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Propositions

1. Complexity of AFLP fingerprints in *Alstroemeria*, which has a large genome, is better controllable by using 8-cutter than 6-cutter restriction enzymes (this thesis).

2. Parental species might be defined by morphological traits as well as genetic distances (this thesis).

3. It is difficult to discern between qualitative and quantitative traits otherwise than by mutations provided loss of functions.

4. Marker technology is also important in ornamentals (this thesis).

5. The development of molecular marker systems, enabling marker-assisted breeding, is in addition to transgenesis another revolution in the short history of molecular plant breeding.

6. Gene banks are not used for conservation of the entire ecological biodiversity but mainly for the conservation of agrobiodiversity.

7. Molecular taxonomists are replacing traditional taxonomists, but it is more and more clear that both are needed for the conservation of biodiversity.

8. The combination of genomics, proteomics and phenotyping will be the promising future in biological research.

The propositions are part of the thesis, "Use of genetic markers in *Alstroemeria*"
by Tae-Ho Han, Wageningen University, Tuesday 9 January 2001.

Use of genetic markers in *Alstroemeria*

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Tae-Ho Han

Use of genetic markers in *Alstroemeria*

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Use of genetic markers in *Alstroemeria*

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Bibliographic Abstract: This thesis describes the results of various applications of the AFLP marker technique in *Alstroemeria*. The aim of this study was 1) to adapt the AFLP technique for *Alstroemeria* species which has a large genome size by increasing selective nucleotides and by using rarer restriction enzyme; 8-cutter instead of 6-cutter, 2) to study the genetic diversity of 22 *Alstroemeria* species of Chilean and Brazilian origin, and *Bomarea salsilla* and *Leontochir ovallei* as outgroup including F_1 hybrids in order to identify parental genotypes, 3) to construct genetic linkage maps of the *A. aurea* genome, and to allocate the pollen colour trait and 4) to map in *A. aurea* QTLs involved in ornamentally important traits. In conclusion, the perspectives of marker-assisted breeding in ornamentals were discussed.

Contents

To my wife and parents

1 Introduction

The use of ornamentals

In human history the presence of ornamentals has been noticed over more than 2,000 years. In Asia the cultivation of orchids was mentioned in Confucius's writings (551 – 479 B.C.) indicating that the Chinese were using the orchid flowers for decorating their homes (Withner 1959). In Europe the cultivation of carnation was known in Theophrastus's writings about 300 B.C, as the Greeks used the carnation flowers in crowns for their athletes (Larson 1980).

In those times, no particular ornamental breeding for commercialisation was performed but indigenous selections by growers must have been carried out. Likewise, no mass cultivation as nowadays by farmers did occur in ornamentals at that time. Flowers were rather grown in private gardens or obtained from nature. In the appearance of the industrialisation and urbanisation, people could not access the ornamentals any more as before from nature. Therefore, the demand of ornamentals has been increased continuously ever since the urbanisation of mankind and the economic growth of the western countries. The majority of ornamentals are nowadays obtained from the market, cultured by professional growers often in greenhouses. The development of mass propagation systems supported by tissue culture techniques and artificial environmental control enabled the ornamental breeding companies to react on such demands. This type of mass propagation was generally followed by scientific investigations including modern plant breeding techniques. However, the scientific

input in ornamentals was limited to a considerably smaller scale as compared to that in the main food crops because of the luxurious need in human life.

Ornamental breeding

Genuine ornamental breeding programs started approximately 200 years ago, together with breeding programs for food crops. For example, rose and chrysanthemum got attention by European and American breeders early in the 18th century and orchids were bred since the 17th century (Larson 1980). The classical breeding program crossed desirable parental genotypes and selected the marketable plants. Mutation breeding resulted also in successful cultivars by obtaining various types of genetic variation. In recent years genetic engineering of ornamentals has been commonly performed to improve the characters of qualitative and quantitative nature. The easiest way to improve the ornamentals is making transgenic plants, for example in petunia (Winefield *et al.* 1999), iris (Jeknic *et al.* 1999) and gerbera (Nagaraju *et al.* 1998). Transgenic ornamentals could be accepted easily by the public and governments compared to transgenic food crops due to the indirect use of the plant. However, the production and consumption of transgenic plants is in a big debate. Therefore, great alerts should be taken when and how to introduce transgenics at the public, governmental and industrial level. In a Korean proverb we say, "Prevention is better than cure". The other but unpopular way till now is the marker assisted selection (MAS) in ornamentals. However, based on the success of MAS in food crops, MAS has been started in recent years in ornamentals like rose (Debener and Mattiesch 1999) and *Rhododendron* (Dunemann *et al.* 1999). In order to perform MAS, the construction of linkage maps is a prerequisite. It allows MAS breeding programmes with the objective to improve morphological characters, such as leaf chlorosis, flower

colour in *Rhododendron* in a quantitative way (Dunemann *et al.* 1999), petal number (double versus single flowers) and flower colour (pink versus white) in rose (Debener and Mattiesch 1999) and basal branching in sunflower (Gentzbittel *et al.* 1999).

Alstroemeria

Alstroemeria, also known as the Peruvian or Inca lily, is a genus in the family of *Alstroemeriaceae* consisting of approximately 60 species of rhizomatous, herbaceous plants, most of which are endemic to Chile and Brazil (Bayer 1987; Ravenna 1988; Aker and Healy 1990). The family of *Alstroemeriaceae* includes several related genera, such as *Bomarea* Mirbel, the monotypes *Leontochir ovallei* Phil. and *Schickendantzia* Pax (Dahlgren and Clifford 1982; Hutchinson 1973). *Alstroemeria* is grown in the Netherlands as the ninth economically important cut flower on the auction. The popularity of *Alstroemeria* as cut flower can be attributed to its extensive range of colourful flowers, its long vase-life and its ability to grow at low greenhouse temperatures.

There are two main varieties of *Alstroemeria* discriminated; the 'Butterfly'-type, which has year-round blooming, short stems and peduncles, and the 'Orchid'-type, with season blooming, long stems and peduncles. The first *Alstroemeria* breeding goes back to 1948 when the first variety called 'Walter Fleming' was introduced (Anonymus, 1949). It was an interspecific hybrid (2n=2x) between the Chilean species *A. aurea* R. Graham and *A. violacea* (Bayer 1987; Vonk-Noordegraaf 1981). During vegetative propagation of this variety, a spontaneously doubled allotetraploid (2n=4x) plant was found, which produced viable 2x gametes. This plant has been crossed with diploid *Alstroemeria* species, thus producing triploid (2n=3x) offspring

of which the triploid 'Orchid'-hybrids originated. Interspecific hybrids between the Chilean species *A. pelegrina* L. or *A. violacea* and the Brazilian species *A. psittacina* or *A. inodora* formed another group of commercial varieties, the 'Butterfly'-types. After doubling of the genomes, these hybrids were allotetraploids (2n=4x) and produced viable 2x gametes (De Jeu *et al.* 1992).

The *Alstroemeria* breeding program has mainly been focused on the production of cut flowers. In recent years there is also a demand for *Alstroemeria* pot and garden plants. According to such demand breeders have developed compact cultivars suitable for use as indoor pot plants and outdoors in plantations and in landscapes. In addition, the attempts to develop fragrant *Alstroemeria* varieties resulted in a success (http://www.plants-magazine.com/newplants/newplant4.shtml).

Marker technology in ornamentals

The majority of ornamental plants are still unknown at the genetic level. Therefore, molecular markers have been developed for a relatively limited number of ornamentals as compared to the large number of plants from the main food crops, such as potato (Struik *et al.* 1999; Sandbrink *et al.* 2000), tomato (Moreira *et al.* 1999), and barley (Qi *et al.* 1999). Applications of molecular markers in ornamentals have been studied for cultivar identification (Loh *et al.* 1999), pedigree analysis (Friesen *et al.* 1997) and germplasm variability (Zhang *et al.* 1997). Different applications of molecular markers have been recognised in a relatively slow phase in ornamental breeding such as construction of linkage maps (Dunemann *et al.* 1999; Debener and Mattiesch 1999; Gentzbittel *et al.* 1999) and gene tagging (Scovel *et al.* 1998).

Use of marker technology in *Alstroemeria*

The utilisation of molecular marker techniques has principally been hindered in *Alstroemeria* because of its large genome size ranging from 37 to 79 pg (2C-value) (Bharathan *et al.* 1994; Buitendijk *et al.* 1997). At first the progressive utilisation of marker techniques, RAPD in *Alstroemeria* stopped after the study of discerning the genetic variation and relationship of *Alstroemeria* cultivars, hybrids and species with RAPD markers (Anasstassopoulos and Keil 1996; Dubouzet *et al.* 1997; Picton *et al.* 1997). They agreed that the RAPD marker technique was a simple and effective tool to discriminate *Alstroemeria* cultivars, hybrids and species. Anastassopoulos and Keil (1996) however, concluded that the RAPD technique was, as expected, an inappropriate tool to distinguish *Alstroemeria* plants with phenotypically visible X-ray mutations and mutations caused by somaclonal variation.

Recently, the improvement of a marker technique AFLP has become available for plants with a large genome size like *Alstroemeria* and *Allium* (Chap. 2; Van Heusden *et al.* 2000).

Objectives and outline of the thesis

AFLP (Amplified Fragment Length Polymorphism) is a PCR-based multi-locus marker technique, which allows the selective amplification of subsets of genomic restriction fragments (Vos *et al.* 1995). AFLP has been used for multiple purposes such as construction of linkage maps in rose (Debener and Mattiesch 1999) and *Rhododendron* (Dunemann *et al.* 1999), marker saturation at specific genomic regions in barley (Schwarz *et al.* 1999) and rice (Xu *et al.* 2000), analysis of genetic diversity

in rice (Virk *et al.* 2000), molecular phylogeny in *Allium* (Van Raamsdonk *et al.* 2000) and cultivar identification in celery (Li and Quiros 2000).

The common AFLP system uses two restriction enzyme combinations with rare and frequent cutters (6- and 4-cutter) to control the number of AFLP bands per lane and the size of the AFLP bands, respectively (Vos *et al.* 1995). Vos *et al.* (1995) tested the AFLP technique in middle-large genomes and indicated that the AFLP technique could allow accurate amplification of subsets of restriction fragments for plant species with a large genome size. One way was by increasing the number of selective nucleotides added to the core primers, which can amplify accurately even in complex template mixtures (Chap. 2). The second way was by using a more rare cutter (i.e. 8-cutter: *Sse*8387I) at production of the primary templates resulting in simple template mixtures (Chap. 4). In Chapter two and four of this thesis these two ways were applied in *Alstroemeria*, an example of a plant species with a very large genome, in order to obtain reproducible, clear and labor-saving fingerprints. Furthermore, the applicability of AFLP as a molecular marker in species with a large genome was discussed generally.

