentrations can also stabilize any mispriming that may occur between the primer and the template DNA resulting in the production of undesirable product (Kidd and Ruano, 1995). Low magnesium concentrations, less than 0.5 μM, will impare the extension rate of the DNA polymerase since magnesium is needed as a co-factor for the enzyme (Kidd and Ruano, 1995, Ivinson and Taylor, 1993). There is also a portion of the magnesium that is chelated by the raw nucleotides thus making it unavailable for use by the enzyme. Magnesium concentration will normally range from 1.0 to 10.0 μM depending on the organism studied. Optimization of the magnesium concentration is necessary for each organism. Generally species in the same genera and possibly in the same family will have similar magnesium ion requirements.

Both nucleotides and primers are needed throughout the entire PCR reaction at a constant rate. For this to be possible both are added to the reaction in an excess amount. The four deoxynucleotides, thiamine, guanine, cytosine and adenosine are normally added at a concentration of 100-200 μM. This amount of nucleotide will ensure a nearly constant supply over the entire reaction time. To ensure a constant supply of primer, the primer is at an initial excess with respect to the template DNA. A final concentration of 0.1-0.2 μM of primer is added to the reaction mixture. Too high of an amount of primer can also be detrimental to the PCR process as it can lead to primer dimer formation and to excessive mispriming (Kidd and Ruano, 1995; Black, 1993).

The template DNA is the most important portion of the PCR reaction, as it is the item of interest. The purity of the DNA is of paramount importance. Since

genomic DNA naturally exists within a cell it is important to remove all the cell components during the extraction process.

The extraction of the DNA from the cell is a relatively simple process. The process involves grinding the sample material in liquid nitrogen in order to disrupt the cells. Following grinding, the material is placed in an extraction buffer containing a detergent to disrupt cellular membranes and high salt concentrations to prevent enzymatic activity from destroying the genomic DNA. Following incubation of the solution, a solution of chloroform and isoamyl alcohol is added to remove the proteins from the solution. Once the DNA is removed form all other cellular debris it is necessary to treat the DNA with an RNAase and proteinase to destroy any miscellaneous RNA and protein.

The DNA is normally quantified by using a spectrophotometer. Nucleic acid will absorb light waves in the ultraviolet range of the spectrum at around 200 to 300 nanometers. For the quantity and purity of the DNA to be checked, absorbancy readings are taken at 260 and 280 nanometers. The ratio of absorbance at 260 nanometers to absorbance at 280 nanometers will give the purity of the DNA. Pure DNA will have a ratio of 1.8 while RNA will have a ratio of 2.0 when pure. Proteins will produce a ratio of 1.4 at the same wavelengths. Since pure genomic DNA is very difficult to obtain a ratio of 1.7 to 1.9 is considered pure enough for PCR and other analysis techniques. A ratio higher than 1.9 indicates contamination of the DNA with RNA and a ratio below 1.7 indicates an overabundance of protein. In either case, the DNA sample should be treated with the appropriate enzyme. The exact concentration of DNA used varies greatly with the organism and technique

used for analysis. The important concept to be aware of is the appropriate ratio of primer to template. If the ratio is too high, too high a primer concentration, primer dimers can form and mispriming will occur. If the ratio is too low, too much template, the primer will be too thinly spread out and low amplification will occur (Halldén et al., 1996).

The concentration of DNA polymerase is also of importance. If the concentration is too high, the production of DNA from inaccurate primer/template interactions can occur due to the over availability of the enzyme. This over availability can promote the premature amplification of DNA from regions where the primer and template are improperly annealed. This problem can be reduced with an increase in annealing temperature. The recommended concentration of enzyme is 0.25-0.75 units per reaction (Kidd and Ruano, 1995). This amount ensures a concentration high enough to provide adequate amplification but low enough to prevent inaccurate amplifications from occuring (Kidd and Ruano, 1995, Staub et al.,1996). The exact concentrations of enzyme used will vary with the type of enzyme and the organism studied.

The end result of a PCR is a fragment or several fragments of DNA with a defined length. The detection of these fragments is most commonly done using an electrophoretic gel. This process involves the placing of a portion of the PCR product into an agarose or polyacrylamide gel along with an appropriate sized molecular marker and running a current across the gel to separate the different lengths of DNA fragment. The resulting gel is then stained using either a fluorescent dye, usually ethidium bromide, or a light reactive chemical such as silver

nitrate to detect the presence of the DNA. Ethidium bromide is a stain that associates itself within the DNA molecule and will brightly fluoresce when viewed under an ultraviolet transilluminator. Silver nitrate is a molecule that complexes with organic compounds and when exposed to a reducing agent reacts to form elemental silver. Staining with silver has the ability to be 100-1000 times more sensitive to DNA concentrations than ethidium bromide. Thus, silver staining has the ability to detect 1 picogram of nucleic acid per millimeter of gel as opposed to 100 nanograms per millimeter with ethidium bromide (Newton, 1995). Staining with silver allows for a permanent record of the PCR reaction to be kept, where as ethidium bromide staining will degrade after a short period of time. Silver nitrate staining uses a less toxic chemical while ethidium bromide is a known mutagenic agent. Using either detection method, the DNA fragments will show up as distinct bands within the gel. The position of the bands in the gel varies with the particular size of the DNA fragments. Using these bands and banding patterns it is possible to develop a profile unique for a species or cultivar.

Electrophoresis is a chromatographic technique used to separate mixtures of ionic compounds such as nucleic acid or protein. Gel electrophoresis utilizes the elements of separation based on charge, size and conformation. DNA migrates through a gel composed of agarose differently than through a gel composed of polyacrylamide. In an agarose gel, charge and size are the determining factors for fragment migration. In a polyacrylamide gel, charge and fragment size are important but the conformation of the DNA fragment is also important. Since single stranded

DNA has the ability to bind to itself, the DNA fragment forms secondary structures. These secondary structures will inhibit or enhance the migration of the fragment and allow for greater separation of the fragments (Sambrook et al., 1989). Conformation differences may allow the separation of alleles and thus the identification of single base mutations within the genome.

Agarose is an organic substance which is extracted from seaweed. The linear structure of agarose allows it to form into a stiff matrix when heated and cooled. The density of the matrix, and subsequently its separation capabilities, is determined by the percent concentration of agarose in the gel. The higher the concentration of agarose the smaller the DNA fragment that can be differentiated. Agarose has a separation capability of 200 to 50,000 base pairs depending on the concentration of the agarose (Sambrook et al., 1989). Agarose gels on the other hand lack the resolution capabilities to differentiate small differences in DNA fragment length which is sometimes crucial for genetic analysis.

Acrylamide, or more accurately polyacrylamide, is a non-biological substance that when exposed to free radicals and stabilized with TEMED (N,N,N',N'-tetramethylethylenediamine) leads to a reaction in which the monomers of acrylamide will polymerize into long chains. When acrylamide is mixed with N,N'-methylethylenebisacrylamide (bis-acrylamide) and the polymerization reaction is allowed to occur, the result is cross-linked acrylamide chains that form a matrix similar to the agarose gel. The matrix density is determined by the length of the polyacrylamide chains and the degree of cross-linking. The chain length is determined by the percent of acrylamide in the solution and the percent cross-

linking is determined by the concentration of bis-acrylamide. The ratio of bis-acrylamide to acrylamide is normally 1 to 29 parts respectively, although this can be modified depending on the application. The separation abilities of acrylamide is from 1 to 5000 base pairs depending on the polyacrylamide matrix. The resolution of a polyacrylamide gel is much greater than an agarose gel. A polyacrylamide gel has the ability to differentiate DNA fragments that differ by one base pair in length (Sambrook et al., 1989). This ability to separate to one base pair has made the sequencing of DNA possible.

Random amplified polymorphic DNA based PCR (RAPD-PCR) allows for the amplification of random sequences across the entire genome and thus increases the ability to detect differences. Non-targeted or random PCR has become increasingly relevant in recent years for the identification and differentiation of species and cultivars as well as the determination of parental heritages. The amplification of DNA fragments occurs across all regions of the genome thus allowing for less conserved sequences in the DNA to be amplified. The amplification of these DNA sequences increases the relative ability to differentiate individuals since the DNA will contain a relatively higher proportion of variable DNA. These variable sequences can then be used for the creation of species or cultivar specific primers that can be used in further analysis of the subject being studied.

RAPD-PCR is referred to as a non-targeted PCR process, which is non-specific or multiple binding of primers to the genome. For this to occur it is necessary that the primers used for the random amplified polymorphic DNA method

of PCR analysis conform to certain requirements. RAPD's use a single ten base primer with a minimum 50% guanine/ cytosine content for proper binding. This type of primer allows for the non-specific amplification of portions of the genome where possible differences or polymorphisms may be present. It is this ability to detect areas of difference which make RAPDs useful in the differentiation of individuals. It is also necessary that the primers contain non-homologous sequences on either end to prevent the looping of the primer. When two RAPD primers are employed for a single reaction it is important that the primers not contain sequences that are homologous to each other in order to prevent the formation of primer dimers (Black, 1993).

Random amplified polymorphic DNA analysis was first reported by Williams et al. in 1990. The discovery that single primers of small size (5-10 nucleotides) could be used for the fingerprinting of genomic material in much the same way that restriction enzymes are used showed the true value of RAPD analysis (Welsh et al., 1991). As with most research in molecular genetics, the use of arbitrarily primed PCR was initially performed using animal and bacterial DNA and later modified for use with higher plants. This initial research demonstrated that the use of primers smaller than 8 bases in size allowed for excessive priming of the genome and thus made the banding pattern or fingerprint difficult to read. The use of primers larger than 12 bases prevented arbitrary priming and thus detection of polymorphisms was reduced to only large differences in the genomes (Caetano-Anollés et al., 1991). The use of primers with a minimum 50% G+C content was also established (Caetano-Anollés et al., 1991). In 1990, Williams et al. reported that the use of

polymorphisms in human DNA amplified with arbitrary primers of ten bases in size could be used as genetic markers. Williams also showed that these polymorphisms could be inherited. As early as 1991 the term RAPD had been developed and the identification of plant cultivars using random amplified polymorphic DNA analysis had occurred (Hu and Quiros, 1991). The use of RAPD's for cultivar and specific identification has many benefits over traditional identification techniques.

The phenotype of a plant consists of its morphology and the production of secondary compounds such as terpinoids. The use of phenotype for characterization and determination of phylogenetic relationships is unreliable in many species since there is no reliable relationship between genotype and phenotype because phenotype can be profoundly affected by environment. Identification based on morphology has been and still is the main method of cultivar and breeding line identification. This method of identification can lead to some problems in crops where only modest differences exist between lines or where non-morphological differences exist. Morphological characterization is also a high cost and labor intensive process which is highly susceptible to environmental influences. The interaction of genotype within a certain environment will give a morphology specific only to that environment. Therefore, it would be valuable to eliminate the environmental influences that affect the genetic expression of the crop. A controlled environment is one method of reducing environmental affects to a minimum, but is costly and unreliable. The best method to evaluate and identify specific traits is to eliminate the environmental affects and evaluate the DNA of the crop. The use of RAPDs is one of the methods available to do this.

Williams et al. (1990) showed that RAPD markers can be used as effectively as restriction fragment length polymorphisms (RFLP) while reducing costs and labor. An advantage to using RAPDs is the use of small amounts of genomic DNA (25 ng) as opposed to greater than 2 to 10 μg for RFLPs. The speed, ability to detect extensive polymorphisms and simplicity of RAPDs is of great advantage over RFLPs. The use of small amounts of DNA needed for analysis is of advantage since small quantities of cellular material is needed (0.5-4 g). This need for small amounts of material allows for the evaluation of seedlings and seeds. Since seeds can be used for analysis, it is possible to evaluate crosses without having to have a large or mature plant for DNA extraction.

The primary uses so far of RAPDs is for identification of cultivars and species of economically important crops and for marker assisted selection in breeding programs (Orozco-Castillo, 1994; Qu and Hancock, 1997; Striem et al., 1996). The use of genetic fingerprints for crops has become increasingly important in the past decade as plant patent laws have changed to encompass the use of genetic analysis as a means of identification (Scott et al.,1996; Staub and Meglic,1993). As most cultivars of plants are unique crosses, mutations or selections of species it is important to be able to differentiate among them. RAPD analysis has been used in many species for identification including American elm (Kamalay and Carey, 1995), Jamaican yam (Asemota et al., 1996), *Ozothamnus diosmifolius* (Ko and Henry, 1996), poinsettia (Ling et al., 1997), *Chrysanthemum* (Scott et al., 1996) and blueberry (Levi and Rowland, 1997). This ability to identify

cultivars may assist in the creation of a greater diversity of unique specimens, thus increasing diversity of cultivars.

RAPD's can also be used to evaluate the degree of polymorphism or genetic diversity within a species (Asemota et al, 1996; Farnham, 1996; Kump and Javornik, 1996; Virk et al., 1995)). This aspect of RAPD analysis is important in the evaluation of plant germplasm collections. For germplasm collections it is important for the collection to have as high a degree of polymorphism as possible as this will safeguard the bio-diversity of the collection. Knowing the degree of polymorphism will also assist researchers in the determination of the relative ability of the species to provide new genetic combinations for breeding. Warburton and Bliss (1996) compared the diversity of American and Asian grown peaches to determine the genetic diversity within the species. They concluded that the American grown peach germplasm was limited, but the same could not be said about peaches from closer to its center of origin. RAPD analysis has also been used to determine the genetic diversity among and between two morphologically distinct American grape types (Qu et al., 1996), US collard cultivars and land races (Farnham, 1996) and artichoke cultivars and breeding populations (Tivang et al., 1996). Making germplasm collections based on genetic diversity will permit germplasm repositories and breeders to increase their ability to evaluate the diversity within their collections and make proper decisions about collection and breeding.

The use of RAPD analysis is also useful in those plants where no previous genetic information is available. This lack of genetic information may be due to alternate classification techniques such as morphology, isozymes or chromosome

karyotype analysis or due to the relative newness of the crop. Using RAPDs for the initial evaluation of a plant allows the researcher to develop species or cultivar specific primers from the RAPD profile using genetic sequencing of a portion of the RAPD product. In the case of tea (*Camellia sinesis*), Wachira et al. (1995) showed that separation of taxonomic populations using RAPDs was possible without the need for detailed genetic study. Wachira et al. (1995) also demonstrated that populations that are highly out-crossing and therefore highly heterogenous can be separated into taxonomic groupings. RAPD analysis has also been used to study the taxonomical relationships of *Asphodelus* species (Lifante and Aguinagalde, 1996). The identification of clonal populations is also possible using RAPD analysis. Castiglione et al. (1993) showed that RAPD classification of *Populus* clones was possible.

The determination of a cultivar's genetic parentage from wild species or from other cultivars is also possible using RAPD analysis with a high degree of accuracy. When compared to isozyme analysis, RAPD analysis provides a more definitive separation of closely related cultivars (Heun et al., 1994). This ability to differentiate between closely related cultivars or accessions has been valuable in many crops including *Avena sterlis* (Heun et al., 1994), buckwheat (Kump and Javornik, 1996), hybrid rice (Wang et al., 1994), coffee (Orozco-Castillo et al., 1994), solanum potato (Hosaka et al., 1994), mango (Schnell et al., 1995) and strawberries (Parent and Pagé, 1995). Debener et al. (1996) used RAPDs to detect genetic variability between a group of rose cultivars and selected wild rose species to determine

possible parentages for the cultivars. RAPD analysis has also been used for the deduction of a putative parent for the 'Braeburn' apple (Gardiner et al., 1996) and in determining the genetic relationships in *Malus* species and cultivars (Dunemann et al., 1994). The accurate evaluation of cultivars from a narrow germplasm can be accomplished with the use of as few as two RAPD primers (Parent et al., 1993) but will normally range from 5 to 25 primers.

RAPDs can also be used to detect genetic change in clonally propagated crops (Taylor et al., 1995) and to test the purity of F_1 hybrid crops (Rom et al., 1995). Genetic variability or somaclonal variation can occur in species that have been clonally propagated for long periods of time (Taylor et al., 1995). This variation is of value to breeders and researchers while developing new lines and cultivars but is normally undesirable in existing cultivars. The assessment of somaclonal variation using RAPD analysis is only valuable when large mutations to the genome occur. Since a somaclonal variant has the possibility of being an alternate expression of existing genes, there can be difficulties in analysis. In the case of *Alstroemeria*, Anastassopoulos and Keil (1996) showed that the use of RAPD's was valuable in the differentiation of species and cultivars produced using traditional breeding techniques but was ineffective in determining variation from mutation induced variation. This finding is supported by the research of Taylor et al. (1995) and Wallner et al. (1996) whose research came to the same conclusion as Anastassopoulos that only large changes in the genetic code can be detected using RAPD analysis techniques. In those crops that naturally undergo spontaneous genetic mutation such as carnation and *Chrysanthemum*, the use of RAPD analysis

is effective in distinguishing the mutations (Wolff et al., 1993; Wolff, 1996). In the case of sugar beet, where spontaneous mutation is low, the detection of somaclonal variants with RAPD analysis was ineffective (Munthali et al., 1996). Isabel et al. (1996) were unable to differentiate between *varigata* and normal white spruce after screening 250 RAPD markers. Thus, the use of RAPD analysis for the detection of mutation based variation is species dependent.

The use of RAPD's as a means of purity control of F_1 hybrids is possible as demonstrated by Rom et al. (1995) with their research with tomato hybrids. This purity control aspect of RAPD analysis is useful with all F_1 hybrid produced crops as the RAPD markers are inherited in a mendelian fashion (Heun and Helentjaris, 1993). De Filippis et al. (1996) were able to identify somatic hybrids of tobacco as well generated from protoplasts and electrofusion using RAPDs.

RAPD analysis can also be used for the identification of plants for the purposes of plant patenting (Welsh et al., 1995). This is a relatively new aspect of genetic analysis and is still developing. It is of value to breeders and seed companies to develop genetic fingerprints for their crops as part of the patenting process. Traditionally, cultivars and species are identified by the use of measurable traits which are controlled by genes. As stated previously, phenotype is the interaction of genotype with environment. Since this is true, the use of the plant's genome is of value in the characterization of a species or cultivar. As each species and cultivar will have a unique genetic profile or fingerprint, it is possible to identify species based on this genetic profile if the correct analytic methods are used. Castiglione et al. (1993) showed that the use of RAPD analysis could be used as

a convenient method of defending plant breeders' rights with their study of *Populus* clones.

RAPD based analysis has also recently been used for the creation of gene linkage maps (Thompson et al., 1997; Yu and Pauls, 1994). Since RAPD markers can be used in a similar manner as RFLP markers it is possible to associate certain DNA fragments with genetic traits with RAPD markers being inherited as a dominant marker and RFLP markers being inherited as co-dominant markers (Halldén et al., 1996). Qu and Hancock (1997) were able to create an extensive genetic linkage map for blueberry, *Vaccinium* species with the use of inter-specific crosses of diploid and tetraploid species. A genetic linkage map was created for walnut with the use of RAPD markers and back crossing in 1996 by Woeste et al. Molecular genetic markers have also been found for seedlessness in grape (Striem et al., 1996), scab resistance in apple (Yang et al., 1997), *Fusarium lateritium* resistance in sweet potato (Buffone et al., 1996), and golden mosaic virus resistance in common bean (Urrea et al., 1996). The use of RAPD markers rather than RFLP markers can be of benefit to researchers due to the shortened analysis period and the need for smaller amounts of genomic DNA. The RAPD markers can then be further examined and gene specific primers can be developed with little difficulty.

The number of genetic differences or polymorphisms distinguishable depends largely on the primer used for analysis but is also highly dependent on the conformation of the genome itself. With RAPD's, polymorphisms can easily be identified through the presence or absence of bands in the electrophoretic gel.

These presences or absences can be the result of mutations at the annealing site of a primer in the genome that results in changes in the annealing of the primer or from mutation within the region of amplification resulting in a larger or smaller DNA fragment being produced. One of the potential problems associated with RAPD-PCR is that the product size is generally limited to 300 to 3000 bases in size. Although this size range is limited for most species studied, it is sufficient for the characterization of the species.

Chapter 3

MATERIALS AND METHODS

A total of 39 species and cultivars were examined (Tables 3-1 and 3-2). The majority of the plants were container grown in a greenhouse at the W.D. Holly Plant Environmental Research Center in Fort Collins Colorado under natural day length and temperatures ranging from 10°C in the winter and 28°C during the summer. Three of the plants studied were derived from tissue culture grown plants. Stems were harvested during the fall and placed in a 4°C cooler for short term storage. Leaf samples were collected from the healthiest young stems and only the top portion of the stem was used for DNA extraction. All samples were weighed, washed in distilled water, patted dry using laboratory wipes, wrapped in plastic food wrap and placed in a -70°C freezer until DNA extraction was performed. For the tissue culture grown material, extraction was performed using fresh material only. DNA was extracted for all samples at least twice and the best results as determined by relative DNA purity used for RAPD analysis.

As there is a good deal of confusion about the identity of several of the *Alstroemeria* species, alternate names are provided in Table 3-1. The label used

Table 3-1: *Alstroemeria* species and abbreviations, source and synonyms used for analysis.

Species or cultivar	Synonym	Source	Origin	Abbrev
A. aurantiaca Don-Yellow Flower	*A. aurea* *A. lutea*	Unknown	Chile	aur441
A. aurantiaca Don-Orange Flower		Unknown	Chile	aura
A. caryophyllae		Dr. G. Pisen, Utah	Chile	cary
A. chilensis Cree	*A. ligtu* ssp *ligtu*	U.K.	Chile	chil
A. haemantha Ruiz and Pavon	*A. ligtu* ssp *simsii*	U.K.	Chile	haem
A. ligtu hybrid		Holland	Chile	lh
A. ligtu L. species	*A. chilensis* ?	Chile	Chile	li341
A. magnifica spp *maxima* Philipi		Fred Meyer	Chile	magm
A. psittacina Lehm	A. pulchella A. braziliensis	Jardin Alpin-Commun de Meyrin, France	Chile	psit
A. pulchella L.	A. psittacina	Univ. Botanical Garden, Dresden, Germany	Chile	pulc
A. pulchra ssp *lavandulaceae**		Fred Meyer	Chile	pulv
A. versicolor Ruiz and Pavon*		Fred Meyer	Chile	vers

* Tissue culture derived samples.

Table 3-2: *Alstroemeria* cultivars and abbreviations used for analysis.

Cultivar	Abbreviation
Alnba	alnb
Appel Bloesem	appl
Arizo	ariz
Canaria	cana
Carmen	carm
Claudia	clau
Eureka	eure
Jubilee	jubi
King Cardinal	kica
Luceana	luce
Marina	mari
Mona Lisa	mona
Monika	moni
Neva	neva
Orange Beauty	orbe
Orchid	orch
Orego	oreg
Pink Triumph	pint
Red Surprise	reds
Regina	regi
Rosario	rosa
Rosita	rosi
Sarah	sara
Texas	texa
Yellow King	yelk

in this report is the one provided by the original source of the plant or seed (Stephens, 1995). Included in the species studied are two forms of *A. aurantiaca*, two different *A. versicolor* derived from different seed sources and specimens of *A. psitticina* and *A. pulchella*. The latter two species are commonly considered to be different names for the same species (Stephens, 1995). Three *A. ligtu* hybrids were also studied with the species.

Total Genomic DNA Extraction

Leaf samples totaling 2 grams were collected from young vegetative shoots. Six samples from different genotypes were extracted at each session. Samples were removed from the freezer and placed on ice until grinding in liquid nitrogen. Samples were unwrapped and placed in a pre-chilled mortar and liquid nitrogen was poured over the sample to flash freeze it. The frozen sample was then ground with a pestle until a fine powder was formed. The sample was then placed in a centrifuge tube containing 20 mls of CTAB extraction buffer (Table A-1) preheated to 65°C. The samples were gently inverted to mix with the extraction buffer. The samples were then placed in a 65°C water bath and incubated for 30 minutes with tube inversion every 10 minutes.

After incubation for 30 minutes the samples were removed and 15 mls of a 24:1 chloroform:isoamyl alcohol mixture (Table A-1) was added to each sample. The samples were then gently mixed for 15 minutes at room temperature. After mixing, the samples were balanced against each other in a polypropylene Oak Ridge centrifuge tube (Fisher Scientific) using the chloroform:isoamyl alcohol

mixture. The samples were centrifuged in a Baxter Scientific Biofuge 17R centrifuge at 6,000 RPM at 4°C for 10 minutes. The supernatant was then removed and filtered through sterile miracloth into another centrifuge tube. The chloroform:isoamyl alcohol extraction was then repeated to remove further proteins from the solution. After centrifuging for a second time, the supernatant was filtered through sterile miracloth into a clean centrifuge tube. Fifteen to twenty mls of ice-cold 95% ethanol was then added to the centrifuge tube to precipitate the nucleic acid. This solution was then incubated for a minimum of an hour in a -20°C freezer to allow for maximum nucleic acid precipitation.

After incubation, the samples were moved to a laminar air flow hood and the nucleic acid was removed using a sterile glass hook and placed into a sterile 50 ml beaker. The nucleic acid was then washed in 15-20 mls of ice cold 70% ethanol. The nucleic acid was then removed and placed into a sterile 1.5 ml microcentrifuge tube and allowed to dry in the laminar air flow hood. Once dry, usually 15 to 20 minutes, the nucleic acid was then redissolved in 500 μls of TE buffer (Table A-1). The nucleic acid was dissolved using gentle agitation and if necessary incubation in a 65°C water bath for a few minutes.

Once dissolved, 10 μls of a 10 mg/ml RNAase stock and 2 μls of a 10 mg/ml Proteinase K stock was added to each sample and gently mixed followed by incubation at 37°C in a water bath for 20 minutes. After incubation, 50 μls of a 3M sodium acetate solution was added and gently mixed. One ml of ice cold 95% ethanol was added to re-precipitate the DNA followed by incubation for a minimum of 30 minutes in a -20°C freezer. The samples were then centrifuged in a Baxter

Scientific Biofuge 17R at 10,000 RPM for 15 minutes at 4°C to separate the DNA from the rest of the solution. The supernatant was poured off and discarded. The DNA was rinsed in ice cold 70% ethanol and then allowed to air dry in the laminar air flow hood. The dry DNA was then redissolved in 500 μls of TLE buffer (Table A-1). Twenty μls of the DNA solution was added to 580 μls of sterile distilled water and used for spectrophotometric analysis. The rest of the solution was stored in a -20°C freezer until ready for use.

The diluted sample was used to check the purity and quantity of the DNA using a Beckman DU640 spectrophotometer. Samples that had 260:280 ratios of less than 1.7 or greater than 1.95 or whose concentration was less than 300 μg per ml were re-extracted. The original DNA solution was then used to make a solution with a concentration of 12.5 μg per ml for use in the RAPD reactions. The samples were diluted into sterile distilled water for the creation of the RAPD stock solution.

PCR Amplification

All initial liquid manipulations were performed in a laminar air flow hood. All components of the PCR reaction were kept frozen at -20°C and then thawed in ice prior to use. Each PCR reaction was a total of 25 μls. A total of 35 samples was run at a time. For each individual reaction 15 μls of sterile distilled water, 2.5 μls of 10X PCR buffer containing 1.5 mM magnesium chloride (Perkin-Elmer), 0.25 μls of each deoxynucleotide, 0.5 μls of 25 mM magnesium chloride (Promega) were added to make a total of 2.0 mM in the solution and 2 μls of 2.52 mM primer (University of British Columbia, Genosys) diluted from a 15.15 mM stock. To ensure

the proper concentration of all components, a master mix was created by multiplying the number of samples plus one for error in pipetting by each of the amounts given above. Twenty-one μls of the master mix was added to each 500 μl PCR tube and labeled according to primer and DNA sample to be added. Prior to the addition of the sample DNA the PCR tubes were placed on a ultra-violet transilluminator and irradiated for 10 minutes to reduce the extent of outside contamination. After irradiating, 2 μls of sample DNA was added to the appropriate tube and overlaid with two drops of mineral oil (Sigma Chemical Company). The PCR tubes were then placed in a Perkin-Elmer PE480 thermocycler and heated to 95°C for 5 minutes and then cooled to 80°C. During the heating step, the DNA Taq polymerase was diluted to 0.75 units Taq per reaction tube from a solution of 5 units per μl. For 35 samples, 5.4 μls of Taq (Perkin-Elmer) was added to 66.6 μls of sterile distilled water. Two μls of the Taq solution was added to each reaction tube while soaking at 80°C. After the Taq was added, the reaction tubes were heated to 94°C for 7 minutes and then run through 45 cycles of heating to 94°C for one minute, cooling to 36°C for 1 minute and heating to 72°C for 2 minutes. After the cycles were completed the samples were held at 72°C for an additional five minutes and then cooled and held at 4°C until placed in a -20°C freezer or run on an electrophoretic gel.

University of British Columbia RAPD primer sets 4 and 5 were evaluated for usable primers. Thirty primers were randomly picked and screened for amplification. Ten of the primers showed amplification and of those five showed banding that could be used for analysis. Primers 402, 405, 411, 414 and 415 were initially used plus the addition of an additional custom made primer purchased from Genosys

(Table 3-3). Primers 414 and 415 were later discarded due to excessive amplification in the control lane and poor amplification and reproducibility. Screening of primers involved running multiple primers using two arbitrary species and a cultivar to ensure that amplification was possible with both species and cultivars. Those primers that were found to amplify and produce bands were further narrowed to those primers that produced strong bands and did not amplify in the control lane. Controls consisted of sterile distilled water in place of the sample DNA.

Each DNA sample was replicated during a single PCR reaction so two reactions were included in each PCR. Each primer was repeated so a minimum of four separate reactions were performed in two RAPD cycles. The DNA used in all the reactions was from a single extraction to ensure conformity in the reactions.

Gel Formation and Electrophoresis

Large format polyacrylamide gels were used for the separation of the RAPD product. The glass plates on which the gels were formed measured 35 cms in width and 45 cms in length and 0.4 mm thick. Forty-six or 70 RAPD samples were run on each gel along with a 1 Kb molecular marker placed at the outermost wells and in the center of the gel to check uniformity in migration of the RAPD products through the gel.

An Owl Scientific Nugeneration sequencing gel apparatus was used. Two glass plates were used in the formation of the gel, one notched plate (Owl Scientific) and one solid plate (Owl Scientific and Blacks Glass). The solid plate was throughly cleaned with soap and 95% ethanol to remove organic compounds.

Five μls of bind silane (γ-methacryloxy-propyl-trimethoxysilane, Sigma Chemical Company) was added to one ml of a 95% ethanol 0.5% glacial acetic acid solution (Table A-2) and mixed well. The bind silane solution was applied to the plate and spread evenly over the entire plate. The plate was allowed to dry for 5 minutes before continuing. The plate was washed three times using 95% ethanol to remove excess bind silane. After washing the plate was set aside and covered while the notched plate was prepared.

The notched plate was throughly cleaned with soap and 95% ethanol to remove organic compounds. One ml of Rain-X (Unelko Corp.)was poured on the plate and wiped across the entire plate until a haze developed. The application of the Rain-X was repeated.

Spacers 0.4 mm in width were placed on either edge of the gel and the solid plate was placed on the notched plated with the bind silane side facing the notched plate. A spacer was inserted into the bottom gap of the sandwich and aligned with the bottom edge of the plates and clamped into place. The plates were then sealed on three sides with gel sealing tape (Gibco). Special care was taken to form tight seals along the entire length of the plate sandwich and "military corners" were formed to ensure leakage was kept to a minimum.

A 25 ml pipette was used to pour the acrylamide solution into the plate. Any bubbles that formed were removed by tilting the plate away from the bubble location until the bubble was reached and removed. The plate was filled to the lip of the notched plate and the sharks tooth comb was inserted smooth side down approximately 5 mm into the gel. The plate was then placed on the lab counter and

propped at a 5 degree angle to minimize leakage. The space left above the notched plate was covered with a portion of the acrylamide solution to prevent bubbles from forming near the top of the gel.

Excess acrylamide was poured into a disposable 50 ml centrifuge tube, sealed, and then placed on its side for observation. The gel polymerized within 30 minutes of pouring and could then be used immediately or stored for several hours until ready to use. Polymerization was identified in two ways. First, the formation of condensation on the sides of the centrifuge tube indicated that polymerization had occurred. Second the formation of schlieren type bands at the interface of the comb and gel. A schlieren band is a horizontal band that gradually blends into the main body of the gel.

Once the gel had fully polymerized, it was placed in the vertical gel housing and clamped into place. 1X TBE buffer was placed in the lower buffer chamber and any air pockets that existed between the gel and the buffer were removed with a pasteur pipette. The upper buffer chamber was then filled with 1.5X TBE buffer and the sharks tooth comb was carefully removed and set aside with careful note of the original orientation of the comb in the gel. The interface between the gel and the buffer was then cleaned using a pasteur pipette and gentle pressure to remove any un-polymerized acrylamide. The sharks tooth comb was then reinserted with the teeth toward the gel. The teeth were placed so that they slightly dented the gel surface but did not penetrate it. This was done so that the space between the teeth formed the sample wells.

The RAPD products were then removed from the freezer or thermocycler and 7.5 μls of tracking dye was added to each sample and mixed. Five μls of each sample was placed in a well on the gel. A 1KB molecular marker was placed in the wells on the edge of the gel and in a central well to check for uniformity of DNA migration. When all the samples were loaded the gel was run for 18 hrs at 14 milliamps at room temperature.

Staining

Silver staining was performed using the protocol developed by Antolin et al. (1996). When the gel had completed its electrophoretic run, the gel was removed from the housing and placed on the counter. The sharks tooth comb was removed from the gel and the two glass plates separated. When the two plates separated the side spacers and the notched plate were set aside and the plate with the gel adhered to it was placed in a tub. The acetic acid solution (Table A-4) was poured over the gel and the tub was placed on a shaker table and gently agitated (50 rpm) for 20 minutes or until the blue marker dye band was no longer visible. The gel was then removed from the tub and rinsed three times in distilled water for 2 minutes per rinse. The gel was then placed in a tub containing the silver nitrate solution (Table A-4), covered and gently agitated for 30 minutes. The gel was then rinsed for 10 to 15 seconds with distilled water and placed in a tub ready to be developed. Prior to developing the gel, the lights directly over the gel were turned off and blinds closed. The developing solution (Table A-4) was then poured onto the plate and agitated by hand until the DNA markers began to darken. When the 1 KB fragments began

to darken the lights were turned back on and developing continued until sample bands became evident. The developing reaction was terminated before excessive darkening of the background could occur. To terminate the reaction the fixing solution from the first part of the process was poured onto the plate and agitated until effervescing stopped. The plate was then placed in distilled water and agitated for 10-15 minutes with a transfer to fresh distilled water and soaking for 10 minutes. The plate was then removed from the water and placed against a wall and allowed to air dry. While drying, 10 grams of sodium chloride was added to the silver nitrate solution to precipitate the silver as silver chloride. The silver chloride was then placed in a plastic beaker and allowed to dry into a powder and was set aside and stored.

Scoring

After the gel had dried it was placed on a light table for band scoring. Only those bands clearly visible and repeated in both lanes of the sample were scored as being present. The distance from the origin to the band was measured and recorded in cms. The molecular marker was measured and recorded in the same manner. The bands were compared with the repeated samples on the additional run. Only those bands that appeared on a minimum of three of the sample lanes were counted as being present for final analysis. Due to the non-conformity of the migration of the bands, visual observation between the plates was made to confirm the bands found corresponded to each other. Once the bands were verified, a rough molecular weight was determined using the equation developed by Schaffer and

Sederoff (1981). The bands were then converted into a matrix of 1 and 0 indicating presence or absence of the band respectively. This matrix was completed for all specimens and primers studied.

The complete matrix was analyzed using the cluster analysis program of Statistica using un-weighted pair-group average with Euclidian distances. A phylogenetic tree was produced for interpretation. The algorithm used for the cluster analysis was $d_{ku}=(T_i d_{ki} + T_j d_{kj})/ (T_i + T_j)$ with the algorithm used for calculating distance as $D=\{\sum_l (x_i - y_i)^2\}^{1/2}$ (Swofford et al., 1996).

Table 3-3: Sequence and name of primers used for RAPD analysis.

Primer	**Sequence**
UBC 402	3' CCC GCC GTT G 5'
UBC 405	3' CTC TCG TGC G 5'
UBC 411	3' GAG GCC CGT T 5'
Alst-19	3' ACC CCC GAA G 5'

Chapter 4

SPECIES RESULTS

The RAPD assay employed used a total of 30 primers from the 400 series of UBC primers plus one custom primer to screen the 15 species and hybrids. Ten of the primers used in the study produced amplification when viewed on agarose gels stained with ethidium bromide. Of these ten primers only six produced reproducible bands. Two of the six primers consistently produced segments in the control reactions and had poor amplification resulting in weak bands. These two primers were eliminated from further examination of the species due to the possibility of misinterpreting banding results in the gel.

The RAPD-PCR was customized for the species studied. After testing several concentrations of magnesium chloride (1.0, 1.5, 2.0, 2.5, 3.0, 3.5 mM), a final concentration of 2.0 mM was found to produce the strongest bands. Various concentrations of Taq were also tested (0.25, 0.5, 0.75, 1.0 units per reaction) and 0.75 units per sample was found to be adequate for amplification. Concentrations of template DNA were not varied and remained at 25 ng per reaction during the entire experiment. The use of 25 ng of template DNA, 2.0 mM magnesium chloride

and 0.75 units of Taq gave consistent results for the primers and produced clear readable bands that were reproducible.

Initial trials at DNA extraction resulted in the extraction of phenolic compounds and little if any DNA. The addition of 2% PVP-40 to the extraction buffer allowed for the extraction of total genomic DNA. When tested on the spectrophotometer, the DNA was revealed to be of high purity and relatively high quantity when examined using a spectral absorbance curve (Figure A-1). The ratio of OD 260/280 resulted in purity ratios that ranged from 1.71 to 1.97 (Table A-5). A ratio less than 1.86 indicates contamination with proteins and a ratio greater than 1.91 indicates contamination with RNA. Excessive contamination by proteins may inhibit the ability of the DNA to be amplified. Excessive RNA contamination may result in the amplification of DNA segments specific to the RNA and not the sample DNA. The inclusion of an Rnase in the extraction protocol will reduce this problem. Minimum or maximum acceptable OD 260/280 ratios are 1.70 and 2.0. All samples used showed a ratio that fell within an acceptable range for purity. The concentration of DNA ranged from 94 to 1469 μgs per ml. The lowest concentrations were derived from tissue culture samples of *A. pulchra* ssp *lavandulaceae*, *A. versicolor* 46-666 and 94-11. These results indicated that tissue culture samples are not ideal sources when this extraction process is used.

Source material was also examined when it came to determining ideal sources of leaf material. Older leaf material from the midstem yielded less DNA and was highly contaminated with proteins which made amplification less reliable. The

best performance was achieved with DNA derived from the youngest portion of the stem and included the growing point.

Preliminary evaluations of tissue and length of time frozen indicates that the storage of the material was also found to be important in terms of the quality and quantity of the DNA extracted. Leaf material stored at -70°C for a long period of time yielded less DNA of lower quality than fresh material. These results indicate that the use of material collected directly from the plant and stored for the shortest period of time was the best for DNA quantity and quality. Overall the results indicated that the extraction process works well with *Alstroemeria* when 2% PVP-40 is added to the extraction buffer and when fresh material is used.