In Chapter three the genetic diversity of 22 *Alstroemeria* species, one interspecific hybrid and the distantly related species *Bomarea salsilla* and *Leontochir ovallei*, was assessed by AFLP analysis. The species classification in *Alstroemeria* is based on an evaluation of morphological traits of the flower, stem, leaf, fruit and rhizome, which is rather difficult because morphological characteristics can vary to a large extent in different environmental conditions (Bayer 1987). The available biosystematic information on *Alstroemeria* species is mainly restricted to the Chilean species but

little is known about the classification of the Brazilian species. The Chilean and Brazilian species used in this chapter are commonly used in the breeding programme of *Alstroemeria*. Therefore, identification of genetic relationships at species level could be very useful for breeding in supporting the selection of cross combinations between large sets of parental genotypes, thus broadening the genetic basis of breeding programmes (Frei *et al.* 1986).

In Chapter four and five, linkage maps of *A. aurea* (A002 × A003) were constructed by using AFLP markers and QTL analysis for 13 morphological traits was conducted, respectively. *Alstroemeria* has several features that could complicate the molecular genetic studies, such as the already mentioned large genome size, heterozygosity due to the outbreeding nature of the species, inbreeding depression during maintenance by selfed seed, the long generation cycle and polyploidy of the cultivars. Yet, linkage maps and marker assisted selection for quantitative characters or traits expressed in a late stage of plant development, such as flower shape and flower colour, would be useful in our ornamental breeding program. Double-pseudo-testcross analysis was conducted because of the allogamous nature of the *Alstroemeria* species (Grattapaglia and Sederoff 1994; Hemmat *et al.* 1994). Finally, Chapter six represents a general discussion on the implications and expectations of the AFLP marker technology on *Alstroemeria* as an organism with a large genome size, the utilisation of AFLP for biodiversity studies, the construction of linkage maps, QTL mapping, and marker-assisted breeding of *Alstroemeria* and in ornamentals generally.

2 Optimization of AFLP fingerprinting of organisms with a large genome size: A study on *Alstroemeria spp.*

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Abstract

The recently introduced PCR-based DNA fingerprinting technique AFLP (Amplified Fragment Length Polymorphism) allows the selective amplification of subsets of genomic restriction fragments. AFLP has been used for multiple purposes such as construction of linkage maps, marker saturation at specific genomic regions, analysis of genetic diversity and molecular phylogeny and cultivar identification. AFLP can be tailored by varying the number of selective nucleotides added to core primers and can allow accurate amplification, even in complex template mixtures generated from plant species with very large genomes. In this study *Alstroemeria*, a plant species with a very large genome was tested for adapting the AFLP protocol. The results indicated that the estimated number of amplification products was close to the observed number, when eight selective nucleotides were used, but seven selective nucleotides did not increase the number of amplification products fourfold. However, we found reproducibility in both +7 and +8 fingerprints. Various distributions of selective nucleotides over the various rounds of preamplifications were tested. Preamplification with four selective nucleotides followed by final amplification with eight selective nucleotides produced clear and reproducible AFLP patterns. The effects of GC content of primers and multiple preamplification steps were also discussed.

Key words: AFLP genetic marker, *Alstroemeriaceae*, Inca lily, Multiple cascade PCR, Preamplification

Introduction

In the last decade a large number of DNA marker techniques have been developed. These techniques are based either on DNA-DNA hybridization, like restriction fragement length poltmorphism (RFLP) markers (Beckman and Soller 1983), or on the amplification of specific DNA fragments using specific or random primers like sequence-tagged sites (STS, Shin *et al.* 1990), sequence-characterized amplified region (SCAR, Paran and Michelmore 1993), random amplified polymorphic DNA (RAPD, Rafalski *et al.* 1991) and amplified fragment length polymorphism (AFLP, Vos *et al.* 1995). These marker techniques can be easily performed on species with a small to moderately large genome size, such as *Arabidopsis* (145 MB), barley (1,500 MB) (Bennett and Leitch 1995) and mammal genomes (3,000 MB). For Southern hybridization the detection limit is determined by the DNA binding capacity to membranes used for Southern blotting and labeling efficiency of the DNA probes. For polymerase chain reaction (PCR)-based marker techniques, however, the amount of template DNA needed for amplification is at least 1,000 times less than for Southern hybridization. The most important factor that might interfere with the amplification of specific fragments is the competition with other fragments that are amplified by random priming due to one or more mismatches.

The recently introduced PCR-based DNA fingerprinting technique AFLP (Vos *et al.* 1995) allows the selective amplification of subsets of genomic restriction fragments. AFLP has been used (1) for the construction of linkage maps, as in *Arabidopsis* (Alonso-Blanco *et al.* 1998), barley (Becker *et al.* 1995; Qi *et al.* 1998; Waugh *et al.*1997) and potato (Van Eck *et al.* 1995); (2) for marker saturation at specific genomic regions in barley (Büschges *et al.* 1997), potato (Van Voort *et al.* 1997), rice

(Maheswaran *et al.* 1997) and tomato (Thomas *et al.* 1997); (3) for the analysis of genetic diversity in nematodes (Folkertsma *et al.* 1996) and for molecular phylogeny in potato (Kardolus *et al.* 1998); (4) for cultivar identification in barley (Ellis *et al.* 1997) and in potato (Milbourne *et al.* 1997). AFLP can be tailored according to the complexity of the pool of the restriction fragments by varying the number of selective nucleotides added to core primers which hybridize to adaptors ligated to the restriction fragments. Vos *et al.* (1995) tested the AFLP technique in middle-large human genomes and indicated that the AFLP technique could allow accurate amplification of subsets of restriction fragments, even in complex template mixtures generated from plant species with very large genomes, just by increasing the number of selective nucleotides added to the core primers.

An example of a plant species with a very large genome is *Alstroemeria*, also known as the Peruvian or Inca lily. It is a genus in the family of *Alstroemeriaceae* consisting of approximately 60 species of rhizomatous, herbaceous plants, most of which are endemic to Chile and Brazil (Bayer 1987; Ravenna 1988; Aker and Healy 1990). The nuclear DNA content (2C-value) ranges from 37 to 79 pg for Chilean species and from 50 to 56 pg for Brazilian species (Bharathan *et al.* 1994; Buitendijk *et al.* 1997). The haploid genome size (1C) of 25 pg, which equals to approximately 25,000 Mbase pairs will render approximately 12,000,000 different *Eco*RI/*Mse*I fragments, assuming random distribution of restriction sites, whereas only 36,000 fragments are expected from an *Arabidopsis* genome (1C= 0.04 pg).

If species with a large genome contain abundantly occurring repetitive sequences, the number of different amplification products will be reduced. Still, species with a large

genome size are expected to generate more AFLP amplification products than species with small genomes. Until now little is known about the fine-tuning of AFLP for the analysis of species with such large genomes.

In this chapter, our aim was to adapt the AFLP protocol for *Alstroemeria*. Various numbers of selective nucleotides and preamplification steps were tested in order to obtain reproducible, clear and labor saving fingerprints. Furthermore, the applicability of AFLP as a genetic marker in species with a large genome was discussed.

Material and Methods

Plant material

Sixteen accessions of in total seven diploid (2x = 2n = 16) non-inbred *Alstroemeria* species from Chilean or Brazilian origin were used to acquire general *Alstroemeria* AFLP fingerprints with a wide genotypic background. Several species commonly used as breeding parents were selected and crossed with each other (*A. aurea* × *A. inodora* and *A. inodora* × *A. psittacina*) (Table 1).

The AFLP protocol

Genomic DNA was isolated from fresh leaf tissue of greenhouse grown plants by using the CTAB method according to Rogers and Bendich (1988). The AFLP method was performed as described by Vos *et al.* (1995). A slight modification on this general protocol was with respect to the number of selective nucleotides, and the number of preamplification steps. The general protocol included four steps: (1) restriction of genomic DNA with *Eco*RI and *Mse*I, and ligation of adaptor sequences to the

Table 1. Accessions and origins of *Alstroemeria* species for AFLP analysis

Plant material	Accession/source[a]
Chilean Species	
A. aurea R. Graham	A001, A002, A003, A004, A011, A017, 96A001-1
A. diluta	AD5K
A. hookeri Loddiges *spp. Hookeri*	AP6K
A. ligtu L. *spp. Ligtu*	AL1S
A. pelegrina L.	C100, C057-2
Brazilian Species	
A. inodora Herb.	P002, P004-20, P008-1
A. psittacina Lehm	D032
Interspecific hybrids (F_1)	
A. inodora × *A. psittacina*	PD4 (P002 × D032)
A. aurea × *A. inodora*	A1P2 (A001 × P002)
F_2	PD4⊗-2, PD4⊗-3, PD4⊗-4, PD4⊗-5, PD4⊗-6, PD4⊗-7, PD4⊗-8, PD4⊗-9
BC_1	SK004, SK011 ((A1P2) × P002) SK017 (P002 × (A1P2))

[a] Codes from accessions of species maintained at the Department of Plant Breeding, Wageningen Agricultural University.

restriction fragments in order to generate the primary template, (2) selective preamplifications of this primary template with AFLP primers having various additional 3′ selective nucleotides, (3) selective amplification with ^{33}P-labeled *Eco*RI primers having three or four 3′ selective nucleotides and *MseI* primers with four 3′ selective nucleotides, and (4) separation of labeled amplification products on a denaturing polyacrylamide sequencing gel. The anodal buffer was supplemented with 0.5 M sodium acetate to generate a salt gradient, which contributes to a better separation of the larger fragments. The gels were dried on Whatmann 3MM paper, and X-ray films (Konica, Tokyo, Japan) were exposed at room temperature for 1 - 7 days depending on the signal intensity.

Results and discussion

Reducing the number of amplification products

According to Vos *et al.* (1995) the number of amplification products generated by the AFLP technique is related to the size of the genome and the number of selective nucleotides added to the 3' end of the *Eco*RI and *Mse*I core primers. In view of the large genome size of *Alstroemeria*, the number of selective nucleotides added to the core primers varied from six to eight. Theoretically, +6, +7 and +8 fingerprints named by their selective nucleotides should have roughly around 3,000, 720 and 180 bands, respectively, in consideration of the genome size of *Alstroemeria*. However, fingerprints with on average 109 (66 -154) amplification products per lane were obtained using six selective nucleotides (*Eco*RI + 3/*Mse*I + 3) (Table 2). When using seven selective

Table 2. Number of bands according as GC contents for 6, 7 and 8 selective nucleotides.