Levels of Polymorphism

Thirty decamer primers were tested for production of bands with the 15 lines studied. Only 50% of these primers produced amplification products. Only 6 of the 15 primers produced scorable bands with four of them producing strong bands and two producing weak bands. A scorable band was one that was repeatable and that was clearly visible with the use of a light table. This research also tested three Taq polymerases from three sources (Boehringer Mannheim, Promega and Perkin Elmer) to see if significant differences occurred with regards to repeatability. No differences were found to exist among the polymerases used. Perkin Elmer proved to be the least expensive on a per unit basis and was therefore used for amplification in this study.

The primers used yielded highly polymorphic bands which were useful in the characterization of the species. Amplified DNA fragments or bands of different sizes and numbers were produced for each of the species studied. The number of bands produced ranged from 1 to 5 depending on the species and primer used (Table 4-2). Many more bands were produced than were scored since many of these bands were not repeatable or clearly visible. The production of large numbers of bands is associated with the use of polyacrylamide and silver staining. Fragment size ranged from 200 to 1500 bases with the majority of the bands falling between 507 and 1018 bases as indicated by the 1 KB molecular marker. A total of 73 bands were produced across all species and primers. Individual species and primers produced fewer bands. All primer results are presented in tabular form at the end of this chapter (Table 4-2).

Primer 402

For the species and hybrids studied, primer 402 produced eleven reproducible bands across the fifteen subjects (Figure 4-1, 4-2). Bands ranged in size from 560 bases to 1040 bases. The two color variants of *A. aurantiaca* had four of five bands in common. There were many common bands between two of the *A. ligtu* hybrids with the third being polymorphic at one band. A high degree of common bands was also found to exist between *A. psitticina* and *A. pulchella* with all four bands scored occurring at the same locations relative to one another. A high degree of homology existed between the two accessions of *A. versicolor* with all three bands present at the same locations. A high degree of polymorphism existed

among the rest of the species with as few as one and as many as three bands in common. The highest degree of common bands among the species occurred with the band at 730 bases in size which was common to eight of the fifteen lines.

Primer 405

Primer 405 produced a total of 10 repeatable bands across all species and hybrids studied (Figure 4-1, 4-3). Band sizes ranged from 660 bases to 1260 bases. The two color variants of *A. aurantiaca* had 2 common repeatable bands. A great deal of diversity existed between the two accessions of *A. versicolor* with only one out of four bands in common. *Alstroemeria pulchella* and *A. psitticina* exhibited a single common band. The *A. ligtu* hybrids exhibited a greater degree of polymorphism than with primer 402, with only one of the three bands scored common across the three lines. The remaining species showed a high degree of polymorphism among them with the highest degree of common bands occurring at a band of 810 bases.

Primer 411

Primer 411 produced a total of 26 reproducible bands across all species and hybrids studied (Figure 4-4). Band sizes ranged from 510 to 1030 bases. Only one of four bands produced was common to the two color variants of *A. aurantiaca* . A higher degree of likeness was observed in the two accessions of *A. versicolor* with three of four bands at the same relative location within the gel. *Alstroemeria pulchella* and *Alstroemeria psitticina* showed a high degree of likeness with one another in that four of five bands were common. The three *A. ligtu* hybrids also

exhibited a degree of likeness with four common bands across all three hybrids. A high degree of variation in banding existed among the remaining species, with the exception of the band at 510 bases that was shared among 11 of the 15 species and hybrids.

Primer Alst-19

A total of 26 reproducible bands were produced using primer Alst-19 for all species and hybrids studied (Figures 4-5 and 4-5a). Band sizes ranged from 395 to 1080 bases. *Alstroemeria aurantiaca* showed a high degree of likeness between the two color variants studies with four of five bands in common. Only two of six bands in the 2 accessions of *A. versicolor* were common indicating a high degree of variation in the species. Three of four bands were common to *A pulchella* and *A psitticina*. Only two of seven bands were common across all three *A ligtu* hybrids with five of seven bands being shared between any two of the hybrids. A high degree of variation in banding existed among the remaining species, with only a slight degree of likeness existing among some of the species and hybrids.

***Alstroemeria aurantiaca* color variants**

A total of 13 bands were produced for the orange color variant of *A. aurantiaca* and 11 bands for the yellow variant. Of these bands eight were common across both variants. Four reproducible bands were produced with primer 402 for the orange variant and only one was observed for the yellow. Two bands were produced for both variants and were common with primer 405. Two bands were

observed for the orange variant using primer 411 while four bands occurred for the yellow variant with the same primer. Five bands were produced using primer Alst-19 for the orange variant, four of which were observed in the yellow variant.

Alstroemeria caryophylla

A total of eight bands were observed for *A. caryophylla* using all four primers. Only one reproducible band was produced for primer 402 and primer 405, four using primer 411, and two bands for primer Alst-19. Of these eight bands, only one was found to be unique to the species. The remaining bands were similar in size with at least one other species or hybrid studied.

Alstroemeria chilensis

A total of eleven bands were produced for *A. chilensis* using all four primers. Two reproducible bands were produced using primer 402, no bands with primer 405, 5 bands using primer 411, and four bands using primer Alst-19. All bands produced had homology in size with at least one of the other species or hybrids studied.

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A total of eleven reproducible bands were produced using the four primers. Two reproducible bands were produced for both primer 402 and 405, three bands with primer 411 and four bands with primer Alst-19. All bands produced were common with at least one of the other species studied with five bands common to *A. caryophylla*.

***Alstroemeria ligtu* hybrids**

The three *A. ligtu* hybrids studied produced a total of 46 bands among them using all four primers. Hybrid 86H-1-5 had a total of 14 reproducible bands, while hybrid 86H-21-19 and 86H-34-14 had a total of 16 bands each. Two reproducible bands were observed with primer 402 for all three hybrids with one of the bands in hybrid 86H-21-19 being unique. Two reproducible bands were observed with primer 405. The two bands in hybrids 86H-1-5 and 86H-21-19 were alike but were not common to with two bands produced in hybrid 86H-34-14.

Six bands were produced by hybrid 86H-1-5 using primer 411, four were produced for hybrid 86H-21-19 and five for hybrid 86H-34-14. A total of four of the bands were alike among all three hybrids with the remaining bands in hybrids 86H-1-5 and 86H-34-14 being unique. Four bands were produced for hybrid 86H-1-5 using primer Alst-19 with seven bands observed in both hybrid 86H-21-19 and hybrid 86H-34-14. Two of these bands were in common across all three hybrids. Five of the bands were alike between hybrids 86H-21-19 and 86H-34-14 but were not common to 86H-1-5.

Alstroemeria ligtu

A total of ten bands were observed for *A. ligtu* using all four primers. No reproducible bands were produced for primer 402, three for primer 405, four for primer 411 and three for primer Alst-19. Although five of the bands observed were common with at least one of the *A. ligtu* hybrids studied, only one band was common across all three hybrids.

Alstroemeria magnifica* ssp. *maxima

A total of eleven bands were observed for *A. magnifica* spp. *maxima* using all four primers. No reproducible bands were produced using primer 402, only one for primer 405, three for primer 411 and seven using primer Alst-19. Two of the bands observed were unique to the species with the remaining bands being shared with at least one of the other species studied.

Alstroemeria psitticina* and *Alstroemeria pulchella

Since *A. psitticina* and *A. pulchella* are commonly considered to be synonyms for the same species and thus will be discussed together. A total of 14 reproducible bands were observed for *A. psitticina* and 12 for *A. pulchella*. Eleven of the bands produced by both species were alike. Three bands were found to be unique to *A. psitticina* and one band was found unique to *A. pulchella*. Five of the bands were found to be repeated in at least one of the other species studied.

Alstroemeria pulchra* ssp *lavandulaceae

A total of eleven bands were produced using all four primers. One reproducible band which was unique to this species was produced using primer 402. Primer 405 produced two reproducible bands, one of which was found to be unique to this species, with the other being common to five other species. Three bands were produced using primer 411, and five with primer Alst-19, none of which were unique to the species but were common with at least one of the other species studied.

Alstroemeria versicolor accessions

A total of twenty-seven bands were observed for the two *A. versicolor* accessions with accession 46-666 producing 14 bands and accession 94-11 producing 13 bands. A great deal of diversity was evident as 7 bands were common to the two accessions, while the remaining seven bands for accession 46-666 and six bands for accession 94-11 were not common. Two bands in accession 46-666 were unique to the accession and one band was unique to accession 94-11. All other bands produced were common to at least one other species studied.

Cluster Analysis

The data, using the 73 bands, generated a dendogram with four distinct clusters when it was subjected to cluster analysis using un-weighted pair group method (Figure 4-6). The two *A. versicolor* accessions clustered together while *A. pulchella, A. psittacina, A. pulchra* ssp *lavandulaceae*, and *A. magnifica* ssp *maxima* formed a second general cluster. The three *A. ligtu* hybrids clustered with *A. chilensis*, *A. haemantha* and *A. caryophylla*, but did form a distinct group from the other three species. *Alstroemeria ligtu* clustered with the two color variants of *A. aurantiaca.* The linkage distance of the species as indicated in the dendogram (Figure 4-6) gives an idea of the relatedness of the species. All species and hybrids studied were separated using the 73 bands produced by the four primers used.

As indicated on the dendogram, the two *A. versicolor* accessions are related with a linkage distance of 3.6. *Alstroemeria pulchella* and *A. psittacina* are linked at

a distance of 2.0 while *A. magnifica* ssp *maxima* and *A. pulchra* ssp *lavandulaceae* have a linkage value of 4.0. *Alstroemeria pulchella* and *A. psittacina* have a linkage distance to *A. magnifica* ssp *maxima* and *A. pulchra* ssp *lavandulaceae* of 4.4. The three *A. ligtu* hybrids are linked at a distance of 3.6 while hybrid 86H-21-19 and 86H-1-5 have a linkage of 3.4. *Alstroemeria chilensis* had a linkage distance of 3.8 when compared to *A. caryophylla*. A linkage distance of 3.0 occurred between *A. haemantha* and *A. caryophylla*. The *A. ligtu* hybrids had a linkage distance of 3.9 when compared to the other species in its cluster. *Alstroemeria ligtu* was linked at a distance of 4.0 when compared with the two color variants of *A. aurantiaca.* The two color variants of *A. aurantiaca* had a linkage distance of 2.8 when compared with each other.

Alstroemeria versicolor had a linkage distance of 4.6 when compared to the rest of the species and hybrids studied. The cluster containing *A. ligtu* and the two color variants of *A. aurantiaca* had a linkage distance of 4.5 when compared to the rest of the species and hybrids studied. The cluster containing all the species and hybrids except *A. versicolor, A. ligtu* and the two color variants of *A. aurantiaca* had a linkage distance of 4.4 when compared across all the species and hybrids studied.

The examination of the relationships of the *Alstroemeria* species could only be done in a preliminary way as there was a limited number of genotypes for each species, one in most cases, available for RAPD analysis. Further analysis of the species, including species unavailable for this study, must be done in order to firmly

establish the relationships. Analysis using more primers is also necessary for the establishment of relationships.

Table 4-1: Sizes in base pairs of DNA fragments observed for four primers used in *Alstroemeria* species and hybrids.

Sample *	UBC 402	UBC 405	UBC 411	Alst-19
Aura	840, 762, 685, 658	959, 708	897, 639	819, 580, 532, 484, 407
A441	658	959, 708	828, 663, 639, 510	819, 532, 484, 395
Cary	729	820	1030, 597, 535, 510	762, 395
Chil	807, 729		1030, 816, 805, 768, 609	786, 774, 711, 657
Haem	729, 685	820, 763	1030, 816, 510	786, 774, 407, 395
L15	729, 658	852, 763	1030, 805, 794, 616, 609, 510	762, 711, 598, 395
L2119	729, 605	852, 763, 560	805, 616, 609, 510	819, 805, 786, 774, 598, 464, 395
L341		852, 763, 708	897, 731, 586, 510	819, 786, 701
L3414	729, 658	820, 735	816, 805, 616, 609, 510	819, 786, 774, 598, 566, 532, 395
Magm		708	794, 704, 626	1080, 937, 819, 711, 547, 484, 395
Psit	1040, 807, 729, 587	820, 560	816, 794, 704, 510	1080, 735, 598, 421
Pulc	1040, 807, 729	820	1007, 816, 794, 704, 510	1080, 735, 598
Pulv	560	820, 585	897, 882, 768	1080, 937, 735, 711, 407
Vers46	1040	1260, 657	882, 616, 535, 510	981, 937, 819, 805, 774, 701, 522
Vers94	1040	1260, 820, 763	616, 574, 535, 510	981, 819, 711, 657, 410

* For explanation of abbreviations see Table 3-1.

Figure 4-1. RAPD bands of *Alstroemeria* species using primers 402 and 405 run on a polyacrylamide gel using silver staining.

Lanes 1 and 38: 1 KB molecular marker with weights indicated.
Lanes 2-19: **Primer 402**,

- Lanes 2 and 3: Aura441
- Lanes 4 and 5: Aura
- Lanes 6 and 7: Chil
- Lanes 8 and 9: Lg341
- Lanes 10 and 11: Magm
- Lanes 12 and 13: Pulv
- Lanes 14 and 15: Vers46
- Lanes 16 and 17: Vers94
- Lanes 18 and 19: Eure

Lanes 20-37: **Primer 405**

- Lanes 20 and 21: Aura441
- Lanes 22 and 23: Aura
- Lanes 24 and 25: Chil
- Lanes 26 and 27: Lg341
- Lanes 28 and 29: Magm
- Lanes 30 and 31: Pulv
- Lanes 32 and 33: Vers46
- Lanes 34 and 35: Vers94
- Lanes 36 and 37: Eure

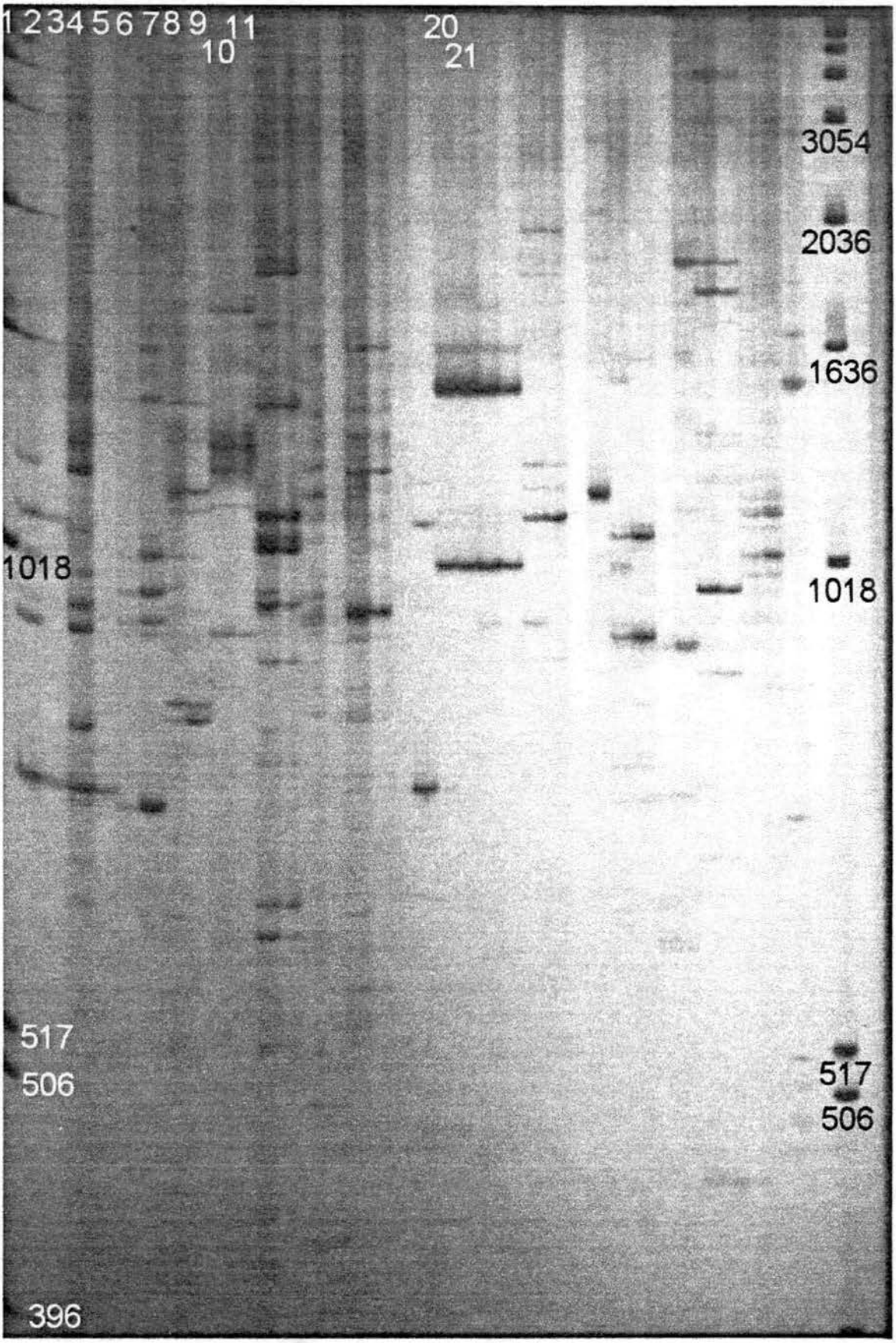

1 2 3 4 5 6 7 8 9 10 11
20 21
3054
2036
1636
1018
1018
517
506
517
506
396

Figure 4-2. RAPD bands of *Alstroemeria* species and cultivars using primer 402 run on a polyacrylamide gel using silver staining.