GC content effects	Primer combinations	Species	Average Bands	Range	Standard Deviation
Six selective nucleotides	16	7	109 a[1]	66-154	17
33%	2	6	121a[2]	83-145	19
50%	8	7	109b	66-154	17
67%	4	6	110b	81-149	15
83%	2	5	94c	74-117	13
Seven selective nucleotides	24	7	87 b	47-158	23
29%	3	6	111a	88-137	14
43%	9	7	98b	63-146	18
57%	8	6	81c	49-158	22
71%	4	6	73c	47-124	19
Eight selective nucleotides	80	2	91 b	36-135	20
38%	4	2	84a	66-107	14
50%	16	2	90a	55-121	15
63%	28	2	87a	36-107	21
75%	24	2	94a	52-135	22
88%	8	2	97a	60-133	20

[1] Results of Duncan test ($\alpha = 0.05$) among band numbers of 6, 7 and 8 selective nucleotides.
[2] Results of Duncan test ($\alpha = 0.05$) among band numbers of GC contents for each selective nucleotide classes.

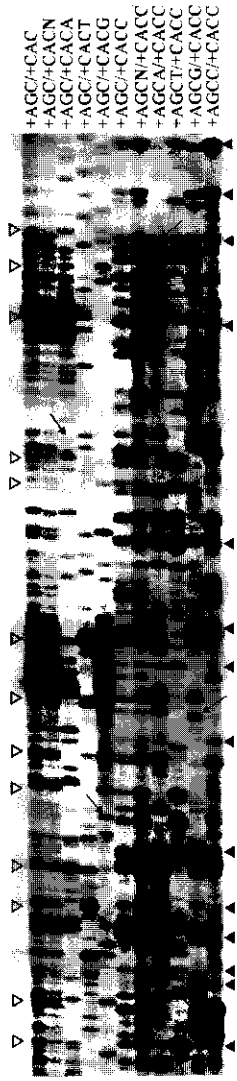

Fig 1. The +6, +7 and +8 fingerprints of *A. inodora* (P002) and the mixture of corresponding fingerprints. *Arrows* indicate bands present but absent in the mixture acnd preceding fingerprints. *Arrowheads* indicate the homeologous bands present in two lanes.
[a] The mixture of +AGC/+CACA, +AGC/+CACC, +AGC/+CACG and +AGC/+CACT
[b] The mixture of +AGCA/+CACC, +AGCC/+CACC, +AGCG/+CACC and +AGCT/+CACC

nucleotides (*Eco*RI + 3/*Mse*I + 4) the number of amplification products decreased significantly. The average number of amplification products per lane was 87 (47 - 158). Similarly, with eight selective nucleotides (*Eco*RI + 4/*Mse*I + 4), 91 products (36 - 135) were amplified. These results indicated that the expected number of amplification products was close to the observed number when eight selective nucleotides were used, always considering the rough estimation of the number of amplification products. However, the observed number of amplification products from six and seven selective nucleotides did not meet the expected number of amplification products. The number of amplification products dramatically reduced with six and seven selective nucleotides may be due to the presumed high frequency of repetitive DNA and due to technical limitations, such as separation of nearly comigrating bands.

The effect of reducing one extra selective nucleotide on the number of bands per lane was studied in greater detail by comparing the fingerprints generated with a total of seven selective nucleotides (the +7 fingerprint) with the four corresponding fingerprints generated with primers with eight selective nucleotides (corresponding +8 fingerprints), as well as the fingerprint of a mixture of equal volumes of the corresponding +8

Table 3. Fourteen various preamplification steps with final +4/+4 selective nucleotides on 6 genotypes (P002, D032, PD4, PD4⊗-2, PD4⊗-8, PD4⊗-9)

Code	Preamplification steps	Example
ST0	PT[a]→+4/+4	PT→+AGCC/+CACG
ST1	PT→+1/+1→+4/+4	PT→+A/+C→+AGCC/+CACG
ST2	PT→+2/+2→+4/+4	PT→+AG/+CA→+AGCC/+CACG
ST3	PT→+3/+3→+4/+4	PT→+AGC/+CAC→+AGCC/+CACG
ST4	PT→+1/+1→+2/+2→+4/+4	PT→+A/+C→+AG/+CA→+AGCC/+CACG
ST5	PT→+1/+1→+3/+3→+4/+4	PT→+A/+C→+AGC/+CAC→+AGCC/+CACG
ST6	PT→+2/+2→+3/+3→+4/+4	PT→+AG/+CA→+AGC/+CAC→+AGCC/+CACG
ST7	PT→+2/+2→+2/+3→+4/+4	PT→+AG/+CA→+AG/+CAC→+AGCC/+CACG
ST8	PT→+2/+2→+3/+2→+4/+4	PT→+AG/+CA→+AGC/+CA→+AGCC/+CACG
ST9	PT→+2/+2→+3/+3→+4/+4	PT→+AG/+CA→+AGC/+CAC→+AGCC/+CACG
ST10	PT→+1/+1→+2/+2→+2/+2→+4/+4	PT→+A/+C→+AG/+CA→+AG/+CA→+AGCC/+CACG
ST11	PT→+1/+1→+2/+2→+2/+3→+4/+4	PT→+A/+C→+AG/+CA→+AG/+CAC→+AGCC/+CACG
ST12	PT→+1/+1→+2/+2→+3/+2→+4/+4	PT→+A/+C→+AG/+CA→+AGC/+CA→+AGCC/+CACG
ST13	PT→+1/+1→+2/+2→+3/+3→+4/+4	PT→+A/+C→+AG/+CA→+AGC/+CAC→+AGCC/+CACG

[a] Primary templates. +1/+1, +2/+2, +2/+3, +3/+2, +3/+3 and +4/+4 indicate the selective nucleotides for each primer. Comparable procedures were followed for other primer combinations (+AGCA/+CACA, +AGCA/+CACC, +AGCA/+CACG, +AGCA/+CACT, +AGCA/+CCCA, +AGCA/+CCCC, +AGCA/+CCCG, +AGCA/+CCCT, +AGCC/+CACC, +AGCG/+CACC and +AGCT/+CACT).

fingerprints. Likewise, the +6 fingerprints were compared with the corresponding four +7 fingerprints with seven selective nucleotides and their mixture (Fig. 1). Occasionally, we observed that a band found in the +7 fingerprint was amplified in two out of the corresponding four +8 fingerprints instead of the expected one lane (Fig. 1, indicated with arrowheads). Bands of +6 fingerprints were frequently found; two comigrating bands in the corresponding +7 fingerprints as well (Fig. 1, indicated with open arrowheads). This result, suggesting two comigrating alleles with one nucleotide difference, can explain the non-fourfold increase in bands. In addition, some bands of +8

fingerprints, that were missing on the lanes of the mixture and the preceding +7 fingerprint are indicated with arrows inside of the fingerprint (Fig. 1). The same holds true for the +7 fingerprints compared to the mixture and the preceding +6 fingerprints.

In conclusion, on the one hand, we recognized that the preceding fingerprint and the fingerprint of the mixture were largely identical, suggesting that a reduction of selective nucleotides did not generate artifacts. On the other hand, we failed to recognize a fourfold increase due to one less extra selective nucleotide. These results indicate that AFLP technique is reproducible, allowing us to work with +7 and +8 selective nucleotides regardless of the theoretical expectation of the amplification products.

The GC content effect was shown on 120 primer combinations with 16 accessions (Table 2). The data were analyzed with the Duncan test ($\alpha = 0.05$). In +6 fingerprints, the average band numbers of 33 % and 83 % GC contents showed significant differences, 121 and 94 bands respectively. In +7 fingerprints higher GC contents significantly reduced band numbers as in 29%, 43%, 57% and 71% GC contents showed 111, 98, 81 and 73 bands, respectively. This indicates the importance of determining the primer combinations. However, the GC contents of +8 fingerprints did not give any influence band numbers. In soybean, which has an unusually high AT contents, the number of AFLP bands generally increased for primer combinations with AT-rich selective nucleotides. Inversely, GC-rich selective nucleotides reduced the number of bands and increased the quality of bands (Keim *et al.* 1997). The determination of the quality of the bands was based on band intensity and separation from fragments of similar size. *Alstroemeria* was proven to be AT rich in repetitive DNA in the case of A. *aurea* (56 %) (De Jeu *et al.* 1997) and Brazilian species (61 %) (Kuipers, personal communication).

Fig 2. The +8 fingerprint of F_2 (PD4⊗-9) with seven different preamplification steps (See Table 2 for details).

Our data support that *Alstroemeria* is AT-rich and GC contents of primers influence the number of bands.

We conclude that a total of six, seven or eight selective nucleotides could produce clear and reproducible AFLP patterns (less than 100 bands) depending on the primer combinations and species.

The effect of preamplification steps on AFLP fingerprints

In the previous paragraph we determined the influence of the number of selective nucleotides used during the final PCR on the amplification products. In this section we focus on (1) the influence of the number of selective bases of the primers on the preamplification and (2) the influence of the number of preamplification steps on the accuracy of amplification, which affects the reproducibility and the clearness of the bands.

For plant species with a large genome we should include a selective preamplification of the template with moderate selectivity of the primers to avoid mismatches. In case of the average-sized genomes, the preamplification steps with *Eco*RI+1/*Mse*I+1 have been commonly used to avoid mismatches (Vos *et al.* 1995; Qi and Lindhout. 1997). Next to that, the final selective PCR amplification with the radioactively labeled primer combination should also have moderate selectivity. In this

experiment we applied various distributions of the selective nucleotides across the various rounds of preamplifications on 11 related genotypes (parents: P002, D032; F_1 plant: PD4; F_2 plants: PD4⊗-2, PD4⊗-3, PD4⊗-4, PD4⊗-5, PD4⊗-6, PD4⊗-7, PD4⊗-8, PD4⊗-9) (Table 1). Eight selective nucleotides (*Eco*RI+4/*Mse*I+4) at final PCR rendered 14 different preamplification steps (Table 3). Previous experiments on small genomes have shown that the selective amplification of subsets of fingerprints was accurate when no more than three selective nucleotides were added onto the core primer. Application of four selective nucleotides without any preamplification was no longer accurate because mismatches were tolerated at the first selective base (Vos *et al.* 1995).