Lanes 1 and 38: 1 Kb molecular marker with sizes indicated.
Lane 2: Blank
Lanes 3 and 4: Aur441
Lanes 5 and 6: Aura
Lanes 7 and 8: Cary
Lanes 9 and 10: Haem
Lanes 11 and 12: Lh15
Lanes 13 and 14: L2119
Lanes 15 and 16: L3414
Lanes 17 and 18: Psit
Lanes 19 and 20: Pulc
Lanes 21 and 22: Alnb
Lanes 23 and 24: Appl
Lanes 25 and 26: Ariz
Lanes 27 and 28: Cana
Lanes 29 and 30: Carm
Lanes 31 and 32: Clau
Lanes 33 and 34: Eure
Lanes 35 and 36: Jubi
Lane 37: Control

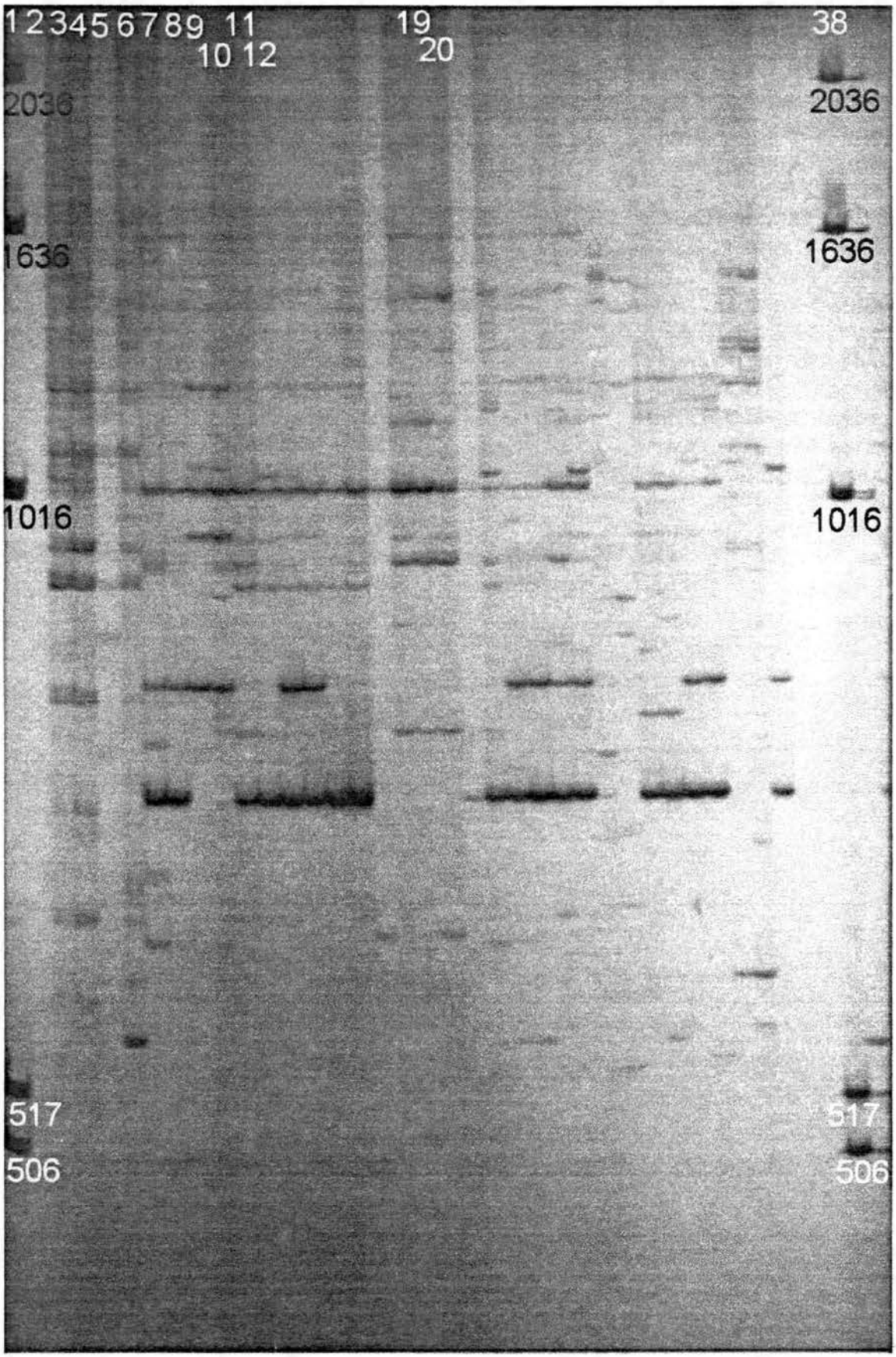

1 2 3 4 5 6 7 8 9 10 11 12
19 20
38
2036
1636
1016
517
506
2036
1636
1016
517
506

Figure 4-3. RAPD bands of *Alstroemeria* species and cultivars using primer 405 run on a polyacrylamide gel using silver staining.

Lanes 1 and 32: 1 Kb molecular marker with sizes indicated.
Lanes 2 and 3: Aur441
Lanes 4 and 5: Aura
Lanes 6 and 7: Cary
Lanes 8 and 9: Haem
Lanes 10 and 11: Lh15
Lanes 12 and 13: L2119
Lanes 14 and 15: L3414
Lanes 16 and 17: Psit
Lanes 18 and 19: Pulc
Lanes 20 and 21: Alnb
Lanes 22 and 23: Appl
Lanes 24 and 25: Ariz
Lanes 26 and 27: Cana
Lanes 28 and 29: Carm
Lanes 30 and 31: Clau
Lane 33: Control
Lanes 34 and 35: Eure
Lanes 36 and 37: Jubi

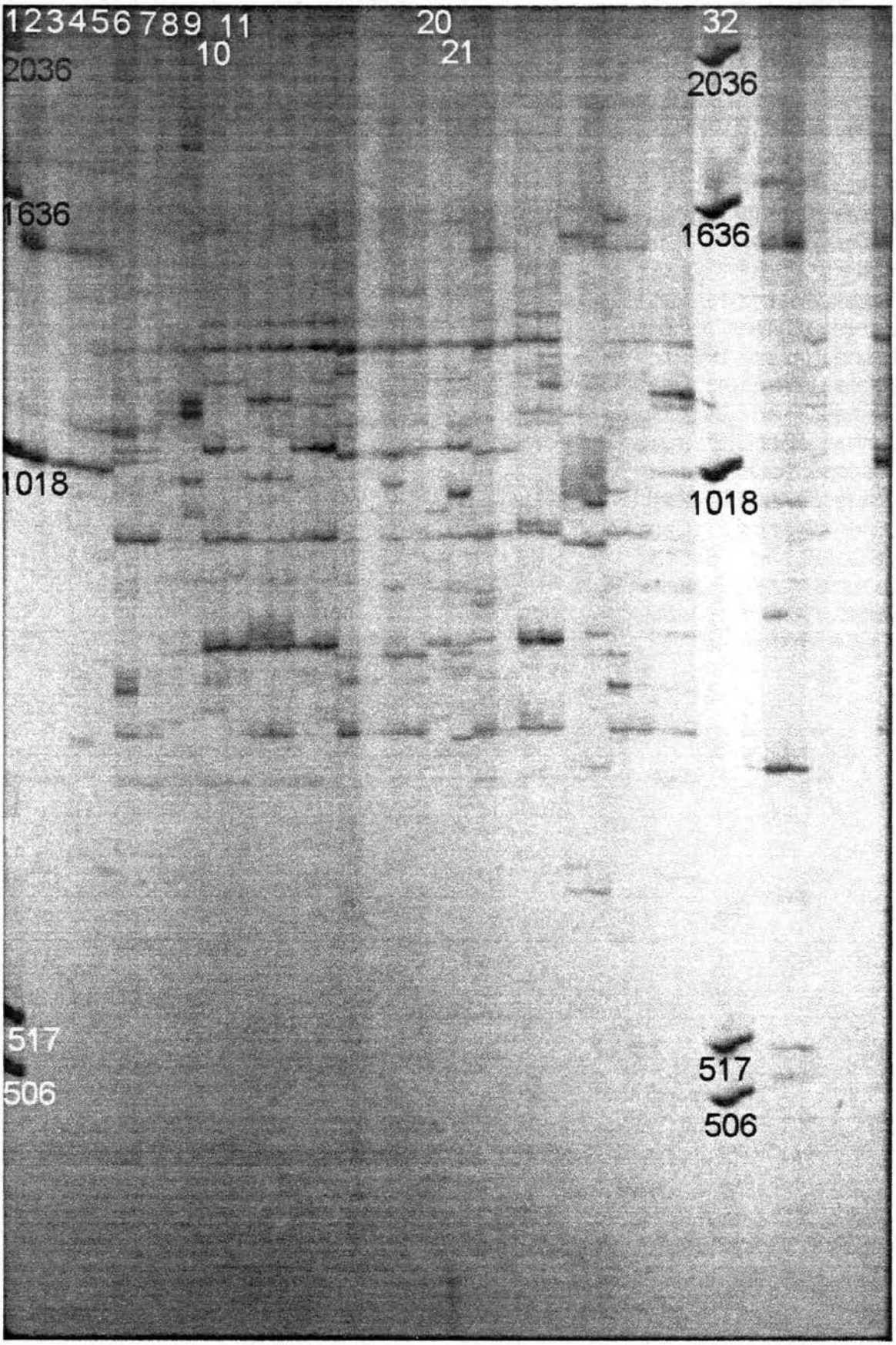
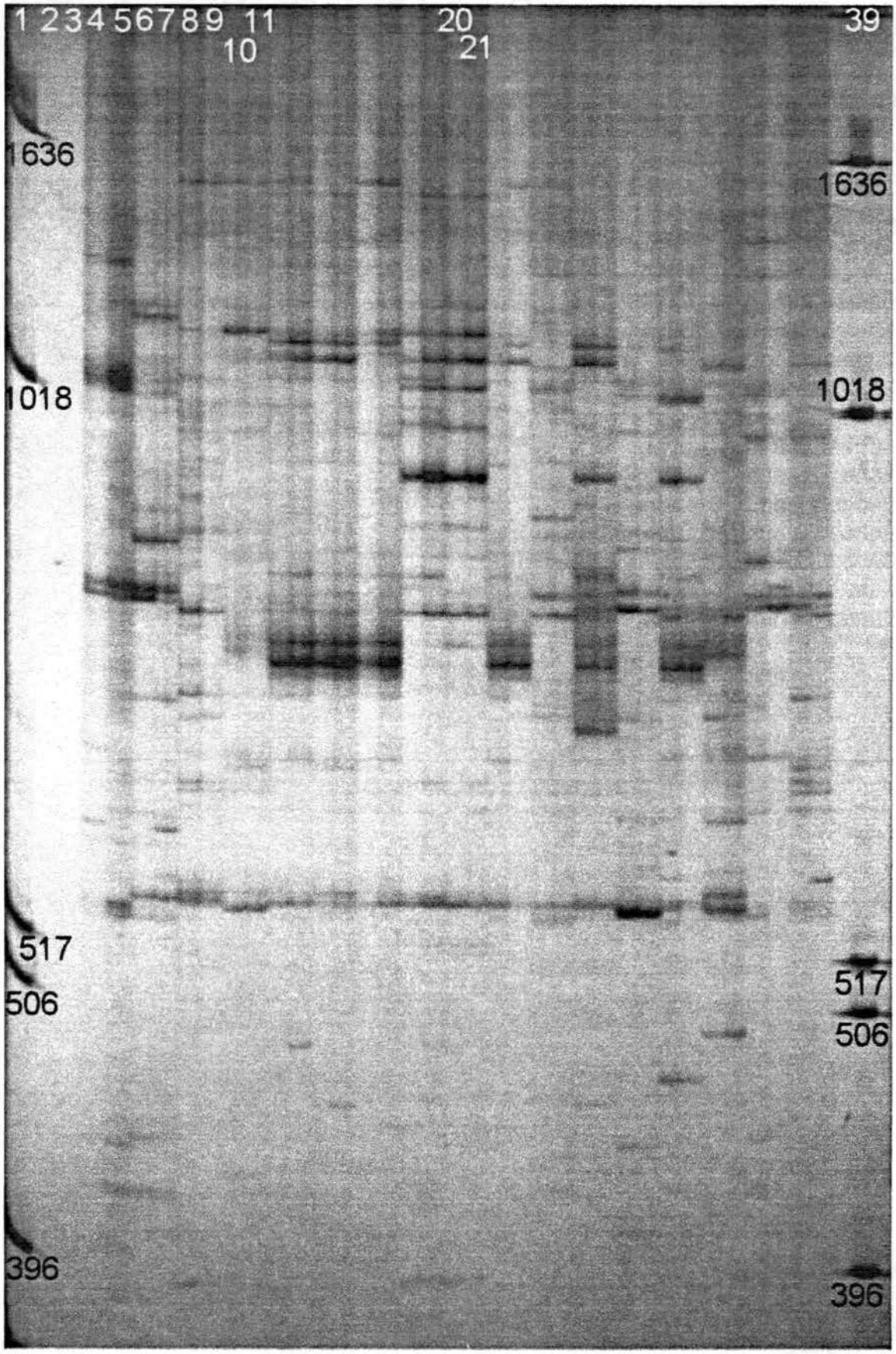

1 2 3 4 5 6 7 8 9 10 11
20 21
32
2036
1636
1018
517
506
2036
1636
1018
517
506

Figure 4-4. RAPD bands of *Alstroemeria* species and cultivars using primer 411 run on a polyacrylamide gel using silver staining.

Lanes 1 and 39: 1 Kb molecular marker with sizes indicated.
Lane 2 and 3: Blank
Lanes 4 and 5: Aur441
Lanes 6 and 7: Aura
Lanes 8 and 9: Cary
Lanes 10 and 11: Haem
Lanes 12 and 13: Lh15
Lanes 14 and 15: L2119
Lanes 16 and 17: L3414
Lanes 18 and 19: Psit
Lanes 20 and 21: Pulc
Lanes 22 and 23: Alnb
Lanes 24 and 25: Appl
Lanes 26 and 27: Ariz
Lanes 28 and 29: Cana
Lanes 30 and 31: Carm
Lanes 32 and 33: Clau
Lanes 34 and 35: Eure
Lanes 36 and 37: Jubi
Lane 38: Control


1 2 3 4 5 6 7 8 9 10 11
20 21
39
1636
1018
517
506
396
1636
1018
517
506
396

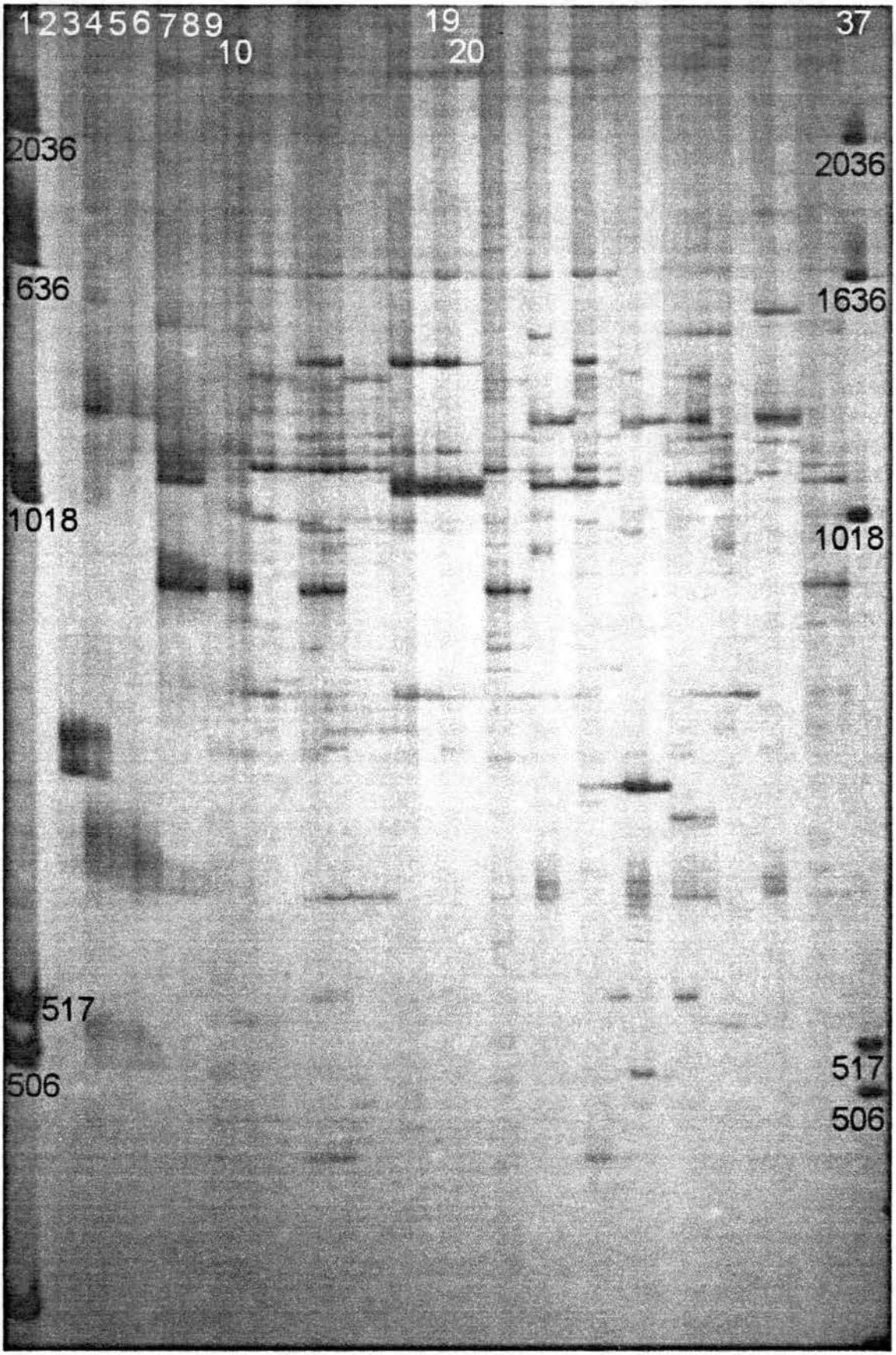
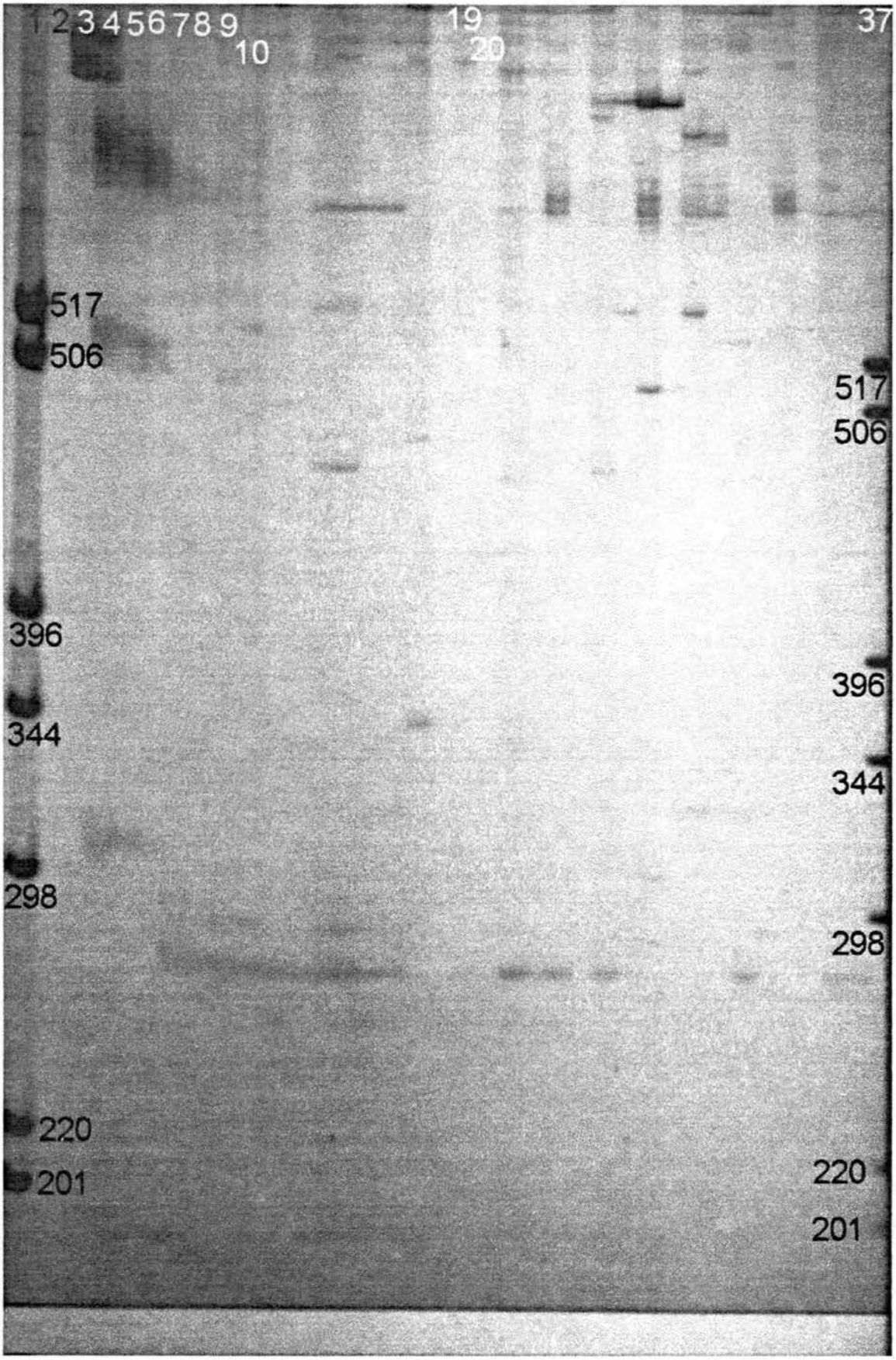
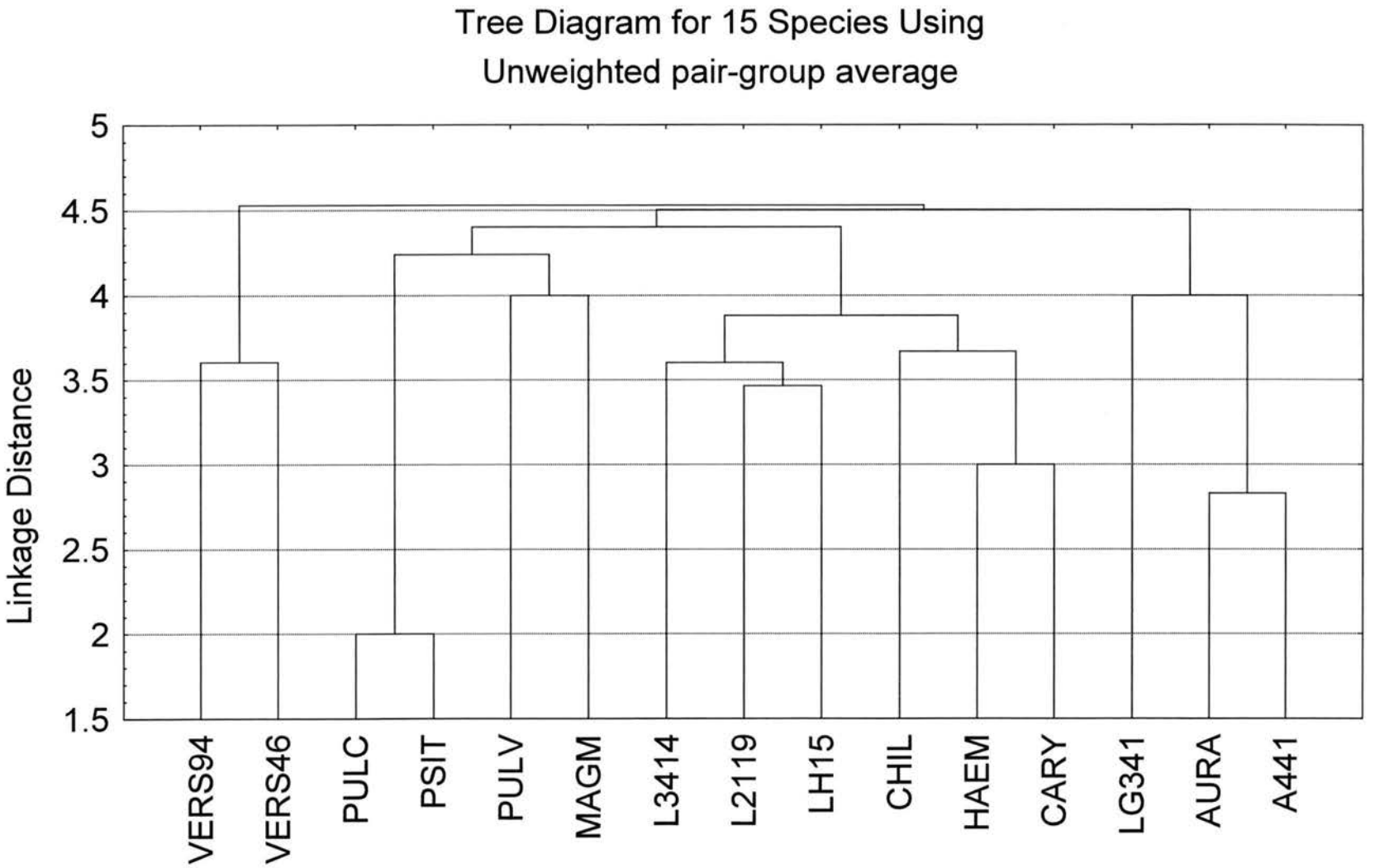