The overall band intensity of the ST0 (PT→ +AGCC/+CACG) fingerprint was very weak resulting in the absence of the bands which were amplified in other preamplification steps (Fig. 2). This is possibly because of the extreme templates competition. However, only a few mismatches were accompanied as a result of the eight selective nucleotides in our study (Data not shown). Unexpectedly, the overall band intensity of the ST1 (PT→ +A/+C→ +AGCC/+CACG) fingerprint was increased, producing an AFLP pattern with extra bands and smears. We are uncertain as to whether these extra bands and smears should be considered as background untill genetic studies are carried out. ST2 (PT→ +AG/+CA→ +AGCC/+CACG) fingerprint produced a clear and reproducible AFLP pattern (Fig. 2 and 3). A severe disappearance of bands was detected on the ST3 (PT→ +AGC/+CAC→ +AGCC/+CACG) fingerprint, resulting in an increase in the intensity of some bands. A contrary AFLP pattern was noticeable between the ST1 and ST3 steps because both steps had six selective nucleotides jump. The distinctive differences in fingerprints due to the preamplification steps proved the

importance of choosing them carefully. In conclusion, we chose the ST2 fingerprint for use in further studies because of its reproducibility and efficiency.

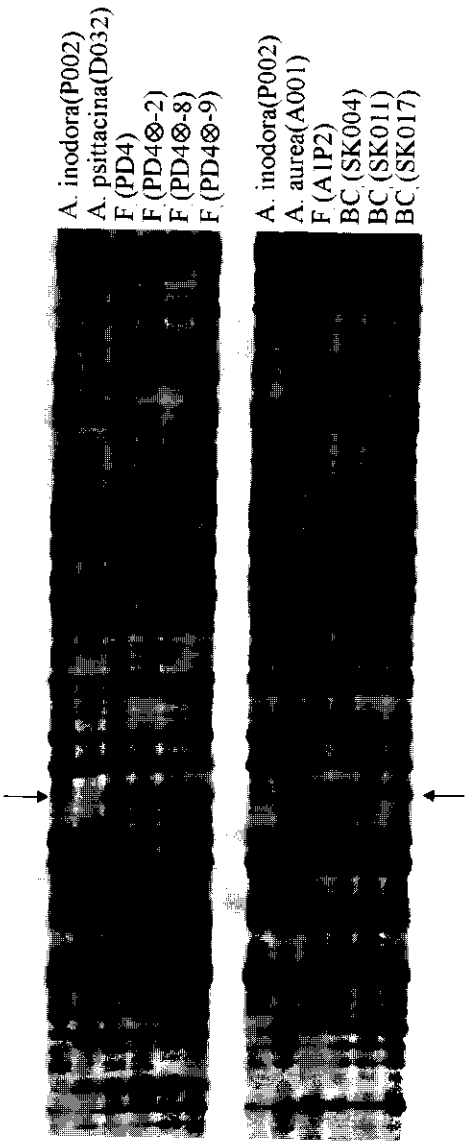

Fig 3. An example of the AFLP segregation pattern of the two populations (F_2 and BC_1) by +AGCA/+CACC primer combination.
The arrows indicate the only bands that are not explainable.

The application of two (ST4, ST5, ST6, ST7, ST8, ST9) or three (ST 10, ST11, ST12, ST 13) preamplification steps resulted in a general decrease in the intensity of the fragments larger than 350-400 nucleotides compared to one preamplification step, indicating that smaller fragments have an advantage over larger fragments during PCR (data not shown). The intensity of several products had minor differences. In few cases the intensity changed in such a way that the band was present or absent

The independent AFLP pattern was detected on the basis of the preamplification steps even though the final selective bases were identical. However, a multiple preamplification (2 to 4 times) in general was reproducible although it was not

preferable because of the loss of large fragments and extra labor. It provide the opportunity to increase the selective bases as long as one or two selective nucleotides for both primers are used.

Are AFLP products good genetic markers?

Both of the two segregating populations (F_2 and BC_1) used to choose the optimized AFLP protocol are genetically related. Therefore, the markers obtained in these progenies could be used to validate the accuracy of AFLP fingerprinting, because fragments observed in offspring genotypes should be present in at least one of the parental genotypes (Table 1). The theoretically expected segregation patterns were demonstrated, and most of the bands from progenies were traceable through their parents when performed with the +2/+2 preamplification step (ST2) (Fig. 3). In the F_2 population from the *A. inodora* × *A. psittacina* cross, bands were evenly derived from each parent and the bands of backcross progenies were mostly from the recurrent parent (P002). Only 1 band from the progenies was not present in the parents' fingerprints suggesting possible mismatches (Fig 3. arrow indicated). Further linkage analysis will allow verification of true AFLP markers. Furthermore, the intensity difference in some bands indicated the possibility of zygosity determination as a codominant marker (Van Eck *et al.* 1995; Staub and Serquen, 1996).

In conclusion, various primer combinations of +7 and +8 fingerprints should be tested before any investigation depending on the species and the purpose of the study. The ST2 preamplification step (PT → +2/+2 → +3/+4 or +4/+4) was suitable for genetic studies in *Alstroemeria* species because of its robustness and efficiency. Consequently,

AFLP markers can be applied for species with large genomes as long as the preamplification step and the final selective nucleotides are well defined by the users.

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3 Genetic diversity of Chilean and Brazilian *Alstroemeria* species assessed by AFLP analysis

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Abstract

One to three accessions of 22 *Alstroemeria* species, an interspecific hybrid (*A. aurea* × *A. inodora*), and single accessions of *Bomarea salsilla* and *Leontochir ovallei* were evaluated using AFLP-marker technique to estimate the genetic diversity within the genus *Alstroemeria*. Three primer combinations generated 716 markers and discriminated all *Alstroemeria* species. The dendrogram inferred from the AFLP fingerprints supported the conjecture of the generic separation of the Chilean and Brazilian *Alstroemeria* species. The principal co-ordinate plot showed the separate allocation of the *A. ligtu* group and the allocation of *A. aurea*, which has a wide range of geographic distribution and genetic variation in the middle of other *Alstroemeria* species. The genetic distances based on AFLP markers determined the genomic contribution of the parents to the interspecific hybrid.

Key words: *Alstroemeriaceae*, *Bomarea*, classification, Inca lily, *Leontochir*, Monocotyledonae

Introduction

The genus *Alstroemeria* includes approximately 60 described species of rhizomatous, herbaceous plants, with Chile and Brazil as the main centres of diversity (Uphof, 1952; Bayer, 1987; Aker and Healy, 1990). The Chilean and Brazilian *Alstroemeria* are recognized as representatives of different branches of the genus. The family of Alstroemeriaceae, to which *Alstroemeria* belongs, includes several related genera, such as *Bomarea* Mirbel, the monotype *Leontochir ovallei* Phil. and *Schickendantzia* Pax (Dahlgren and Clifford, 1982; Hutchinson, 1973).

The species classification in *Alstroemeria* is based on an evaluation of morphological traits of the flower, stem, leaf, fruit and rhizome (Bayer, 1987). The available biosystematic information on *Alstroemeria* species is restricted to the Chilean species as described in the monograph of Bayer (1987). Little is known about the classification of the Brazilian species (Meerow and Tombolato, 1996). Furthermore, morphology-based identification is rather difficult because morphological characteristics can vary considerably in different environmental conditions (Bayer, 1987).

The immense genetic variation present in the genus *Alstroemeria* offers many opportunities for the improvement and renewal of cultivars. Therefore, identification of genetic relationships at the species level could be very useful for breeding in supporting the selection of crossing combinations from large sets of parental genotypes thus broadening the genetic basis of breeding programmes (Frei *et al.* 1986). The species used in this study are commonly used in the breeding programme of *Alstroemeria* for cut flowers and pot plants.

Molecular techniques have become increasingly significant for biosystematic studies (Soltis *et al.* 1992). RAPD markers were used for the identification of genetic relationships between *Alstroemeria* species and cultivars (Anastassopoulos and Keil, 1996; Dubouzet *et al.* 1997; Picton and Hughes, 1997). In recent years a novel PCR-based marker technique, AFLP (Vos *et al.* 1995), has been developed and used for genetic studies in numerous plants including lettuce (Hill *et al.* 1996), lentil (Sharma *et al.* 1996), bean (Tohme *et al.* 1996), tea (Paul *et al.* 1997), barley (Schut *et al.* 1997), and wild potato species (Kardolus *et al.* 1998). These studies indicated that AFLP is highly applicable for molecular discrimination at the species level. The technique has also been optimized for use in species such as *Alstroemeria* spp., which are characterized by a large genome size (2C-value: 37 - 79 pg) (Chapter 2).

In this chapter, we produced AFLP fingerprints of 22 *Alstroemeria* species, one interspecific hybrid (*A. aurea* × *A. inodora*) and the distant related species *Bomarea salsilla* and *Leontochir ovallei*, and we analysed their genetic relationships. The interspecific hybrid was included in our study in order to investigate the possibility of identifying the parental genotypes.

Materials and methods

Plant material

Seeds and plants of 22 *Alstroemeria* species were obtained from botanical gardens and commercial breeders. The collection has been maintained for many years in the greenhouse of Unifarm at the Wageningen Agricultural University. When available, three accessions were selected for each *Alstroemeria* species, and both *B. salsilla* and

L. ovallei were chosen as outgroups. One interspecific hybrid (*A. aurea* × *A. inodora*) was obtained from earlier research (Buitendijk *et al.* 1995) (Table 1). All accessions were identified according to their morphological traits (Uphof, 1952; Bayer, 1987).