1 2 3 4 5 6 7 8 9
10
19
20
37
2036
636
1018
517
506
2036
1636
1018
517
506

Figure 4-5a. RAPD bands of *Alstroemeria* species and cultivars using primer Alst-19 run on a polyacrylamide gel using silver staining. (Lower portion of gel)

Lanes 1 and 37: 1 Kb molecular marker with sizes indicated.
Lane 2: Control
Lanes 3 and 4: Aur441
Lanes 5 and 6: Aura
Lanes 7 and 8: Cary
Lanes 9 and 10: Haem
Lanes 11 and 12: Lh15
Lanes 13 and 14: L2119
Lanes 15 and 16: L3414
Lanes 17 and 18: Psit
Lanes 19 and 20: Pulc
Lanes 21 and 22: Alnb
Lanes 23 and 24: Appl
Lanes 25 and 26: Ariz
Lanes 27 and 28: Cana
Lanes 29 and 30: Carm
Lanes 31 and 32: Clau
Lanes 33 and 34: Eure
Lanes 35 and 36: Jubi


1 2 3 4 5 6 7 8 9 10
19 20
37
517
506
396
344
298
220
201
517
506
396
344
298
220
201


Tree Diagram for 15 Species Using
Unweighted pair-group average
Linkage Distance
5
4.5
4
3.5
3
2.5
2
1.5
VERS94
VERS46
PULC
PSIT
PULV
MAGM
L3414
L2119
LH15
CHIL
HAEM
CARY
LG341
AURA
A441

Chapter 5

CULTIVAR RESULTS

The RAPD assay employed used the same primers that were used for the species assay. The RAPD-PCR was customized for the cultivars studied in the same manner as for the species. A final magnesium chloride concentration of 2.0 mM was used for the production of the strongest bands. A Taq concentration of 0.75 units per reaction was used for the PCR amplification. Template DNA concentration was set at 25 ng per reaction for all reactions and cultivars.

The extraction of the DNA for the cultivars was performed using the same protocol as for the species. The addition of 2% PVP-40 improved the quality and quantity of the DNA extracted. When the DNA was tested on the spectrophotometer, it was found to have high purity and relatively high quantity, although this varied with the cultivar (Figure A-2). The ratio of OD 260/280 resulted in purity ratios that ranged from 1.83 to 2.03 (Table A-6). Several of the samples indicated contamination with RNA. These samples were re-extracted in an attempt to get a purer DNA sample. After several extractions, little improvement in purity was achieved and the least contaminated samples were used for DNA amplification.

Purity of the DNA was also affected by the length of storage in the freezer. The longer the leaf material was stored the lower the purity (data not shown).

DNA concentrations ranged from 379 to 2552 μgs per ml. Most samples ranged from 500 to 1000 μgs per ml. All samples were extracted from the youngest portion of a vegetative stem which included the growing point. The leaf material was either extracted from fresh leaf material or from leaf material that had been stored in a -70°C freezer. The material stored in the freezer generally yielded a lower quantity of DNA. Overall the DNA extracted for the cultivars was of similar quality to the species and hybrids studied although the quantity was higher for the cultivars than the species and hybrids. The differences in the DNA quantity may be due to the ploidy level of the cultivar but this was not investigated. The DNA was diluted to 12.5 mgs per ml for the PCR amplification.

Levels of Polymorphism

Of the twenty decamer primers tested for amplification only 50% of the primers produced amplification products. Only six of the primers were capable of producing scorable bands with four of these primers producing strong bands and two weak bands. A band was scorable only if it was repeatable on a second gel from a second amplification run and clearly visible with the aid of a light table.

The primers yielded highly polymorphic bands which were useful in the characterization of the cultivars. Amplified DNA fragments of different sizes and numbers were produced for each of the cultivars studied. The number of bands produced ranged from one to seven bands depending on the primer and cultivar.

Many more bands were produced than were scored because many of these bands were not repeatable or not clearly visible. The use of polyacrylamide gels and silver staining, in general, results in the visualization of high numbers of bands. Fragment size ranged from 200 to 1500 bps with the majority of the bands falling between 507 and 1018 bases as determined with the use of a 1KB molecular marker (Table 5-1). A total of 73 bands were produced across all cultivars and primers. All bands produced were polymorphic for at least one cultivar. All primer results are presented in tabular form at the end of this chapter.

Primer 402

For the cultivars studied, a total of eleven reproducible bands were produced across the 25 cultivars (Figure 5-1). Bands ranged in size from 560 to 1040 bases. One to four bands were produced per cultivar. Some of the bands showed commonality across most of the cultivars studied but no bands were common across all cultivars. The highest degree of likeness occurred with the bands 729, 605, and 560 bases in size. The band 729 bases in size was common to 17 of 25 cultivars studied. The band 605 bases in size was common to 10 of the 25 cultivars. The band 560 bases in size was common to 8 of the 25 cultivars studied. Bands of size 743 and 685 bases were found to be unique to specific cultivars.

Primer 405

Primer 405 produced a total of 10 bands across all cultivars studied. Band sizes ranged form 660 to 1260 bases (Figure 5-2). One to four bands were produced per cultivar with several bands being common to several cultivars but

none were common to all. The highest degree of likeness occurred with the band 820 bases in size. Sixteen of the twenty-five cultivars shared this band. Twelve of the twenty-five cultivars studied shared a band of size 959 bases. Six cultivars shared bands 735 and 560 bases in size. The band 852 bases in size was found to be unique to a cultivar.

Primer 411

A total of 26 bands were produced for the cultivars studied. Bands ranged from 510 to 1030 bases in size (Figure 5-3). One to six bands were produced per cultivar with several of the bands common to several cultivars but none across all. Sixteen out of twenty-five bands showed likeness at the band 510 bases in size. The band 639 bases in size showed commonality among 12 of the 25 cultivars studied. The band 805 bases in size was common to ten of the twenty-five cultivars studied. Nine of the twenty-five cultivars showed a common band at 626 bases in size. Seven of the twenty-six bands were found to be unique to a cultivar.

Primer Alst -19

A total of 26 bands were produced using the Alst-19 primer that were reproducible (Figure 5-4, 5-4a). Bands ranged from 395 to 1080 bases in size. One to six bands were produced depending on the cultivar. The highest degree of likeness occurred with bands 805, 735, and 395 bases in size. The band 735 bases in size was common to 19 of the 25 cultivars studied. Bands 805 and 395 bases in size was shared by 13 of the 25 cultivars. Five of the twenty-six bands scored were unique to a cultivar.

'Alnba'

A total of eleven bands were produced for the cultivar 'Alnba' using all four primers. One reproducible band was produced using primer 402, two each for primers 405 and Alst-19 and six for primer 411. None of the bands observed were found to be unique to the cultivar. All bands produced were common to at least one other cultivar.

'Appel Bloesem'

Eleven bands were produced for the cultivar 'Appel Bloesem' using all four primers. Two reproducible bands were observed using primers 402 and 411, four for primer 405 and three bands were observed with primer Alst-19. None of the bands observed were unique to 'Appel Bloesem'.

'Arizo'

A total of twelve reproducible bands were produced for 'Arizo' using the four primers. One reproducible band was observed for primer 402, three for primer 405, six bands for primer 411 and two bands using primer Alst-19. One band from primer 405 and one from primer 411 were found to be unique to the cultivar. All other bands studied were in common with at least one other cultivar.

'Canaria'

A total of nine reproducible bands were produced for the cultivar 'Canaria' using the four primers. Three bands each were observed using the primers 402 and Alst-19, two with primer 405, while only one band with primer 411. One band from

primer 405 was found to be unique to the cultivar. All other bands produced were common to at least one other cultivar examined in this study.

'Carmen'

A total of thirteen bands were observed for 'Carmen' using all four primers. Three reproducible bands were produced using primers 402, 405, and 411 and four for primer Alst-19. One band using primer Alst-19 was unique to the cultivar. All other bands produced were shared with at least one of the other cultivars used in this study.

' Claudia'

Thirteen reproducible bands were produced for the cultivar 'Claudia' using all four primers. Three bands were observed using primer 402, two with primer 405, four for primer 411 and four bands with primer Alst-19. None of the bands were unique to the cultivar.

'Eureka'

A total of twelve reproducible bands were produced for the cultivar 'Eureka' using all four primers. Two bands were observed using primer 402, one band using primer 405 and three bands with primer 411, one of which was unique to the cultivar. Primer Alst-19 produced five reproducible bands, two of which were unique to the cultivar. All other bands produced were common to at least one other cultivar.

'Jubilee'

Seven bands were produced with the cultivar 'Jubilee' using all four primers. Two bands were produced for each of the primers 402, 405, and Alst-19. One

reproducible band was observed using primer 411. None of the bands produced were unique to the cultivar but were common to at least one other cultivar.

'King Cardinal'

A total of sixteen reproducible bands were observed for the cultivar 'King Cardinal' using all four primers. Five bands were produced using primer 402, one of which was unique to the cultivar, two bands using primer 405, four bands with primer 411, two of which were unique to the cultivar, and five with primer Alst-19.

'Luceana'

A total of eleven bands were observed for the cultivar 'Luceana' using the four primers. One band was produced using each of the primers 402 and 405, six bands using primer 411, and three bands using primer Alst-19. None of the bands observed were unique to the cultivar.

'Marina'

A total of eight bands were observed for the cultivar 'Marina' using the four primers. Two reproducible bands were produced using primer 402, one band using primer 405, three bands using primer 411, and two bands using primer Alst-19. No bands were found to be unique to the cultivar.

'Mona Lisa'

A total of fifteen reproducible bands were observed using the four primers for the cultivar 'Mona Lisa'. Two bands were produced using primer 402, three bands using primer 405, six bands using primer 411, two of which were unique to the cultivar, and four bands using primer Alst-19.

'Monika'

A total of eleven reproducible bands were observed using the four primers for the cultivar 'Monika'. One reproducible band was observed using each of the primers 402 and 405, four bands using primer 411, and five bands using primer Alst-19. None of these bands were unique to the cultivar.

'Neva'

A total of twelve reproducible bands were produced for the cultivar 'Neva' using the four primers. Primer 402 produced one reproducible band with two bands using primer 405, six bands using primer 411 and three for primer Alst-19. None of the bands were unique to the cultivar.

'Orange Beauty'

A total of eleven reproducible bands were observed for the cultivar 'Orange Beauty' using all four of the primers. Two bands were produced using primer 402, two bands using primer 405, three bands using primer 411, and four bands using primer Alst-19.None of the bands produced were unique to the cultivar.

'Orchid'

A total of ten reproducible bands were observed for the cultivar 'Orchid' using the four primers. One band was produced using primer 402, which was unique to the cultivar, three bands using primer 405, two bands using primer 411 and four bands using primer Alst-19. All but one band produced were common with at least one other cultivar used in this study.

'Orego'

A total of thirteen bands were observed using the four primers for the cultivar 'Orego'. One reproducible band was observed using primer 402, four bands using primer 405, four bands using primer 411 and four bands using primer Alst-19. None of the bands were found to be unique to this cultivar.

'Pink Triumph'

Ten reproducible bands were observed for the cultivar 'Pink Triumph' using the four primers. Primer 402 produced two reproducible bands, two bands using primer 405, four bands using primer 411 and two using primer Alst-19. None of the bands were found to be unique to the cultivar.

'Red Surprise'

A total of fourteen reproducible bands were observed using all four primers for the cultivar 'Red Surprise'. Primer 402 produced two reproducible bands, two bands using primer 405, four bands using primer 411 and six bands using primer Alst-19. One band of primer Alst-19 was found to be unique to the cultivar while all other bands were shared with at least one other cultivar used in this study.

'Regina'

A total of fifteen reproducible bands were observed using all four primers for the cultivar 'Regina'. Two reproducible bands were produced using primer 402, three bands using primer 405, six bands using primer 411, and four bands using primer Alst-19. All bands except one using primer 411 were common to at least one other cultivar and were therefore not unique to the cultivar.

'Rosario'

A total of nine reproducible bands were observed using all four primers for the cultivar 'Rosario'. Primer 402 produced three reproducible bands, one band using primer 405, one band using primer 411 and four bands using primer Alst-19. None of these bands were found to be unique to the cultivar.

'Rosita'

Nine total reproducible bands were observed for the cultivar 'Rosita' using all four primers. One band was produced using each of the primers 402 and 405, four bands using primer 411 and three bands using primer Alst-19. None of the bands were unique to the cultivar but were shared with at least one other cultivar.

'Sarah'

A total of ten reproducible bands were observed using all four primers for the cultivar 'Sarah'. Primers 402 and 405 each produced one reproducible band, with four bands using primer 411, and four bands using primer Alst-19. None of the bands produced were found to be unique to the cultivar but were shared with at least one other cultivar in this study.

'Texas'

A total of eight reproducible bands were observed using all four primers for the cultivar 'Texas'. Both primer 402 and 405 produced one reproducible band, with three bands each for primer 411 and primer Alst-19. None of the bands were unique to the cultivar.

'Yellow King'

A total of ten reproducible bands were observed for the cultivar 'Yellow King' using all four primers. One band was observed using primer 402, three bands using primer 405, four bands using primer 411 and two bands using primer Alst-19. None of the bands produced in 'Yellow King' were found to be unique to the cultivar but were shared with at least one other cultivar used in this study.

Cluster Analysis

The data resulting from cluster analysis using un-weighted pair group method for the cultivars, using the 73 bands, generated a dendogram with four distinct clusters (Figure 5-5). The two cultivars 'Eureka' and 'Canaria' clustered together to form one cluster group. The cultivars 'Orchid', 'Regina', 'Rosita', 'Orange Beauty', 'Pink Triumph', 'Yellow King', 'Sarah', 'Monika', and 'Mona Lisa' formed another general grouping. 'King Cardinal' was in a grouping by itself apart from the rest of this group. The remaining cultivars 'Rosario', 'Claudia', 'Red Surprise', 'Marina', 'Carmen', 'Jubilee', 'Appel Bloesem', 'Arizo', 'Orego', 'Neva', 'Luceana', 'Texas', and 'Alnba' clustered into the final grouping. All cultivars studied were separated into distinct groups using the 73 markers produced by the four primers.

The cultivars 'Eureka' and 'Canaria' were the least related of the cultivars as indicated by their linkage distance of 4.2 as compared to the rest of the cultivars. The two cultivars had a linkage distance of 3.6 when compared to one another. The cultivar 'King Cardinal' had a linkage distance of 3.9 when compared to the cluster

containing the cultivar 'Orchid'. The cluster containing 'Orchid', 'Yellow King' and 'Sarah' had the closest linkage distances of 2.0 with the next closest linkage being 2.8 between 'Sarah' and 'Monika'. A smaller cluster containing the cultivars 'Orange Beauty', 'Pink Triumph', 'Yellow King', 'Sarah', 'Monika', and 'Mona Lisa' occurred within the larger 'Orchid' cluster. This smaller cluster had a linkage distance of 3.2.

The final cluster containing thirteen cultivars could be further divided into two sub-clusters. The first sub-cluster contained the cultivars 'Rosario', 'Claudia', 'Red Surprise', 'Marina', and 'Carmen'. The closest linkage distance within this cluster was 2.4 between 'Red Surprise' and 'Marina'. The entire cluster had a maximum linkage of 3.4. The second sub-cluster contained the cultivars 'Jubilee', 'Appel Bloesem', 'Arizo', 'Orego', 'Neva', 'Luceana', 'Texas', and 'Alnba'. Within this group the cultivars 'Neva' and 'Luceana' had the closest linkage distance of 2.0, with the entire cluster having a maximum linkage distance of 3.4. The cultivars 'Jubilee' and 'Pink Triumph' clustered together within this group and had a linkage distance of 2.8.

Along with being able to determine possible relationships, it was also possible to create unique genetic profiles for all of the cultivars studied using RAPDs. Only four primers were needed to create the profile. From the profiles, or fingerprints, it is possible to identify each cultivar. All four primers were needed to characterize the twenty five cultivars.

Table 5-1: Sizes in base pairs of DNA fragments observed for four primers with the *Alstroemeria* cultivars.

Sample *	UBC 402	UBC 405	UBC 411	Alst-19
Alnb	729	820, 735	805, 794, 616, 609, 574, 510	1080, 395
Appl	729, 605	959, 820, 735	1030, 510	1080, 735, 395
Ariz	605	852, 735, 560	805, 794, 704, 609, 586, 510	735, 395
Cana	807, 658, 560	1260, 560	639	786, 476, 401
Carm	840, 729, 560	959, 820, 560	616, 609, 510	937, 786, 735, 598
Clau	729, 605, 560	820, 763	794, 626, 616, 510	786, 735, 598, 395
Eure	807, 605	959	660, 639, 510	981, 805, 786, 774, 547, 401
Jubi	729, 605	959, 820	574	762, 395
Kica	743, 729, 658, 605, 560	959, 820	939, 805, 645, 639	805, 735, 598, 464, 395
Luce	729	735	1030, 805, 794, 704, 663, 616, 609	762, 657, 395
Mari	729, 560	820	805, 616, 609	735, 547
Mona	729, 605	959, 820, 708	882, 816, 783, 639, 626, 510	805, 735, 522, 464
Moni	605	735	828, 639, 626, 510	805, 735, 598, 522, 464

Table 5-1: Continued

Sample*	UBC 402	UBC 405	UBC 411	Alst-19
Neva	729	820, 763	1030, 805, 794, 704, 616, 510	762, 657, 395
Orbe	729, 605	820, 657	828, 639, 510	805, 762, 735, 532
Orch	685	959, 657, 560	882, 639	805, 735, 532,476
Oreg	729	820, 735, 657, 560	805, 794, 616, 609	762, 735, 657, 395
Pint	658, 605	959, 657	805, 639, 626, 510	805, 735
Reds	729, 560	959, 820	805, 616, 609, 510	805, 735, 547, 410, 401, 395
Regi	729, 560	959, 820, 657	828, 768, 639, 626, 535, 510	735, 547, 464, 401
Rosa	840, 729, 560	820	626	735, 657, 464, 395
Rosi	658	820	882, 639, 626, 535	805, 735, 395
Sara	729	959	828, 639, 626, 510	805, 735, 522, 401
Texa	729	820	805, 794, 510	735, 701, 395
Yelk	729	959, 763, 708	828, 639, 626, 510	805, 735

* For explanation of abbreviations refer to Table 3-2.

Figure 5-1. RAPD bands of *Alstroemeria* cultivars using primer 402 run on a polyacrylamide gel using silver staining.

Lanes 1 and 37: 1 Kb molecular marker with sizes indicated.
Lane 2: Control
Lanes 3 and 4: Kica
Lanes 5 and 6: Luce
Lanes 7 and 8: Mari
Lanes 9 and 10: Mona
Lanes 11 and 12: Moni
Lanes 13 and 14: Neva
Lanes 15 and 16: Orbe
Lanes 17 and 18: Orch
Lanes 19 and 20: Oreg
Lanes 21 and 22: Pint
Lanes 23 and 24: Reds
Lanes 25 and 26: Regi
Lanes 27 and 28: Rosa
Lanes 29 and 30: Rosi
Lanes 31 and 32: Sara
Lanes 33 and 34: Texa
Lanes 35 and 36: Yelk

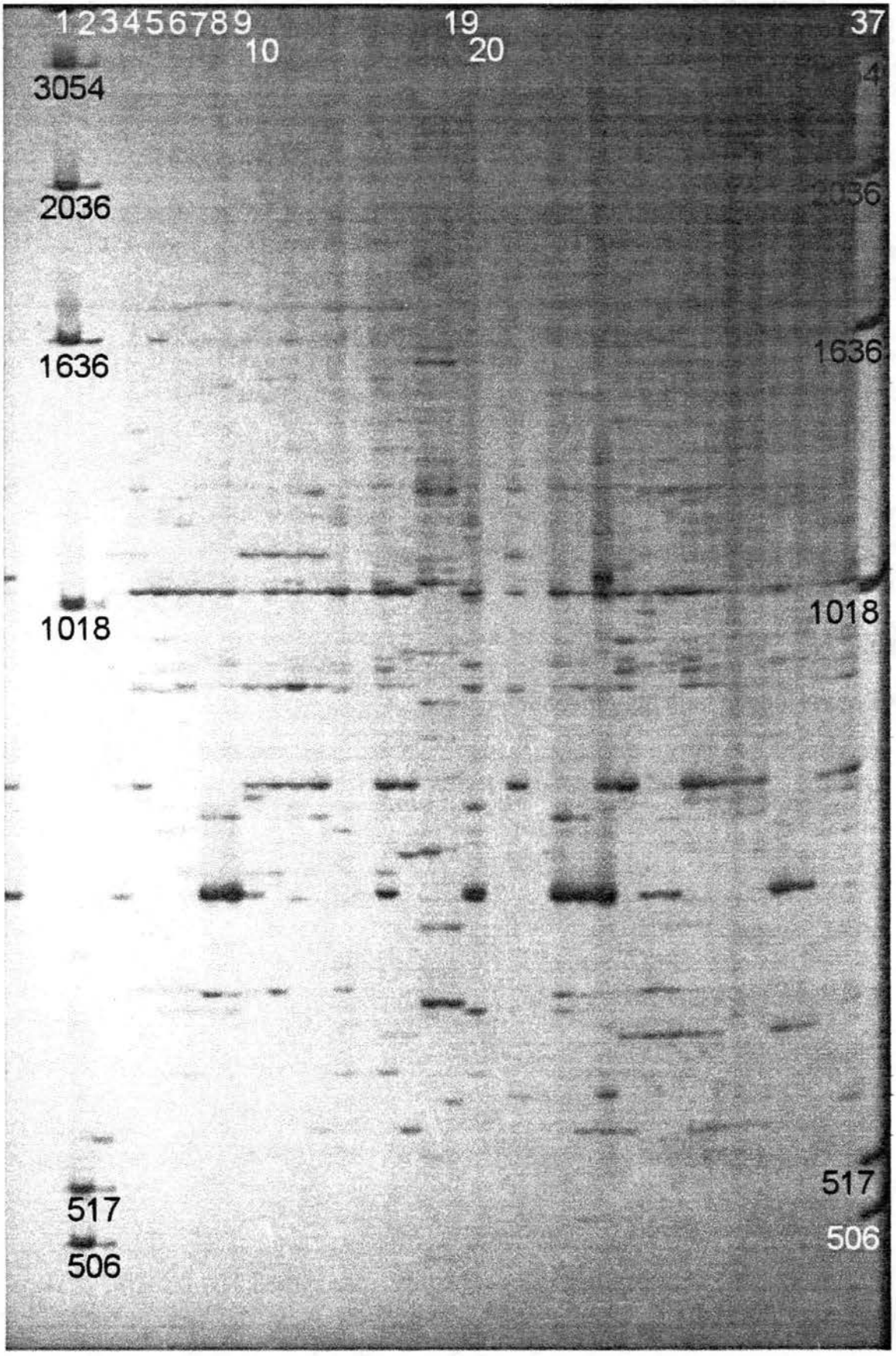
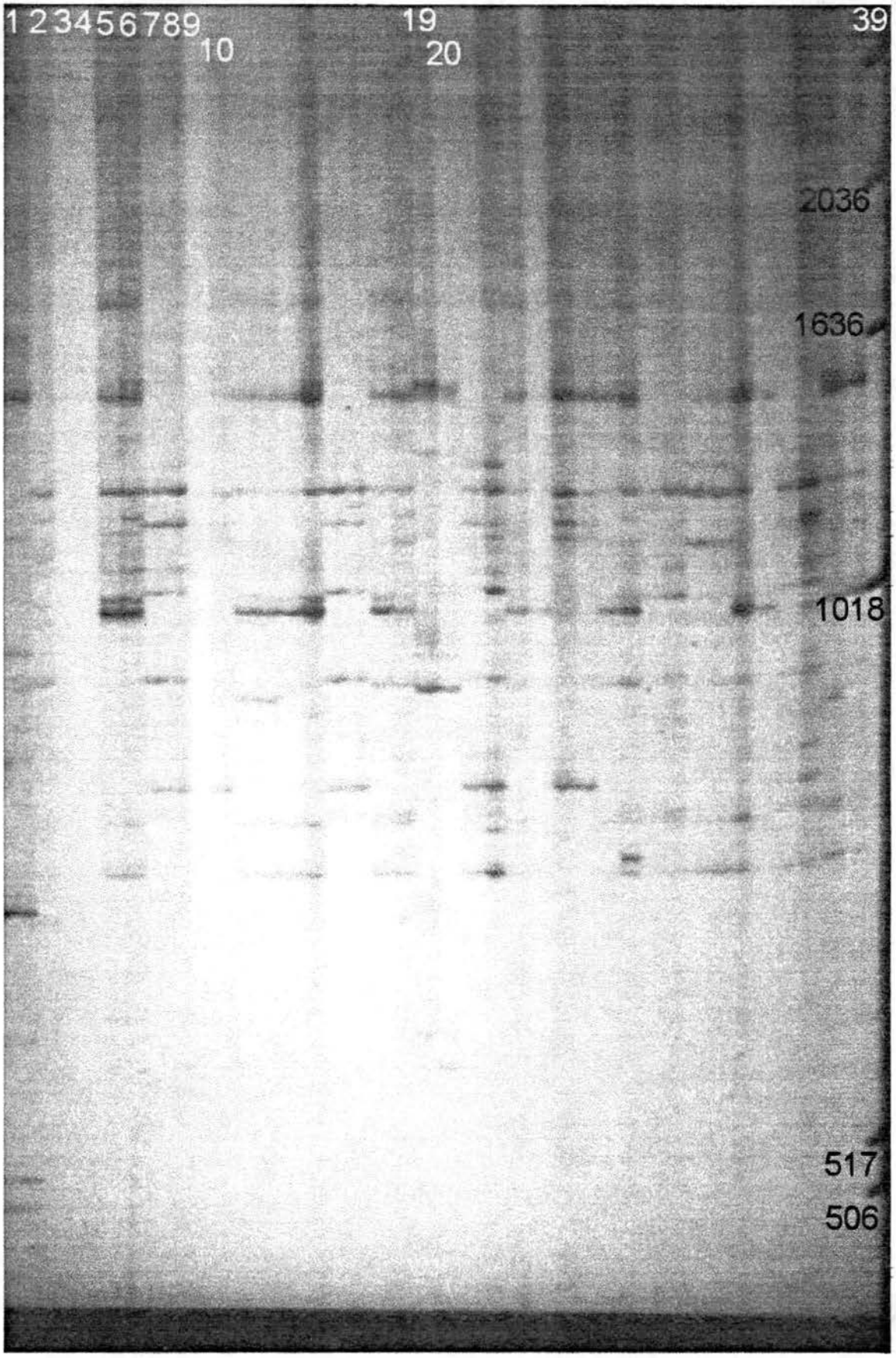
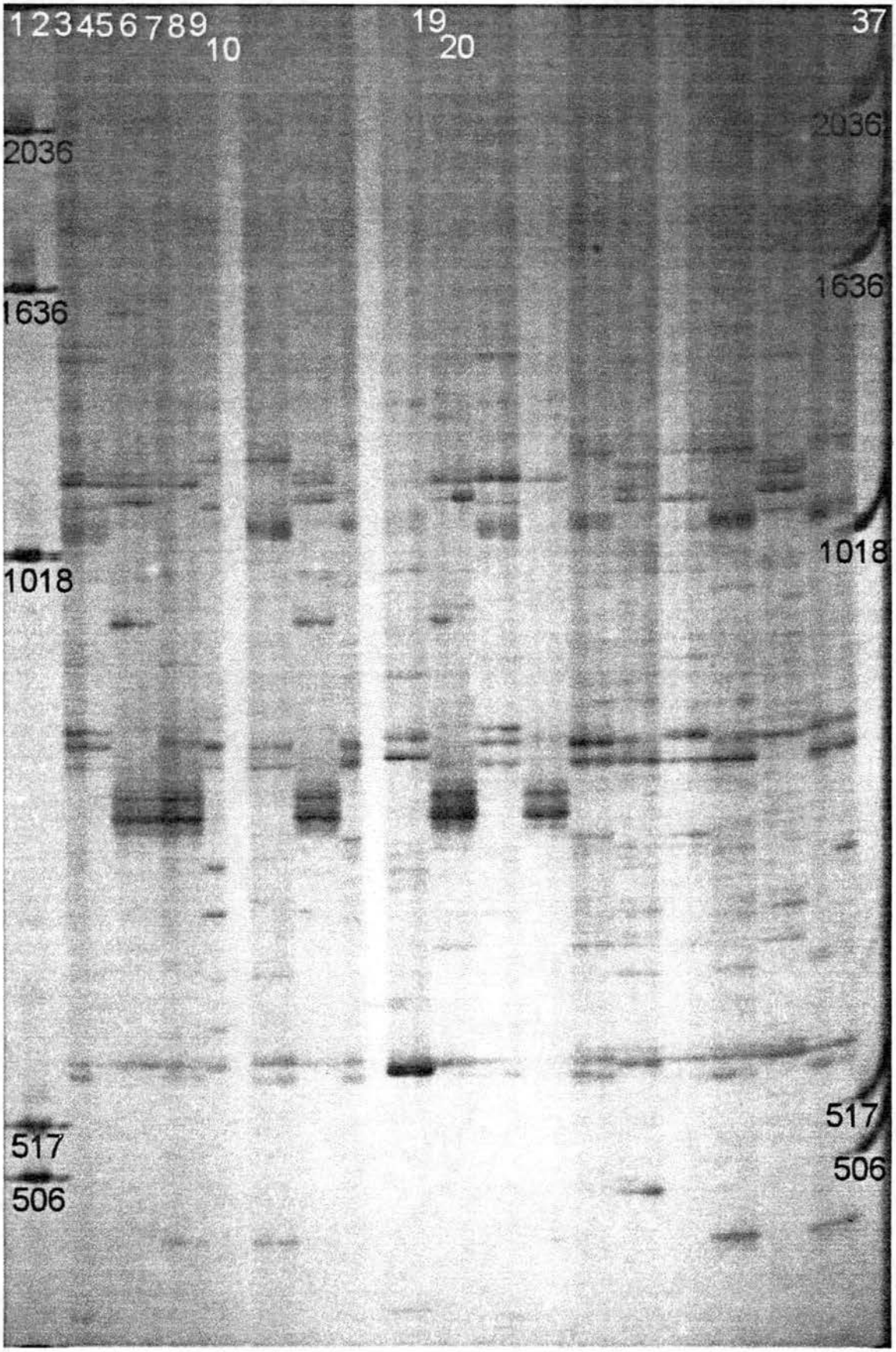

1 2 3 4 5 6 7 8 9
10
19
20
37
3054
2036
1636
1018
517
506
2036
1636
1018
517
506

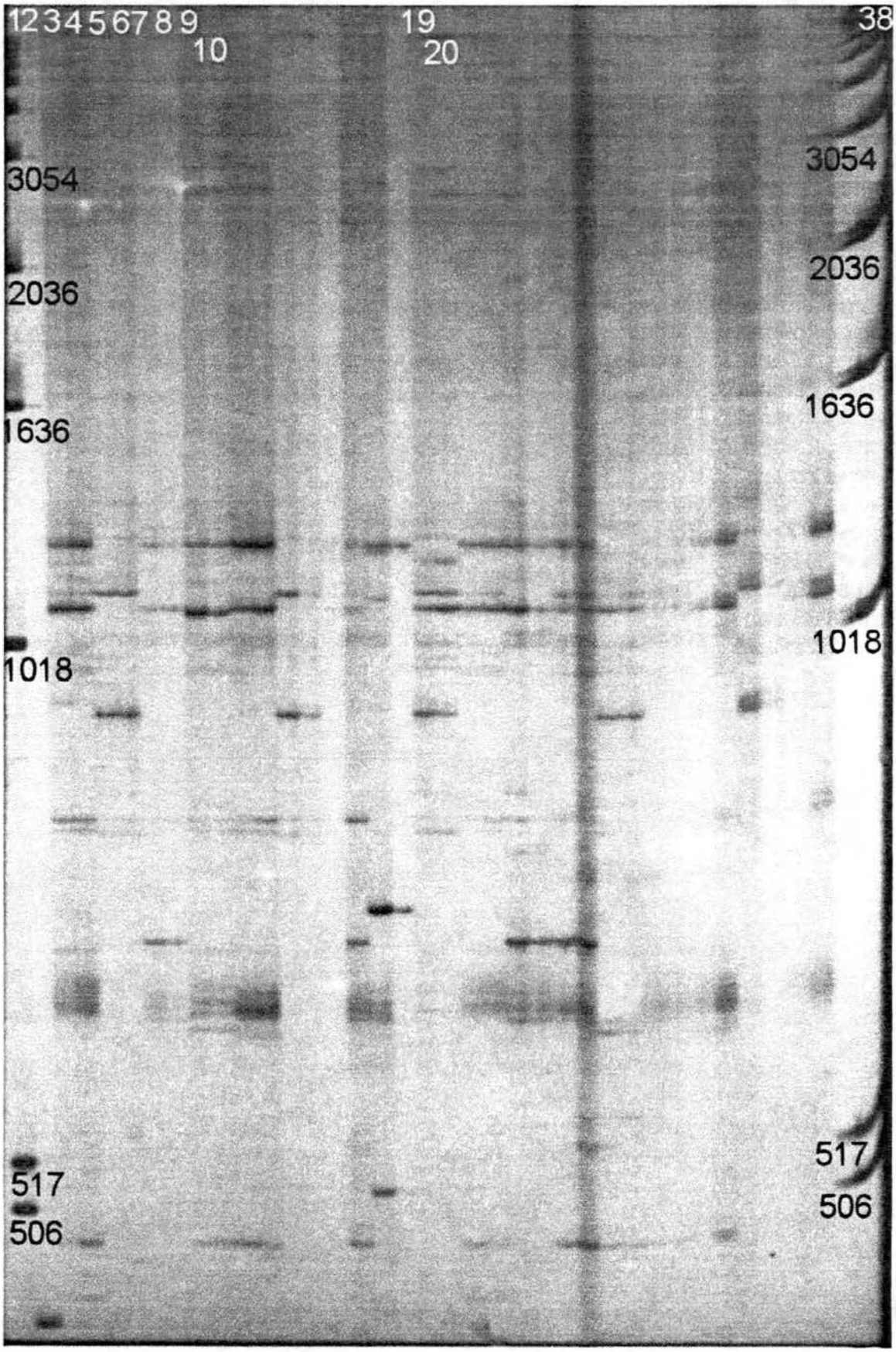
Figure 5-2. RAPD bands of *Alstroemeria* cultivars using primer 405 run on a polyacrylamide gel using silver staining.