Table 1. Accessions and origin of *Alstroemeria* species for AFLP analysis

Code	Plant material	Accession[a]	Distribution/altitude[b]
	Chilean species		
C1	*A. andina* Phil.	IX-2	Chile 26°-31° S.L. 2900-3700 m[1]
C2	*A. angustifolia* Herb. ssp. *angustifolia*	AN1S, AN2S, AN7K	Chile, 33° S.L.,<1000 m[1]
C3	*A. aurea* Grah.	A001, A002, A003	Chile, 36°- 42° / 47° S.L., 200-1800 m[1]
C4	*A. diluta* Bayer	AD2W, AD4K, AD5K	Chile, 29°-31° S.L. 0-100 m[1]
C5	*A. exserens* Meyen	AO2S, AO5S, AO7Z	Chile, 34°-36° S.L. 1500-2100 m[1]
C6	*A. garaventae* Bayer	AH6Z, AH8K	Chile, 33° S.L. 2000 m[1]
C7	*A. gayana* Phil.	XIII-2	Chile 29°-32° S.L. 0-200 m[1]
C8	*A. haemantha* Ruiz and Pav.	J091-1, J091-4	Chile, 33°-35° S.L., 0-1800 m[1]
C9	*A. hookeri* Lodd. ssp. *cumminghiana*	AQ5S, AQ6Z, AQ7Z	Chile. 32°-34° S.L. 0-500 m[1]
C10	*A. hookeri* Lodd. ssp. *hookeri*	AP2S, AP3S, AP8K	Chile, 35°-37° S.L., 0-300 m[1]
C11	*A. ligtu* L. ssp. *incarnata*	AJ7S, AJ12K	Chile, 35° S.L. 1100-1400 m[1]
C12	*A. ligtu* L. ssp. *ligtu*	AL4S, AL6K, AL11K	Chile, 33°-38° S.L.,0-800 m[1]
C13	*A. ligtu* L. ssp. *simsii*	AM6K, AM7K, K101-1	Chile, 33°-35° S.L., 0-1800 m[1]
C14	*A. magnifica* Herb. ssp. *magnifica*	Q001-4, Q001-5, Q007	Chile, 29°-32° S.L.,0-200 m[1]
C15	*A. modesta* Phil.	AK2W, AK3W	Chile 29°-31° S.L. 200-1500 m[1]
C16	*A. pallida* Grah.	AG4Z, AG7K, AG8K	Chile 33°-34° S.L. 1500-2800 m[1]
C17	*A. pelegrina* L.	AR4S, C057-1, C100-1	Chile, 32°-33° S.L.,0-50 m[1]
C18	*A. pulchra* Sims. ssp. *pulchra*	AB3W, AB7S, AB8S	Chile, 32°-34° S.L., 0-1000 m[1]
C19	*A. umbellata* Meyen	AU2Z	Chile, 33°-34° S.L., 2000-3000 m[1]
	Brazilian species		
B1	*A. brasiliensis* Sprengel	BA1K, BA2K, R001-1, R001-2	Central Brazil[2]
B2	*A. inodora* Herb.	P002, P004-6, P008-3	Central and Southern Brazil[2]
B3	*A. pstittacina* (D) Lehm.	D031®, D032, D92-02-1	Northern Brazil[2]
B4	*A. pstittacina* (Z) Lehm.	93Z390-2, 93Z390-4, 96Z390-6	Northern Brazil[2]
O1	Bomarea salsilla *Mirbel.*	M121	Central and Southern South America[3]
O2	*Leontochir ovallei* Phil.	U001	Central Chile[4]
	Interspecific hybrid		
F1	A1P2-2	(A001 x P002)-2	Buitendijk et al. 1995

[a] Codes from accessions of species maintained at the Laboratory of Plant Breeding, Wageningen University and Research centre.
[b] Literature source: 1) Bayer, 1987; 2) Aker & Healy, 1990; 3) Hutchinson, 1959; 4) Wilkin, 1997.

AFLP protocol

Genomic DNA was isolated from young leaves of greenhouse-grown plants using the cetyltrimethylammonium bromide (CTAB) method according to Rogers and Bendich (1988). The AFLP technique followed Vos *et al.* (1995) with modifications of selective bases of pre- and final amplifications (Chapter 2). To assess interspecific variation, autoradiograms were analysed comprising the AFLP fingerprints of a mixture of three accessions per species by pooling 5 µL of the final selective

amplification products according to Mhameed *et al.* (1997). The low level of variation between individual samples showed that pooling accessions was justified. Three primer combinations (E+ACCA/M+CATG, E+ACCT/M+CATC and E+AGCC/M+CACC) were selected from a test of 96 primer combinations, and these produced 272, 211 and 233 bands respectively (Table 2). The choice of the primers used in the study was based upon the visual clarity of banding patterns generated and a preferably low fingerprint complexity. The complexity of the banding pattern is a major limiting factor for scoring AFLP fingerprints of large-size genomes.

Table 2. Sequences of adaptors and primers used

*Eco*RI adaptor		5′-CTCGTAGACTGCGTACC-3′ 3′-CTGACGCATGGTTAA-5′
*Mse*I adaptor		5′-GACGATGAGTCCTGAG-3′ 3′-TACTCAGGACTCAT-5′
*Eco*RI + 0 primer	E00	5′-GACTGCGTACCAATTC-3′
*Eco*RI + 2 primers	E+AC	5′-GACTGCGTACCAATTCAC-3′
	E+AG	5′-GACTGCGTACCAATTCAG-3′
*Eco*RI + 4 primers	E+ACCA	5′-GACTGCGTACCAATTCACCA-3′
	E+ACCT	5′-GACTGCGTACCAATTCACCT-3′
	E+AGCC	5′-GACTGCGTACCAATTCAGCC-3′
*Mse*I + 0 primer	M00	5′-GATGAGTCCTGAGTAA-3′
*Mse*I + 2 primers	M+CA	5′-GATGAGTCCTGAGTAACA-3′
	M+CT	5′-GATGAGTCCTGAGTAACT-3′
*Mse*I + 4 primers	M+CACC	5′-GATGAGTCCTGAGTAACACC-3′
	M+CTAC	5′-GATGAGTCCTGAGTAACTAC-3′
	M+CTAG	5′-GATGAGTCCTGAGTAACTAG-3′

Data analysis

Positions of unequivocally visible and polymorphic AFLP markers were transformed into a binary matrix, with '1' for the presence, and '0' for the absence of a band at a particular position. The genetic distance (GD) between species was based on pair-wise comparisons and calculated according to the equation: $GD_{xy} = 1 - [2N_{xy} / (N_x + N_y)]$, where N_x and N_y are the numbers of fragments to individuals x and y, respectively, and

N_{xy} is the number of fragments shared by both (Nei and Li, 1979). Genetic distances (GD) were computed by the software package TREECON (1.3b) (Van de Peer and De Wachter, 1993). The dendrogram of the 22 *Alstroemeria* species, the interspecific hybrid, *Bomarea* and *Leontochir* was generated based on the genetic distance matrix by using cluster analysis, the UPGMA (unweighted pair group method using arithmetic averages) method with 1000 bootstraps (Felsenstein, 1985; Sneath and Sokal, 1973) (Fig. 1). Principal co-ordinate analysis was performed to access interspecies relationships based on the Nei and Li (1979) coefficient $[2N_{xy} / (N_x + N_y)]$ using the NTSYS-PC program (Rohlf, 1989).

Results and Discussion

The average genetic distance (GD) among species excluding *Bomarea*, *Leontochir*, the interspecific hybrid and *A. umbellata* was 0.65 GD (a table showing the genetic distances between all the species studied is available from the authors on request). *Alstroemeria umbellata* was excluded because the accessions used were found to be highly related and possibly wrongly classified as different from *A. pelegrina*. The average genetic distance (GD) among accessions within a species was 0.32 GD (data not shown). In addition, the average genetic distance between Brazilian species (GD: 0.27) and between Chilean species (GD: 0.33) was not significantly different.

Buitendijk and Ramanna (1996) suggested that the Chilean and Brazilian species form distinct lineages. The genetic diversification of *Alstroemeria* species as detected by the AFLP technique revealed three main clusters with 99 % bootstrap values: the Chilean species, the Brazilian species and the outgroup (Fig. 1). This finding would support an early divergence of these groups and is consistent with the occurrence of interspecific

crossing barriers between the Chilean and Brazilian species (De Jeu and Jacobsen, 1995; Lu and Bridgen, 1997). The variance of the first three principal co-ordinates accounted for 34.9 % of the total variation, differentiated effectively among the species and reflected the main clustering of the dendrogram. From the principal co-ordinate

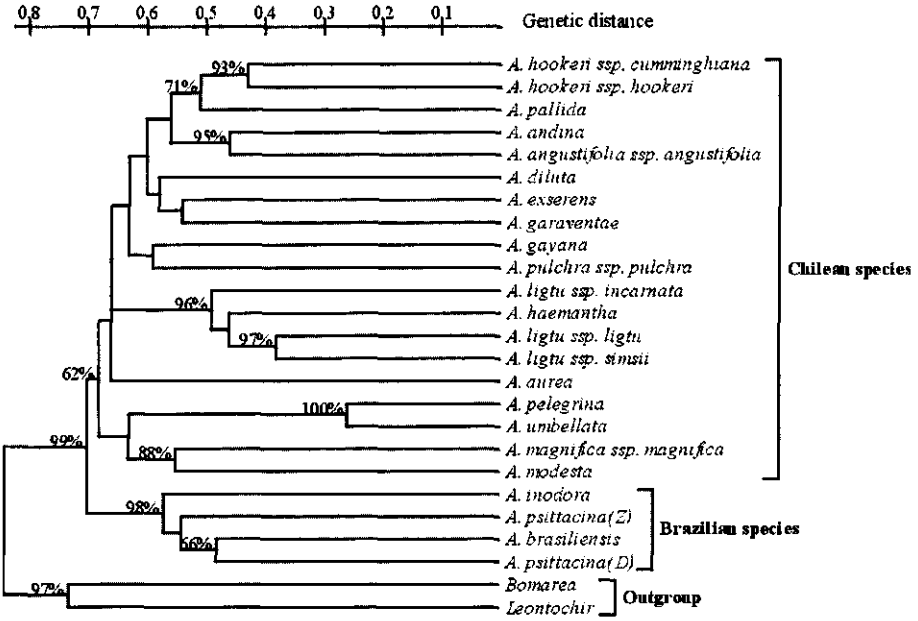

Figure 1. Dendrogram of 22 *Alstroemeria* species, *Bomarea salsilla* and *Leontochir ovallei* resulting from a UPGMA cluster analysis based on Nei's genetic distances obtained from 716 fragment length polymorphism (AFLP) bands. The bootstrap analysis was conducted using TREECON (1.3b) with 1000 bootstrap subsamples of the data matrix. Percentage values for those branches occuring in at least 60% of the bootstrap topologies are shown.

plot, four groups were clearly demarcated: (i) Brazilian group, (ii) Chilean group, (iii) *A. ligtu* group, and (iv) Outgroup (Fig. 2). The Brazilian species (*A. brasiliensis*, *A. psittacina* and *A. inodora)* were consistently assigned to one cluster with 98 % bootstrap values whereas the Chilean species were rather weakly clustered with 62 % bootstrap values containing several subgroups within the Chilean group (Figs. 1 and 2). The dispersion of the Chilean species on the principal co-ordinate plot reflected a wider genetic variation than the Brazilian species. However, the narrow variation of

the Brazilian species might be caused by the limited number of species investigated. Buitendijk and Ramanna (1996) described the similarities between C-banding patterns of *A. inodora* and *A. psittacina*; in our study these species strongly clustered reinforcing this finding (Fig. 1). The similarity between *A. psittacina* and *A. inodora* was also revealed by allozyme analysis (Meerow and Tombolato, 1996) and with a study using species-specific repetitive probes (De Jeu *et al.* 1995). These findings are also supported by the fact that *A. inodora* and *A. psittacina* are easily intercrossable (De Jeu and Jacobsen, 1995).