Lane 39: 1 Kb molecular marker with sizes indicated.
Lanes 1 and 2: Eure
Lanes 3 and 4: Jubi
Lanes 5 and 6: Kica
Lanes 7 and 8: Luce
Lanes 9 and 10: Mari
Lanes 11 and 12: Mona
Lanes 13 and 14: Moni
Lanes 15 and 16: Neva
Lanes 17 and 18: Orbe
Lanes 19 and 20: Orch
Lanes 21 and 22: Oreg
Lanes 23 and 24: Pint
Lanes 25 and 26: Reds
Lanes 27 and 28: Regi
Lanes 29 and 30: Rosa
Lanes 31 and 32: Rosi
Lanes 33 and 34: Sara
Lanes 35 and 36: Texa
Lanes 37 and 38: Yelk

* Control lane in lane not present on figure


1 2 3 4 5 6 7 8 9
10
19
20
39
2036
1636
1018
517
506


1 2 3 4 5 6 7 8 9 10
19 20
37
2036
1636
1018
517
506
2036
1636
1018
517
506


1 2 3 4 5 6 7 8 9
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19
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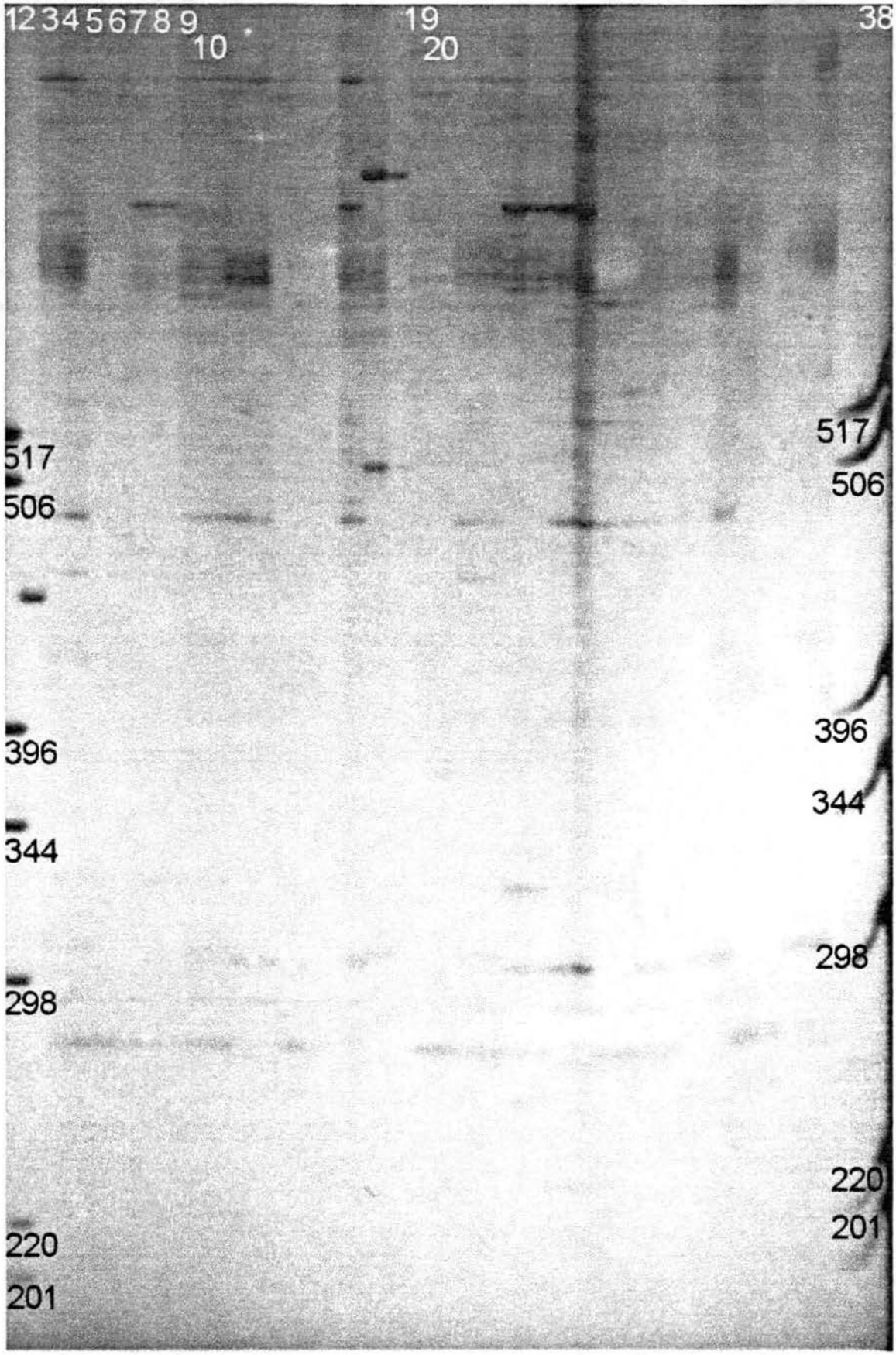
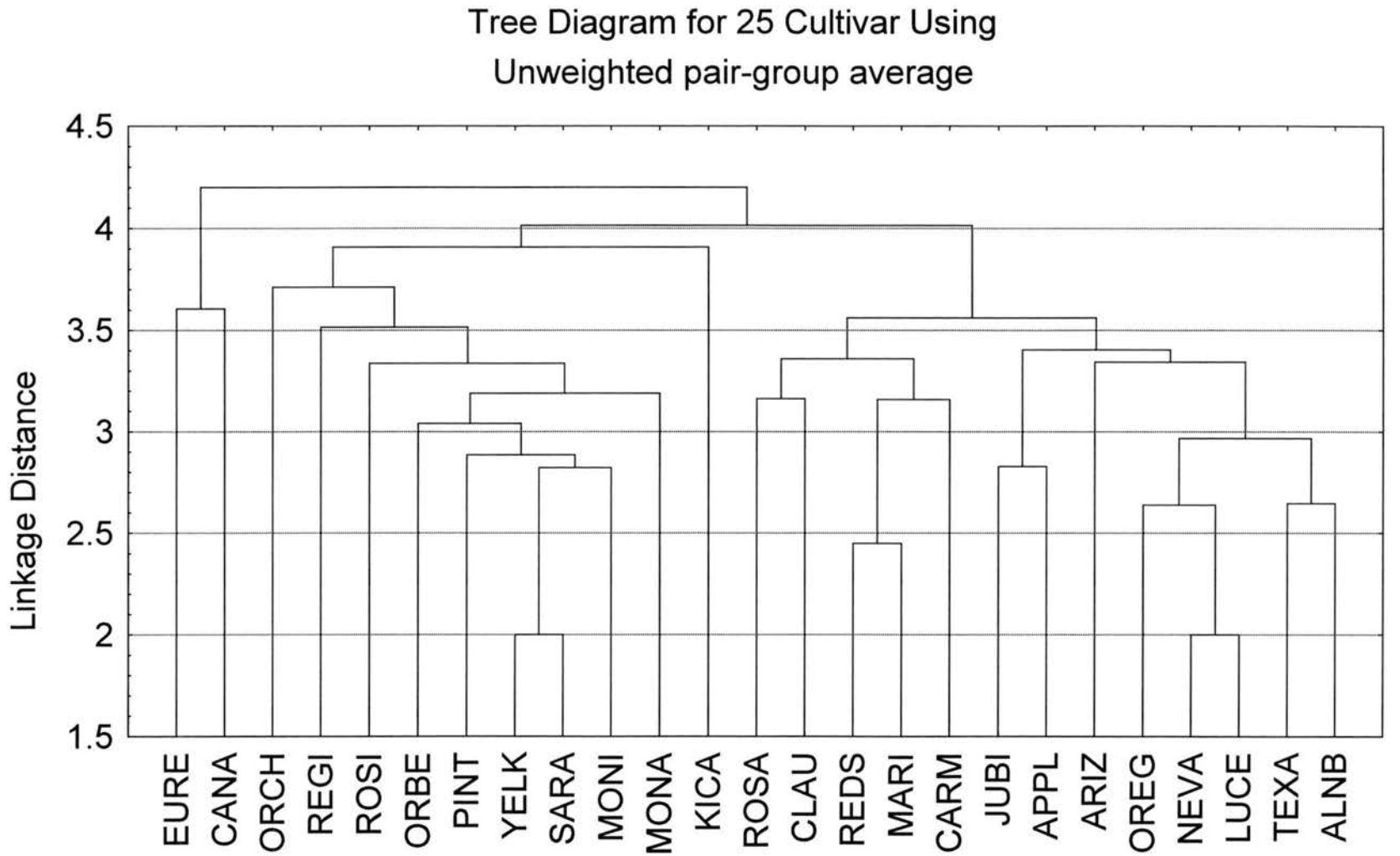
38
3054
2036
1636
1018
517
506
3054
2036
1636
1018
517
506

Figure 5-4a. RAPD bands of *Alstroemeria* cultivars using primer Alst-19 run on a polyacrylamide gel using silver staining. (Lower portion of gel)

Lanes 1 and 38: 1 Kb molecular marker with sizes indicated.
Lane 2: Control
Lanes 3 and 4: Kica
Lanes 5 and 6: Luce
Lanes 7 and 8: Mari
Lanes 9 and 10: Mona
Lanes 11 and 12: Moni
Lanes 13 and 14: Neva
Lanes 15 and 16: Orbe
Lanes 17 and 18: Orch
Lanes 19 and 20: Oreg
Lanes 21 and 22: Pint
Lanes 23 and 24: Reds
Lanes 25 and 26: Regi
Lanes 27 and 28: Rosa
Lanes 29 and 30: Rosi
Lanes 31 and 32: Sara
Lanes 33 and 34: Texa
Lanes 35 and 36: Yelk
Lane 37: Blank


12 3 4 5 6 7 8 9
10
19
20
38
517
506
396
344
298
220
201
517
506
396
344
298
220
201


Tree Diagram for 25 Cultivar Using
Unweighted pair-group average
Linkage Distance
4.5
4
3.5
3
2.5
2
1.5
EURE
CANA
ORCH
REGI
ROSI
ORBE
PINT
YELK
SARA
MONI
MONA
KICA
ROSA
CLAU
REDS
MARI
CARM
JUBI
APPL
ARIZ
OREG
NEVA
LUCE
TEXA
ALNB

Chapter 6

DISCUSSION AND CONCLUSION

For this study, four different ten base primers were used to characterize the fifteen species and hybrids and twenty-five cultivars of *Alstroemeria*. For those primers that did not produce bands it can be concluded that they did not share homology with areas of the template DNA. A high degree of polymorphism in the banding patterns of the species and cultivars was observed across many species. The degree of polymorphism indicates that the genus *Alstroemeria* is highly genetically diverse and the potential breeding for crop improvement is possible using conventional means.

Due to the limited number of specimens available per species there were insufficient numbers for a fingerprint to be created that would characterize intra-specific diversity in a species. In most cases only one or two examples of a species were available for study. Since this was the case, it is likely that all potential bands produced using the RAPD procedure were not represented and further analysis with larger populations that represent the species should be done. All conclusions were made from the fingerprint generated using the limited number of specimens.

The analysis of *A. versicolor* indicated that the species was significantly different from the rest of the species studied. This is supported by taxonomic descriptions of the plant that indicate it is the only species lacking resupinate leaves. Furthermore, there appears to be a high degree of variability present in the two accessions of *A. versicolor* as indicated by the low degree of linkage expressed in cluster analysis.

The two color variants of *A. aurantiaca* were also shown to be closely related as expected. The results also indicated that *A. ligtu* is related to *A. aurantiaca* due to the common banding patterns produced on the gels (see Table 4-1). *Alstroemeria aurantiaca* and *A. ligtu* share a similar leaf morphology which is different than the rest of the species studied. The leaf morphology of the two species is linear to lanceolate leaves with the presence or absence of trichomes depending on the particular specimen. The remaining species and hybrids have an oblong to ovate leaf with no trichomes present.

Alstroemeria pulchella and *A. psittacina* have long been thought to be synonyms for the same species. The results of this study support this hypothesis as indicated by the high degree of linkage in cluster analysis. The fact that the two species did separate in analysis indicates that they are not the same clone but highly related. These two species also possess a similar morphology which differs from the other species studied. The morphology of the two species is one of a low growing, 20 to 30 cms, small leaved type. All other species produce stems 1 to 3

meters in length. The leaves of *A. pulchella* and *A. psittacina* are also ovate in shape and approximately half the size of all other species.

Alstroemeria pulchra ssp *lavandulaceae* and *A. magnifica* ssp *maxima* are believed to be different subspecies of *A. pulchra*. *Alstroemeria magnifica* ssp *maxima* is believed to be a synonym for *A. pulchra* var *maxima* (Kerrys, 1995). This is supported by the linkage distance observed in this study.

The three *A. ligtu* hybrids studied are all accessions from the hybrids created by Dr. Salter in the 1930's. The three *A. ligtu* hybrids were identified as being related since they grouped together. The *Alstroemeria haemantha* used in this study is thought to be a hybrid of *A. haemantha* and *A. caryophylla* based on the findings of Stephens (1995). *Alstroemeria haemantha* is known to cross readily with many species, including *A. caryophylla* and this is what is thought to have happened with the specimen studied. This hypothesis is supported by the results from this study. The linkage between the two species indicates shared parentage. There is also some confusion as to the exact identification of *A. haemantha*. There have been reports that *A. haemantha* is an invalid name for *A. ligtu* ssp *ligtu* which would explain the observation that it grouped close to the *A. ligtu* hybrids. *Alstroemeria chilensis* also appeared to share common genetic background with the *A. ligtu* group. It has also been hypothesized that *A. chilensis* is a synonym for *A. ligtu* ssp *ligtu* (Bayer, 1987) which is supported by the results of this study.

Using only four RAPD primers, it was possible to uniquely characterize all species and hybrids used in this study. Using these same four primers it was also

possible to hypothesize possible relationships between many of the species. The species separated into the same groupings as the distinct morphological groups.

As the breeding of *Alstroemeria* cultivars has involved many different techniques including hybridization, chromosome doubling and mutation breeding, it was expected that the RAPD banding patterns would be variable. Using the four primers, it was possible to uniquely characterize all cultivars studied and to draw possible relationships among them.

The cultivars 'Eureka' and 'Canaria' are believed to have been developed from hybridization of non-homologous genomes (Stephens, 1995). This is supported by the results of this study which separated the two cultivars into a distinct group. Since these two cultivars were developed in a manner different than the rest of the cultivars studied, this significant difference between them and the other cultivars was expected. The remaining cultivars were developed through the use of multiple crosses, chromosome doubling and X-ray mutation.

The American produced cultivars 'Arizo', 'Neva', 'Orego', and 'Texas' all clustered into a single group indicating they were developed from similar parental lines. The cultivar 'Luceana' also clustered in with the American cultivars and showed a close linkage with the cultivar 'Neva', indicating a possible relationship in parentage.

The cultivar 'Appel Bloesem' was developed through the X-ray mutation of the cultivar 'King Cardinal' (Stephens, 1995). Examination of the banding patterns of these two cultivars did not support this finding and indicates that the genetic changes induced in 'Appel Bloesem' were amplified and significant. The linkage

distance observed for these two cultivars was 3.9, indicating little commonality between the two. The cultivar 'Jubilee' was linked closely to 'Appel Bloesem' indicating a possible common heritage.

The two cultivars 'Yellow King' and 'Sarah' had a strong linkage indicating a possible sibling relationship.

Cluster analysis was used to compare the banding patterns for the species and cultivars. With the dendogram produced from the analysis using all 40 species and cultivars studied, possible parentages were evident (Figure 6-1).

Although direct parental and offspring relationships could not be determined using this technique, it was possible to determine which species had likely been used in the initial development of the cultivars. It was found that *A. caryophylla, A. haemantha, A. ligtu, A. chilensis,* and *A. pulchella/A. psittacina* were used in the development of the cultivars. It was not possible to determine whether *A. aurantiaca, A. pulchra* ssp *lavandulaceae,* or *A. magnifica* ssp *maxima* were used in the development of the cultivars. Previous research has indicated that *A. aurantiaca* has been used in the development of many of the cultivars, thus further research with RAPDs should be completed to clarify this point.

Alstroemeria versicolor could not be hypothesized as a possible parent of any of the cultivars studied. The closest linkage to any of the other species or cultivars was 4.5, indicating it was not likely used in the breeding of *Alstroemeria* cultivars.

Alstroemeria pulchella or *A. psittacina* and *A. chilensis* were most likely used for the initial breeding of the modern cultivars as is indicated by their higher linkage

distances. *Alstroemeria ligtu, A. caryophylla,* and *A. haemantha* were most likely used as the final parents of the cultivars.

Many of the reputed parents of the modern cultivars were not available for study during the course of the experiments and should be included in any further studies performed to determine the possible relationships of the cultivars. Further study into the genetic make up of the species and cultivars should be done including further RAPD analysis, sequencing of the RAPD products for the construction of species specific primers, and use of these species specific primers for detailed genetic study.

Of further interest, it was possible to uniquely characterize all the cultivars studied. Breeders need to know the genetic similarity of the potential parents in order to create commercially viable cultivars. Through the use of the banding patterns produced from the four primers studied, a unique genetic fingerprint could be developed for each cultivar. This ability to fingerprint a cultivar will aid the breeder in development of unique new cultivars. This fingerprinting will also aid breeders in the patenting of their cultivars. With the production of a unique genetic fingerprint, a plant may be the same morphologically but possess a unique genetic makeup. This ability to distinguish between different cultivars on the genetic level will also assist in determining if a patent has been infringed.

The use of such a fingerprint can also be used by geneticists to study the evolution of *Alstroemeria* and will allow them to suggest possible hybridizations to breeders. As there has been limited previous research into the production of such fingerprints it is valuable to develop one. From this research it can be shown that

the cultivars of *Alstroemeria* studied can be grouped according to place of origin. This ability to group the cultivars will allow breeders to determine the best choice of breeding material for a given aim.

Summary

The objectives of this study were to uniquely characterize the species and cultivars of *Alstroemeria* using random amplified polymorphic DNA analysis. The data obtained from this research could then be used to determine possible parental relationships among the cultivars and species as well as relationships within the cultivars. The results of this study should assist in the development of a competitive breeding program in the United States.

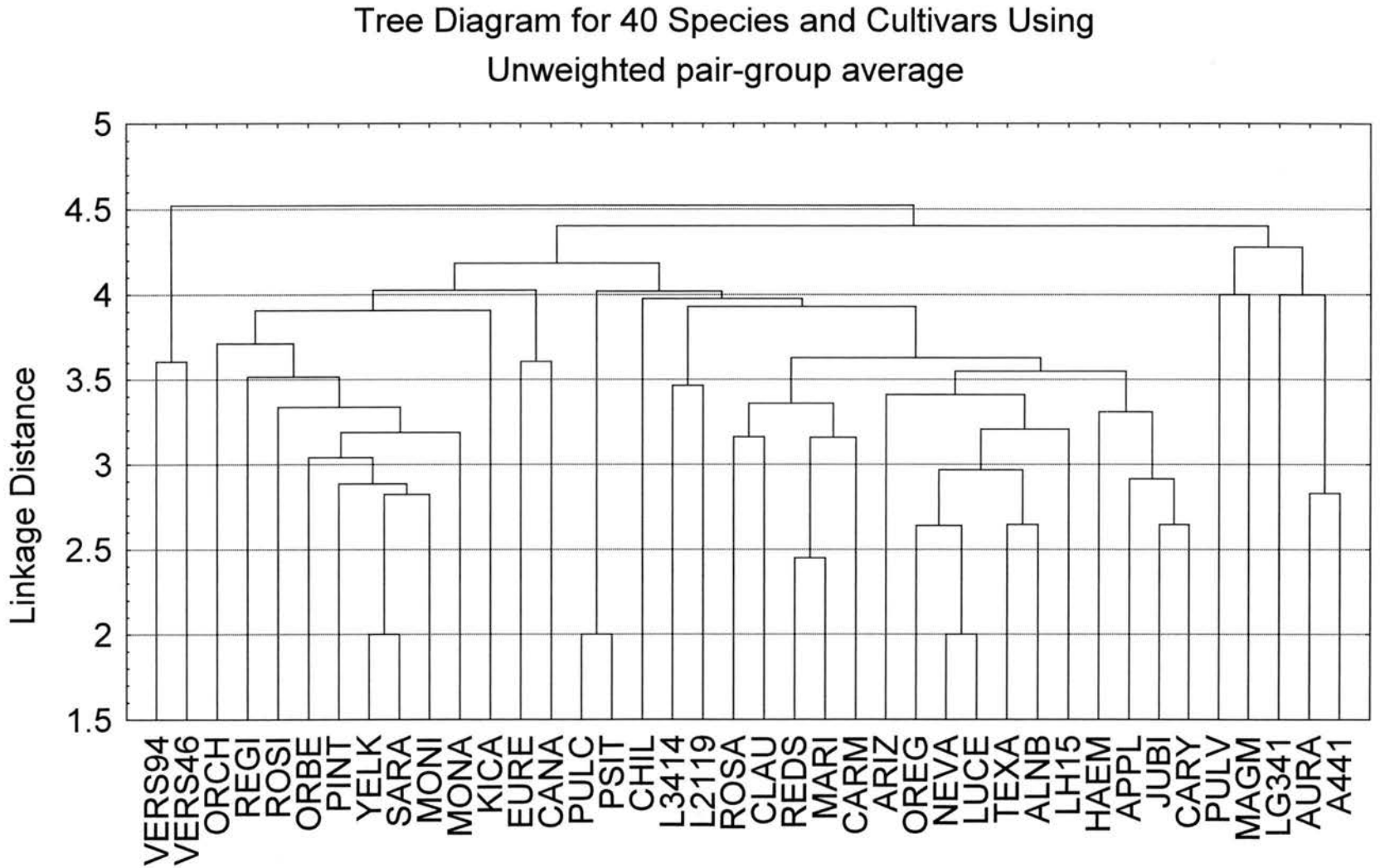
RAPD analysis revealed that *A. pulchella* and *A. psittacina* were genetically similar and could be considered synonyms for one species. Earlier studies (Stephens, 1995) using isozyme and karyotype analysis support this conclusion.

Similarly, *A. ligtu* and *A. chilensis* are thought to be synonymous (Kerrys, 1995). Differences in banding patterns were found to exist between these two species, but when analyzed, they grouped together indicating a high degree of relatedness. It is likely that these species may be closely related or may be biotypes of a single species.

It was possible to uniquely identify all species and cultivars used in this study with the use of only four RAPD primers which produced a total of 73 polymorphic bands. No mono-morphic bands were produced with the four primers utilized. The

use of RAPD analysis for the purposes of plant patenting and parental determination is possible. This study provided further information into the understanding of the relationships among the species and the relationship of the species to the cultivars. With further investigation of more species and genotypes within each species it should be possible to define the parentage of the European cultivars and allow American breeders to develop improved cultivars.

Figure 6-1.Dendogram of 25 cultivars and 15 species and hybrids analysed using cluster analysis.


Tree Diagram for 40 Species and Cultivars Using
Unweighted pair-group average
Linkage Distance
5
4.5
4
3.5
3
2.5
2
1.5
VERS94
VERS46
ORCH
REGI
ROSI
ORBE
PINT
YELK
SARA
MONI
MONA
KICA
EURE
CANA
PULC
PSIT
CHIL
L3414
L2119
ROSA
CLAU
REDS
MARI
CARM
ARIZ
OREG
NEVA
LUCE
TEXA
ALNB
LH15
HAEM
APPL
JUBI
CARY
PULV
MAGM
LG341
AURA
A441

Chapter 7

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APPENDIX

Table A-1: Buffers used in DNA extraction and purification.

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1. **CTAB Extraction Buffer**

	1 liter	**500 mls**	**100 mls**
CTAB	20 grams	10 grams	2 grams
1 M Tris HCl pH 8.0	100 mls	50 mls	10 mls
NaCl	81.816 grams	40.908 grams	8.1816 grams
0.5 M EDTA pH 8.0	40 mls	20 mls	4 mls
PVP-40	10 grams	5 grams	1 gram

20 μl ß-mercaptoethanol was added for every 20 mls of buffer immediately prior to use.

2. **0.5 M EDTA pH 8.0**

	1 liter	**500 mls**
Na_2EDTA	186.1 grams	46.525 grams
H_2O	800 mls	400 mls

Adjust pH to 8.0 with NaOH pellets then bring to volume.

3. **1 M Tris pH 8.0**

	1 liter	**500 mls**	**250 mls**
Tris (Trizma) Base	121.1 grams	60.55 grams	30.275 grams
H_2O	800 mls	400 mls	200 mls

Adjust pH to 8.0 with concentrated HCL then bring to volume.

4. **TE (Tris/EDTA) Buffer**

	1 liter	**500 mls**	**250 mls**
1 M Tris pH 8.0	10 mls	5 mls	2.5 mls
0.5 M EDTA pH 8.0	2 mls	1 ml	0.5 mls
H_2O	988 mls	494 mls	247 mls

5. **TLE (TE Lite) Buffer**

	1 liter	**500 mls**	**250 mls**
1 M Tris pH 8.0	10 mls	5 mls	2.5 mls
0.5 M EDTA pH 8.0	0.2 mls	0.1 mls	0.05 mls
H_2O	989.8 mls	494.9 mls	247.45 mls

Solutions 1 through 5 should be autoclaved after making.

6. **3 M Sodium Acetate**

	250 mls	**100 mls**
Sodium Acetate	61.52 grams	24.61 grams
H_2O	200 mls	75 mls

Once Sodium Acetate has dissolved, bring to volume with H_2O.

7. **Chloroform/Isoamyl Alcohol**

	250 mls
Chloroform	240 mls
Isoamyl Alcohol	10 mls

8. **RNAse Solution**

	1 ml
RNAse	10 mg
0.01M sodium acetate	1 ml

Table A-2: Stock solutions used for polyacrylamide gel electrophoresis.

1. **30% acrylamide stock**

	500 mls	**250 mls**	**100 mls**
Acrylamide	147 grams	73.5 grams	29.4 grams
Bis-acrylamide	3 grams	1.5 grams	0.6 grams
H_2O	500 mls	250 mls	100 mls

Filter acrylamide stock through a Watman #1 filter and store in foil wrapped glass container at 5°C.

2. **5X TBE Buffer**

	4 liters	**1 liter**	**500 mls**
Tris Base	216 grams	54 grams	27 grams
Boric Acid	110 grams	27.5 grams	13.75 grams
0.5 M EDTA pH 8.0	80 mls	20 mls	10 mls
H_2O	3.8 liters	800 mls	400 mls

Once all chemicals are dissolved bring to volume.

3. **25% Ammonium Persulfate**

	1 ml
Ammonium persulfate	250 mg
H_2O	1 ml

4. **Gel Loading Buffer**

	100 mls	**25 mls**	**10 mls**
1 M NaOH	1 ml	0.25 mls	0.1 mls
H_2O	4 mls	1 ml	0.4 mls
Formamide	95 mls	23.75 mls	9.5 mls
Bromophenol blue	50 mg	12.5 mg	5 mg
Xylene cyanol	50 mg	12.5 mg	5 mg

Table A-3: Ingredients used for acrylamide gel and electrophoresis preparation.

1. **5% Polyacrylamide gel**

	150 mls	**100 mls**
30% acrylamide solution	25 mls	16.7 mls
H_2O	87.5 mls	58.3 mls
Glycerol	7.5 mls	5 mls
5X TBE	30 mls	20 mls
25% Ammonium Persulfate	150 μl	150 μl
TEMED	150 μl	150 μl

Solution is filtered through 2 Watman #1 filters prior to adding ammonium persulfate and TEMED.

2. **1X TBE Buffer**

	1 liter
5X TBE Buffer	200 mls
H_2O	800 mls

3. **1.5X TBE Buffer**

	1 liter
5X TBE Buffer	300 mls
H_2O	700 mls

Table A-4: Solutions used for gel staining and development.

1. **Fix/Stop Solution**

	2 liters
Glacial Acetic Acid	200 mls
H_2O	1800 mls

2. **Staining Solution**

	2 liters
Silver Nitrate	3 grams
H_2O	2000 mls

Just prior to use add 3 mls of 37% formaldehyde to solution.

3. **Developing Solution**

	2 liters
Sodium Carbonate	60 grams
H_2O	2000 mls

Solution must be chilled to 5°C and prior to use 3 mls of 37% formaldehyde and 0.4 mls of 1% sodium thiosulfate are added.

4. **1% Sodium Thiosulfate**

	50 mls
Sodium Thiosulfate	0.5 grams
H_2O	50 mls

Table A-5: Relative purity and concentration of DNA extracted from 15 *Alstroemeria* species and hybrids.

Sample	OD260/280 Ratio	Concentration
A. aurantiaca 86H-44-1	1.90	1468.55
A. aurantiaca	1.89	655.20
A. caryophylla	1.83	662.00
A. chilensis	1.90	337.79
A. haemantha	1.94	516.00
A. ligtu 86H-34-1	1.89	408.59
A. ligtu hybrid 86H-1-5	1.77	255.00
A. ligtu hybrid 86H-21-19	1.97	324.00
A. ligtu hybrid 86H-34-14	1.84	215.00
A. magnifica ssp *maxima*	1.91	550.99
A. psittacina	1.88	507.00
A. pulchella	1.92	1158.00
A. pulchra ssp *lavandulaceae*	1.80	93.50
A. versicolor 46-666	1.71	147.17
A. versicolor 94-11	1.81	324.87

Table A-6: Relative purity and concentration of DNA from 25 *Alstroemeria* cultivars.

Sample	OD 260/280 Ratio	Concentration
Alnba	1.95	1422.00
Appel Bloesem	1.99	2315.00
Arizo	1.94	1026.00
Canaria	1.93	2552.00
Carmen	1.88	1689.00
Claudia	1.86	907.00
Eureka	1.86	1053.00
Jubilee	1.92	598.00
King Cardinal	1.92	1022.00
Luceana	1.86	379.00
Marina	1.87	1277.00
Mona Lisa	2.03	644.00
Monika	1.88	464.00
Neva	1.94	466.00
Orange Beauty	1.90	1858.00
Orchid	1.90	1819.00
Orego	1.91	1258.00
Pink Triumph	1.83	500.00
Red Surprise	1.87	658.00
Regina	2.01	719.00
Rosario	1.91	1508.00
Rosita	1.87	483.00
Sarah	1.87	1239.00
Texas	1.88	440.00
Yellow King	1.91	2271.00

Table A-7: RAPD primers screened for amplification

Primer	Sequence
UBC 401	3' TAG GAC AGT C 5'
UBC 402	3' CCC GCC GTT G 5'
UBC 403	3' GGA AGG CTG T 5'
UBC 404	3' TCT CTA CGA C 5'
UBC 405	3' CTC TCG TGC G 5'
UBC 406	3' GCC ACC TCC T 5'
UBC 407	3' TGG TCC TGG C 5'
UBC 408	3' CCG TCT CTT T 5'
UBC 409	3' TAG GCG GCG G 5'
UBC 410	3' CGT CAC AGA G 5'
UBC 411	3' GAG GCC CGT T 5'
UBC 412	3' TGC GCC GGT G 5'
UBC 413	3' GAG GCG GCG A 5'
UBC 414	3' AAG GCA CCA G 5'
UBC 415	3' GTT CCA GCA G 5'
UBC 416	3' GTG TTT CCG G 5'
UBC 417	3' GAC AGG CCA A 5'
UBC 418	3' GAG GAA GCT T 5'
UBC 419	3' TAC GTG CCC G 5'
UBC 420	3' GCA GGG TTC G 5'
UBC 421	3' ACG GCC CAC C 5'
UBC 422	3' CAC CTG CGG G 5'
UBC 423	3' GGG TCT CGA A 5'
UBC 424	3' ACG GAG GTT C 5'
UBC 425	3' CGT CGG GCC T 5'
UBC 426	3' TCT CCC GGT G 5'
UBC 427	3' GTA ATC GAC G 5'
UBC 428	3' GGC TGC GGT A 5'
UBC 430	3' AGT CGG CAC C 5'
UBC 440	3' CTG TCG AAC C 5'

Figure A-1. Absorbance curves for DNA samples of seven *Alstroemeria* species and hybrids obtained with the Beckman DU640 spectrophotometer.

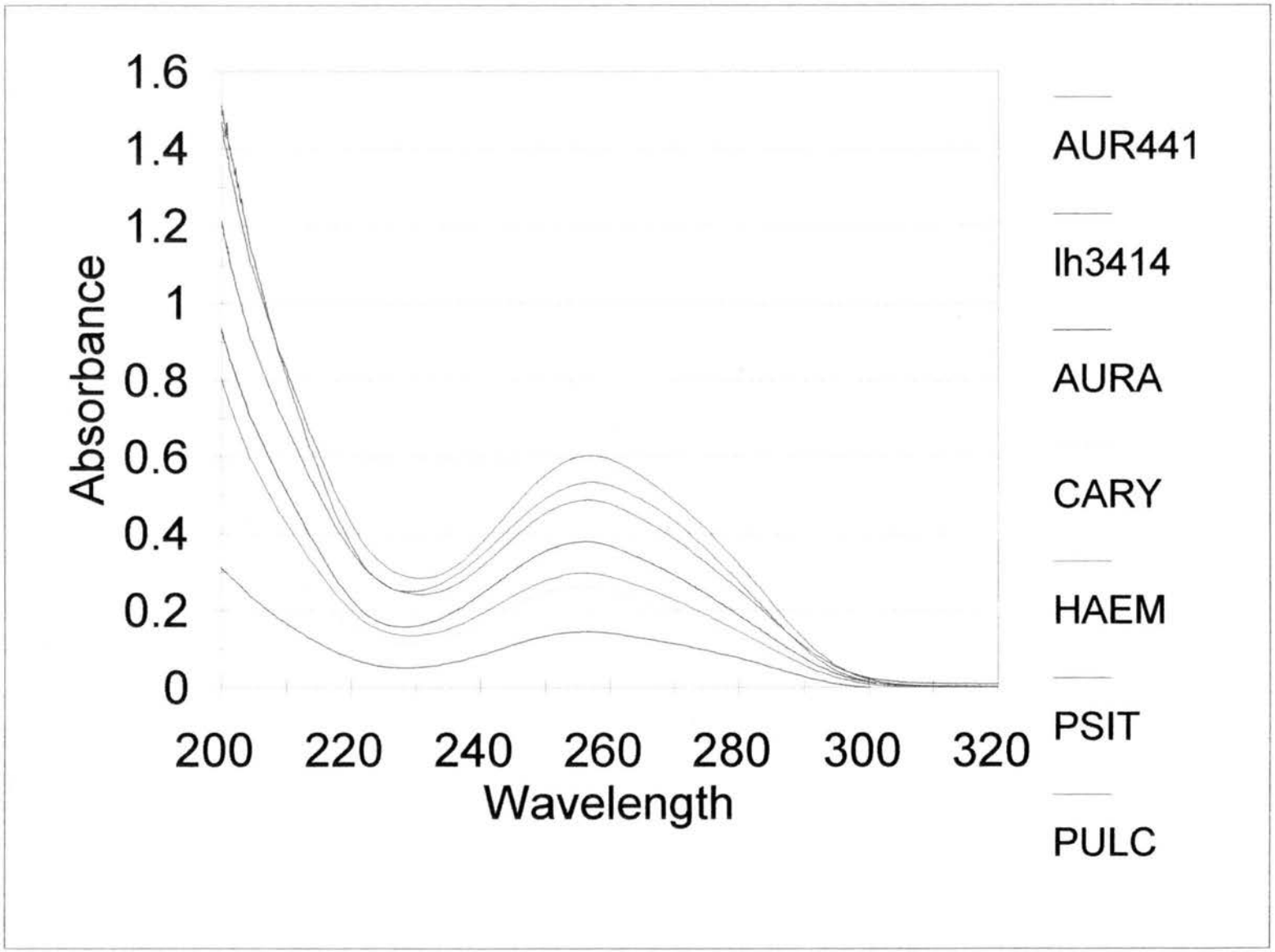
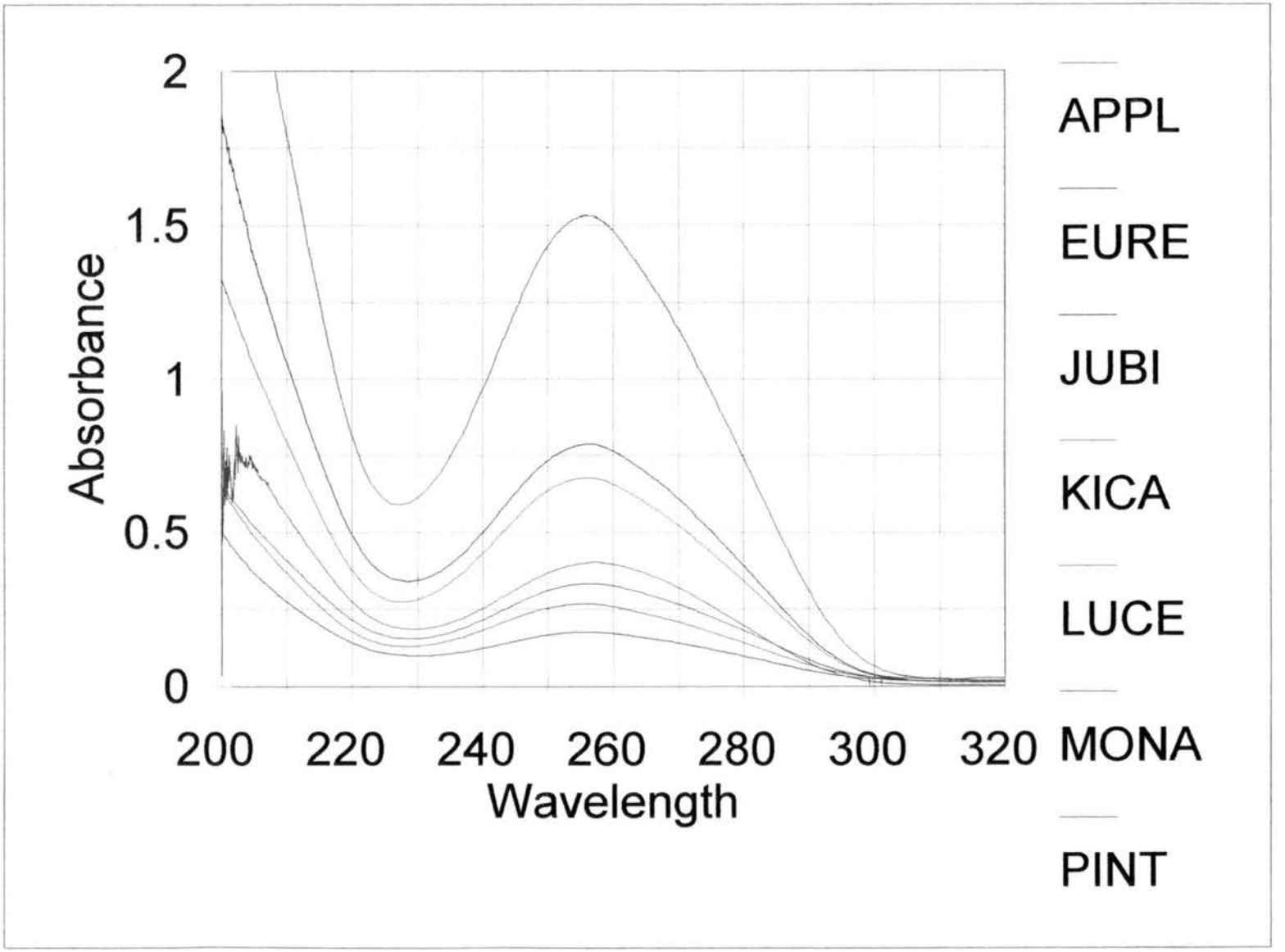

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance
200
220
240
260
280
300
320
Wavelength
AUR441
lh3414
AURA
CARY
HAEM
PSIT
PULC

Figure A-2. Absorbance curves for DNA samples of seven *Alstroemeria* cultivars obtained with the Beckman DU640 spectrophotometer.


Absorbance
2
1.5
1
0.5
0
200
220
240
260
280
300
320
Wavelength
APPL
EURE
JUBI
KICA
LUCE
MONA
PINT