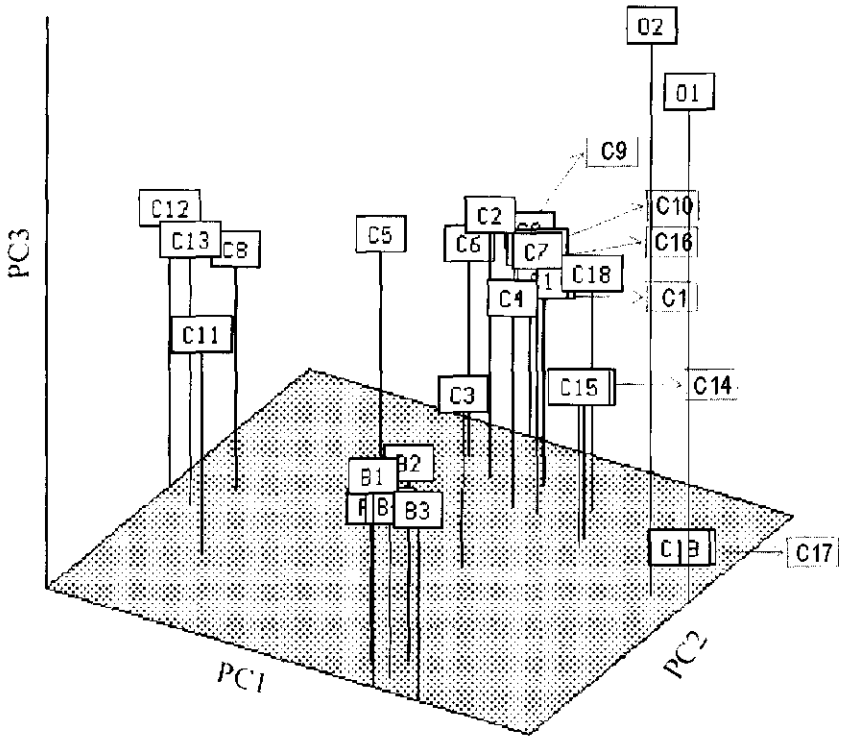

Figure 2. Relationships among 22 *Alstroemeria* species, the F_1 hybrid, *Bomarea salsilla* and *Leontochir ovallei* by principal co-ordinate analysis using fragment length polymorphism (AFLP)-based Nei and Li coefficients. The three principal co-ordinates accounted for 34.9 % of the total variation. PC1, PC2 and PC3: first, second and third principal co-ordinates. See Table 1 for species names.

In addition, the Chilean species *A. aurea* was positioned between three subgroups (Fig. 2). The unique position of *A. aurea*, and the observation that *A. aurea* has a wide geographical spread, suggests that other Chilean species may have evolved from *A. aurea* ecotypes. *Alstroemeria aurea* indeed is a widely spread inhabitant in the regions with higher rainfall at the more southern latitudes between 33 and 47° S in Chile (Bayer, 1987; Buitendijk and Ramanna, 1996). It is not found in Brazil although *A. aurea* plants are found on both sides of the Andes mountains in Argentina supporting the possibility that *A. aurea* ecotypes were also the ancestors of the Brazilian species (A.F.C. Tombolato, personal communication).

Alstroemeria pelegrina and *A. umbellata* were assigned as sister species with a distance of 0.26 showing a remarkable genetic similarity (data available on request). The species we coded under the name *A. umbellata* actually seemed to be an *A. pelegrina* species that did not flower for many years. *Alstroemeria haemantha* was assigned to a group together with *A. ligtu* ssp. *ligtu*, *A. ligtu* ssp. *incarnata* and, *A. ligtu* ssp. *simsii* (Figs. 1 and 2) (Aker and Healy, 1990; Ishikawa *et al.* 1997). Bayer (1987) suggested the synonymous name of *A. ligtu* ssp. *ligtu* for *A. haemantha* Ruiz and Pavon. Our results support this hypothesis. *Alstroemeria exserens* was positioned between the Chilean group and the *A. ligtu* group (Fig. 2). *Alstroemeria andina* and *A. angustifolia* ssp. *angustifolia*, and *A. hookeri* ssp. *cumminghiana* and *A. hookeri* ssp. *hookeri* were clustered together with 95 % and 93 % bootstrap values, respectively.

The interspecific hybrid (A1P2-2) was included in our study in order to investigate the possibility of the identification of the parental genotypes. The F_1 hybrid A1P2-2 showed a 0.45-GD value with *A. inodora* and 0.59 GD value with *A. aurea* showing

genomic contribution of both parents (data available on request). It indicated the feasibility of the AFLP technique as a tool for the identification of parental genotypes (Sharma *et al.* 1996; Marsan *et al.* 1998). *Bomarea* and *Leontochir* showed the mean GD value of 0.83 as outgroup, thus showing large genetic distances within the Alstroemeriaceae family.

In conclusion, the genetic variation and the genetic relationships among *Alstroemeria* species were efficiently rationalized by using AFLP markers for the characterization of germplasm resources. In general, the topologies of the dendrogram and the principal co-ordinate analysis of our study were in agreement with Bayer's views (1987) on the classification of the *Alstroemeria* species. Furthermore, this technique might be useful for the identification of parental genotypes in interspecific hybrids.

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4 The construction of a linkage map of *Alstroemeria aurea* by AFLP markers

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Submitted

Abstract

An AFLP based linkage map has been generated for the ornamental crop species *Alstroemeria aurea*. In view of the large genome size of *Alstroemeria* (25,000 Mb) the number of selective nucleotides for AFLP amplification was increased to *Eco*RI+4/*Mse*I+4 to generate fingerprints of moderate complexity. In addition markers were generated with the enzyme combination *Sse/Mse*, where *Sse*8387I is an 8-cutter and thereby reducing AFLP template complexity. Segregation of 374 AFLP polymorphisms was recorded in the F_1 of an intraspecific *A. aurea* cross (A002 × A003). The map consisted of 8 A002 and 10 A003 linkage groups with 122 and 214 markers covering 306.3 and 605.6 cM, respectively. The two maps were integrated by using the 21% of the AFLP markers that were heterozygous in both parents, and 31% of the markers remained unlinked. Pollen color was assigned on linkage group A002-6. The enzyme combinations *Eco*RI+4/*Mse*I+4 and *Sse*+2/*Mse*I+3 generated 80 and 30 clear bands per lane with 16 and 9 polymorphic markers, respectively. Twenty percent of the *Eco*RI+4/*Mse*I+4 primer combinations resulted in fingerprints that were disturbed by a few excessively thick bands (55 primer combinations out of 288 primer combinations). We conclude that fingerprints and markers generated with the eight-cutter enzyme *Sse*8387I, in combination with +2/+3 selective nucleotides (*Sse*+2/*Mse*I+3) are superior to *Eco*RI+4/*Mse*I+4.

Keywords: AFLP, *Alstroemeriaceae*, double pseudo-testcross, linkage map, *Sse*8387I, pollen color

Introduction

Alstroemeria is a perennial cut flower that belongs to the family of *Alstroemeriaceae*. The *Alstroemeria* genus encompasses approximately 60 described species, all originating from Chile and Brazil (Uphof 1952; Bayer 1987; Aker and Healy 1990). The economic importance is illustrated by figures of the flower auction where *Alstroemeria* is ranked on the ninth position. The increasing popularity of *Alstroemeria* for growers and consumers as cut flower can be attributed to its extensive range of large and colorful flowers, its long vase-life and its ability to grow at low greenhouse temperatures. Recently the demand of *Alstroemeria* pot plants and garden plants on the market has been increased.

The cytogenetics of *Alstroemeria* species have been investigated well, because of the large size of the chromosomes (Kamstra *et al.* 1999). On the contrary, molecular genetic studies are complicated by the large genome size: 4C=107 pg (Bennett *et al.* 1998), which is equivalent to 27,000 megabase per haploid genome. Moreover, genetic studies are complicated by the heterozygosity due to the outbreeding nature of the species and the long generation cycle. Inbreeding depression prevents the generation of stable inbred lines and polyploidy of cultivars should be considered as well.

In recent years, DNA marker techniques have been applied for the identification of genetic relationships among *Alstroemeria* cultivars for instance by using RAPD markers (Anastassopoulos and Keil 1996; Dubouzet *et al.* 1997; Picton and Hughes 1997). The AFLP technique was successfully modified to accommodate the large genome size (Chapter 2) and subsequently applied to study the genetic diversity of Chilean and Brazilian *Alstroemeria* species (Chapter 3).

Linkage maps and marker assisted selection for quantitative characters and traits that are expressed in a late stage of plant development such as flower shape, number of flowering stems and vase life, would be useful tools in an ornamental breeding program. *Alstroemeria* cultivars are generally hybrids between wild species, or arise due to mutations or chromosome doublings of wild species. According to their morphological characteristics such as flower color, flower stem length and flowering time, *Alstroemeria* cultivars are referred to as Orchid, Butterfly and Hybrid types (Dubouzet *et al.* 1997). *Alstroemeria* is usually vegetatively propagated by micropropagation. Because of its ornamental value and relatively high micropropagation rate (Buitendijk 1998) the *Alstroemeria aurea* germplasm is the most commonly used species in *Alstroemeria* breeding programs especially in 'Orchid' types (Tsuchiya *et al.* 1987). The *Alstroemeria aurea* breeding lines are developed and maintained by crossing within similar morphologies, for instance the flower colour in the breeding companies. Nevertheless in view of the allogamous nature of this species, a high level of heterozygosity is expected. This was proven in our preliminary study that showed that enough genetic variations exist within *A. aurea* species based on the AFLP fingerprints of *A. aurea* accessions (Data not shown). Therefore we established an F_1 hybrid mapping population (N = 134) between two diploid *A. aurea* genotypes (A002 × A003; 2n = 2x = 16) in order to construct a linkage map. This type of analysis has been named as double pseudo-testcross or two-way pseudo-testcross analysis because polymorphic loci segregate from both parents involved in the cross (Grattapaglia and Sederoff 1994; Hemmat *et al.* 1994). This method has been used mainly for potato, forest and fruit trees, suggesting its applicability for genetic mapping in any highly heterozygous cross-pollinating plant.

The usefulness of AFLP markers for genetic mapping of outbred crop species has been demonstrated for potato (Van Eck *et al.* 1995), apple (Maliepaard *et al.* 1998), and rubber tree (Lespinasse *et al.* 2000), as well as for mapping a crop species with a large genome size such as onion (Van Heusden *et al.* 2000).

There are two ways to control the complexity of the AFLP fingerprints. One way is by increasing the number of selective nucleotides in the final PCR step (Vos *et al.* 1995; Chapter 2). A higher number of selective nucleotides will selectively amplify a smaller number of restriction fragments, which results in less complex fingerprints to be visualized on the autoradiogram. Our previous study showed that the large genome size of the *Alstroemeria* species resulted in very complex fingerprints with *Eco*RI+3/*Mse*I+3 primer combinations that are commonly used in species with a moderate genome sizes, whereas *Eco*RI+4/*Mse*I+4 resulted in fingerprints with 80-120 bands per lane (Chapter 2). The other way to reduce the complexity of the AFLP fingerprints is by replacing the six-cutter restriction enzyme with an eight-cutter restriction enzyme for the production of the primary templates (Zabeau and Vos 1993). In this study the ability of the eigth-cutter restriction enzyme *Sse*8387I (5'-CCTGCA$^{\downarrow}$GG-3') was tested to control fingerprint complexity, and used to generate markers for the map.

In this chapter the construction of genetic map for *Alstroemeria* is described on the basis of AFLP markers; one kind of AFLP markers is based on *Eco*RI/*Mse*I template with +4/+4 selective nucleotides and the other kind is based on *Sse*/*Mse*I template with

+2/+3 selective nucleotides. The utility of these different enzyme combinations for map construction is discussed.

Material and methods

Plant material for genetic mapping

Based on the level of polymorphism in a pilot study and the number of morphological differences, two *A. aurea* Graham accessions (A002 and A003) were selected and reciprocally crossed in summer 1997. Seeds were sowed individually in vitro and incubated at 4°C until germination. Germination was excellent, so that the progeny will represent an unbiased sample of male and female gametes. From 1998, 134 F_1 hybrids (63 from A002 × A003 and 71 from A003 × A002) were grown in the greenhouse. Fresh leaf samples were collected and stored in -80°C until use. Because both parents are equally used as male and female parents, instead of the term 'male' and 'female' linkage groups we refer to them as 'A002' and 'A003' linkage groups.

Phenotypic analysis

Pollen color was determined visually from the mature anther. Pollen with red and dark red was tentatively genotyped as 'ab' and the pollen with yellow color into 'aa' to allow inclusion of this morphological marker in the molecular marker data set. The pollen color of parent A002 and A003 was red and yellow, respectively. The 52 F_1 genotypes that did not flower were treated as missing values. Since we did not know which parent was heterozygous and thus responsible for the segregation of the trait, the pollen color data was included in both the A002 and A003 data set.

AFLP analysis

DNA isolation was performed according to Rogers and Bendich (1988). Primary template was produced according to Vos *et al.* (1995) using the restriction enzyme combinations *Eco*RI (6-cutter) and *Mse*I (4-cutter), and well as with the restriction enzyme combination *Sse*8387I (8-cutter) and *Mse*I. Pre-amplification of *Sse*8387I/*Mse*I derived primary template was performed with *Sse*+1/*Mse*I+1 or

Table 1. Overview of nucleotide sequences of adaptors and PCR primers for AFLP analysis

Restriction enzyme	Enzyme recognition site	Adaptor
*Eco*RI:	5'-G↓AATT C-3' 3'-C TTAA↓G-5'	5'-CTCGTAGACTGCGTACC-3' 3'-CTGACGCATGGTTAA-5'
*Sse*8387I	5'-CC TGCA↓GG-3' 3'-GG↓ACGT CC-5'	5'-CTCGTAGACTGCGTACATGCA-3' 3'-CATCTGACGCATGT -5'
*Mse*I:	5'-T↓TA A-3' 3'-A AT↓T-5'	5'-GACGATGAGTCCTGAG-3' TACTCAGGACTCAT-5`

Restriction enzyme	Number of selective nucleotides	Primer sequence
*Eco*RI:	+0	5'-GACTGCGTACCAATTC
	+1	5'-GACTGCGTACCAATTC|N
	+2	5'-GACTGCGTACCAATTC|NN
	+3	5'-GACTGCGTACCAATTC|NNN
*Sse*8387I:	+0	5'-TAGACTGCGTACATGCAGG
	+1	5'-AGACTGCGTACATGCAGG|N
	+2	5'-GACTGCGTACATGCAGG|NN
	+3	5'-CTGCGTACATGCAGG|NNN
*Mse*I:	+0	5'-GATGAGTCCTGAGTAA
	+1	5'-GATGAGTCCTGAGTAA|N
	+2	5'-GATGAGTCCTGAGTAA|NN
	+3	5'-GATGAGTCCTGAGTAA|NNN

Sse+2/*Mse*I+2 primer combinations. Final AFLP products were amplified with ^{33}P-radiolabelled primer combinations. Various numbers of selective nucleotides were tested on parental and ten progeny samples to study fingerprint complexity: *Sse*+2/*Mse*I+2, *Sse*+2/*Mse*I+3, *Sse*+2/*Mse*I+4, *Sse*+2/*Mse*I+5, *Sse*+3/*Mse*I+4 and *Sse*+4/*Mse*I+4. The first two primer combinations were tested on +1/+1 pre-amps, and the remaining primer combinations were tested on +2/+2 pre-amplified templates to avoid mismatches during PCR amplifications (Vos *et al.* 1995; Chapter 2). Fingerprints of desirable complexity were obtained with *Sse*+2/*Mse*I+2 and *Sse*+2/*Mse*I+3. In these fingerprints the AFLP bands observed in progeny samples were always related to bands observed in the parental lanes, whereas the other primer combinations resulted in random patterns. For map construction AFLP markers were generated with *Eco*RI+4/*Mse*I+4 and *Sse*+2/*Mse*I+3 primer combinations, using *Eco*RI+2/*Mse*I+2 and *Sse*+1/*Mse*I+1 pre-amplified templates (Vos *et al.* 1995; Chapter 2).

To facilitate the selection of the AFLP primer combinations that show a high number of unambiguous polymorphisms against the two parents 288 *Eco*RI+4/*Mse*I+4 and 28 *Sse*+2/*Mse*I+3 primer combinations were tested. As a result 17 *Eco*RI+4/*Mse*I+4 and 11 *Sse*+2/*Mse*I+3 primer combinations were selected and used to generate AFLP markers for mapping (Table 1 and 2).

The image analysis computer program CrossChecker was used to facilitate the scoring of JPEG scans of the autoradiograms (Buntjer 1999). Using CrossChecker individual plant and marker lanes were semi-automatically indicated, to allow computerized scoring of the absence and presence of the bands. The scoring results as performed by

the computer were verified with the results of a visual interpretation of the original autoradiogram image.

Marker nomenclature

Designation of names to AFLP markers was based on (1) the first letter of the restriction enzyme combination, (2) the letters of the selective nucleotides at the 3′ end of the primers, and (3) the mobility of the amplification products relative to the Sequamark 10-base ladder (Research Genetics, Huntsville, AL, USA). The letter i was included into the marker name when a 3:1 segregation ratio was observed due to heterozygosity in both parents.

Linkage analysis and Map construction

The absence and presence of a band was denoted as 'aa' and 'ab', respectively, for markers segregating from one parent only in an expected 1:1 ratio. For markers that were heterozygous in both parents the single dose presence of a band was denoted as 'ab' and the double dose presence was denoted as 'bb'. In most cases the intensity difference between heterozygous 'ab' or homozygous 'bb' could not be distinguished clearly by the intensity of the bands, and therefor the presence was converted into 'b-' leading to a 3:1 segregation ratio. The data set was converted by using computer software: Splitloc (http://www.spg.wau.nl/pv/pub/splitloc/) to generate JoinMap imput files for separate construction of parental linkage maps, as well as for the construction of integrated linkage maps based on the joint analysis for all marker types. The <ab×ab> markers serve as 'allelic bridge' markers because these markers allow to connect the independent parental maps of genotype A002 and A003. Linkage groups

were named with 'A002- and A003-' for parental linkage groups followed by a number.

Single-locus analysis, grouping of markers and mapping were performed with JoinMap version 2.0 which permits linkage analysis in outbred progenies involving markers with different segregation types (Stam 1993; Stam and Van Ooijen 1995). The Kosambi mapping function was used for converting the recombination fractions into map distances or into centiMorgan (cM) values. Total markers were first divided into linkage groups using a LOD score threshold of 4.4. The JoinMap 2.0 module, JMMAP32 was used to construct linkage maps based on pair wise recombination values obtained with threshold values for LOD ≥ 3 and for REC # 0.35. The order of the allelic bridges on corresponding A002 and A003 linkage groups was compared. In case of an unexpected discrepancy in order of the bridge markers along the A002 and A003 derived linkage group, the marker order of the '1:1' segregating markers only was used as 'fixed orders' to reconstruct the separate parental linkage groups. The order of bridge markers was then inferred manually, by inspection of the number of singletons between the 3:1 and 1:1 segregating marker in the separate A002 and A003 data set. (A singleton is a suspicious data point, because the marker allele is flanked by marker alleles derived from the other homologue, suggesting recombination events at both sides of that marker.) The most accurate order which can be obtained in this way was used as a 'fixed orders' to reconstruct the linkage groups. Consequently the order of the bridge markers coincided in both parental maps (Fig. 1).

Data quality confirmation

In linkage map construction, data quality is essential for the unambiguous estimation of the order of the markers. Scoring errors can result in serious distortions of marker order. However, in view of the generally accepted assumption that the occurrence of double recombinants in a short interval is biologically unlikely to happen due to negative chiasma interference, it is possible to identify suspicious data points (Hemmat *et al.* 1994). Therefore the computer software: Graphical Geno Types (GGT) was employed to display the data according to the marker orders of the map and to inspect the presence of singletons (Van Berloo 1999). After the initial calculation of the map, autoradiograms were re-checked for potential scoring errors, based on singletons displayed with GGT. In this way clear scoring errors were removed or when the quality of the autoradiograms did not allow unambiguous scoring a missing value was assigned. After improving the data, the maps were calculated again.

Results

The diploid parents of the *Alstroemeria* F_1 mapping population, A002 and A003 were screened with 288 *Eco*RI+4/*Mse*I+4 and 28 *Sse*+2/*Mse*I+3 primer combinations (data not shown). The majority of the *Eco*RI+4/*Mse*I+4 primer combinations generated AFLP fingerprints with around 80 clear bands per lane from which around 16 markers were polymorphic, whereas the *Sse*+2/*Mse*I+3 primer combinations generated around 30 clear bands per lane from which around 9 markers were polymorphic. Twenty percent of the *Eco*RI+4/*Mse*I+4 primer combinations resulted in fingerprints that were disturbed by a few excessively thick bands (55 primer combinations out of 288 primer combinations), possibly due to multi copy sequences in the genome. There was no sign

of such disturbance in the *Sse*+2/*Mse*I+3 primer combinations. Primer combinations were chosen on the basis of the high number of polymorphisms found between the parents of our mapping population.

Table 2. Numbers of segregating markers from parent A002, A003 and markers which are heterozygous in both parents, as detected by several *Eco/Mse*I and *Sse/Mse*I primer combinations.

	E+AATC	E+AATT	E+ACCA	E+ACCC	E+ACCG	E+ACCT
M+CGAA			0, 14, 4[1)]			3, 8, 1
M+CGAC				3, 6, 0		
M+CGAG				7, 8, 2		4, 5, 0
M+CGAT		8, 13, 6				
M+CGCA		6, 5, 4				2, 6, 1
M+CGCC			10, 18, 3			1, 3, 6
M+CGCG	11, 22, 11			3, 11, 0	2, 10, 4	2, 8,0
M+CGCT	4, 15, 3				2, 4,2	0, 2, 1
	S+AA	S+AC	S+AG	S+AT		
M+AAA	0, 1,0					
M+AGA		8, 8, 3		2, 0, 1		
M+AGC	3, 2, 2			3, 6, 2		
M+ACC		1, 3, 2				
M+ACT		2, 1, 3				
M+ATG			4, 1, 4	4, 6, 1		
M+ATT		2, 5, 7	5, 3, 4			

[1)] The order of numbers is A002 (ab × aa), A003 (aa × ab) and both (ab × ab).

In total, 274 AFLP markers were obtained from 17 *Eco*RI+4/*Mse*I+4 primer combinations, and 100 AFLP markers from 11 *Sse*+2/*Mse*I+3 primer combinations. Among these markers 48 and 30 markers were heterozygous in both parents (ab × ab) for these enzyme combinations respectively (Table 2). The number of markers which were heterozygous in parent A002 (68 and 35 markers from *Eco*RI+4/*Mse*I+4 and *Sse*+2/*Mse*I+3 primer combinations, respectively) was considerably lower than the number of markers segregating from parent A003 (158 and 35 markers for each enzyme combination).

Distorted segregation was observed in 36 marker loci ($P < 0.01$; Chi-square test) and these are indicated with asterisks in Fig. 1. The A002 and A003 alleles showed distorted segregation in 10 and 11 cases, respectively, whereas in 15 cases the 'ab × ab' type markers showed segregation distortion. Three regions with more than 3 markers that showed a distorted segregation were found on linkage groups, A002-4, A002-5 and A003-10. In case of A002-5 the segregation distortion occurred only in the 'ab × aa' segregation type markers while none of the 'ab × ab' markers showed the segregation distortion.

Map construction

The grouping of the markers into linkage groups was tested by stepwise lowering the LOD score. A stable grouping was obtained at a LOD score of 4.4 and resulted in eight linkage groups for parent A002 and 10 linkage groups for parent A003. For seven linkage groups the A002 and A003 homologues could be identified and aligned on the basis of the 3:1 segregating markers. One linkage group of A002 did not contain any 3:1 segregating marker, and consequently without such an 'allelic bridge' the corresponding A003 homologue could not be identified. The number of linkage groups in A003 exceeded the expected number, since *Alstroemeria aurea* has eight chromosomes. (2n=16). These three A003 chromosomes, without identifiable corresponding A002 homologue, did contain at least one 3:1 segregating bridge marker, but no other 1:1 segregating markers from A002 have been detected with linkage to these allelic bridges. As a consequence these markers remained unassigned to any linkage group during the grouping of A002 markers.

The order of bridge markers showed discrepancies between the maps of the A002 and A003 homologues, except for linkage group 2. In view of the statistical properties of the estimators with respect to the probability of detecting linkage between 1:1 and 3:1 segregating markers in non-inbreds, as described by Maliepaard *et al.* (1997), it is not surprising that such discrepancies are encountered. A simple method to obtain a reasonably accurate impression of the position of the 3:1 segregating allelic bridges is to minimize the number of singletons with flanking markers, and to compare the results obtained in each of the separate parental maps. The number of singletons is counted by using Graphical Geno Types (GGT) software. The most likely positions obtained by this approach are incorporated into JoinMap analysis via a 'fixed sequence file' to obtain, besides information on marker order, the results on the marker distances. The 21 markers, which could not be placed on the linkage map but belonged to the linkage groups were listed below the linkage map (Fig.1).

Map length, marker density and marker distribution

The total map length for A002 was 306.3 cM, and for A003 605.6 cM. This implies a fifty percent reduction of total map length of A002 in comparison to the total map length of A003. Considering the number of markers 122 and 214 in A002 and A003, respectively, this corresponds to an average density of one marker per 2.5 cM for the A002 map and one marker per 2.8 cM for A003 map. From this perspective both maps have an average marker density which is quite similar.

The distribution of markers generated with *Eco*RI/*Mse*I template was compared with the distribution of *Sse*/*Mse*I derived markers. There was no obvious difference in the distribution of markers of these two enzyme combinations along the linkage maps. In

many cases *Eco*RI/*Mse*I and *Sse*/*Mse*I markers mapped to the same locus without recombinations, for example E+AATT/M+CGAT-111, E+AATT/M+CGAT-135 and S+AC/M+AGA-142 on the linkage group A003-4. For some linkage groups clustering of AFLP markers was observed, such as in A003-4 and A003-9. A few widely spaced marker intervals were detected in the current map, for instance in A003-8 with 28 cM between E+ACCG/M+CGCG-124 and E+ACCC/M+CGAC-303.

Pollen color segregated in a 1:1 ratio (37 yellow : 45 red and dark red) and could be localized on A002-6 linkage group. The pollen color was positioned between E+ACCA/M+CGCC-I258 and S+AC/M+AGA-296 with 3.2 and 5.0 cM respectively. In view of the parental phenotypes A002 being red and A003 being yellow, it is assumed that A002 was heterozygous and that red is dominant over yellow.

Fig. 1. Genetic linkage map of *Alstroemeria aurea*. A002 and A003 linkage groups were established with LOD > 4.4 and were connected by allelic bridges indicated by lines. *Asterisks* indicate distorted segregation of markers (chi-squre test). **P = 0.01, ***P = 0.005, ****P = 0.001. ****P = 0.0005, ******P = 0.0001.

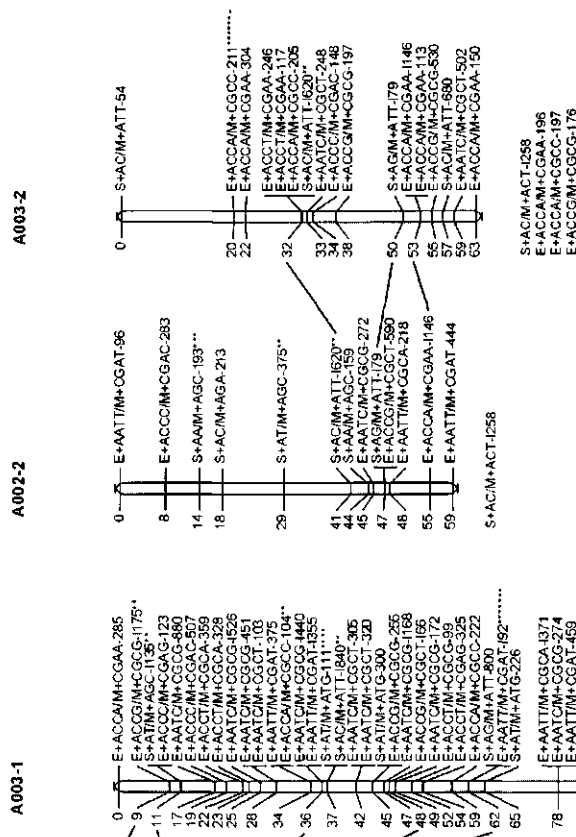


Figure 1, continued

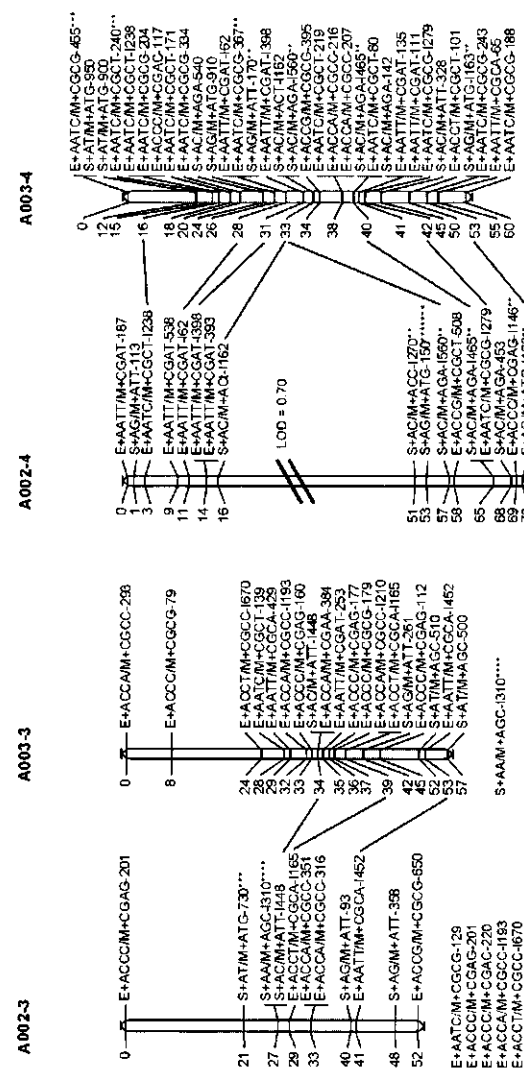


Figure 1, continuded

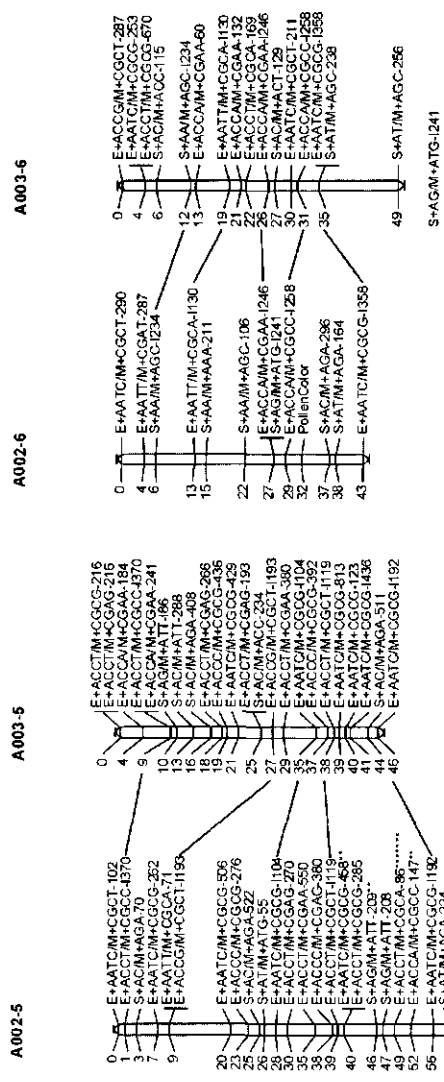


Figure 1, continued

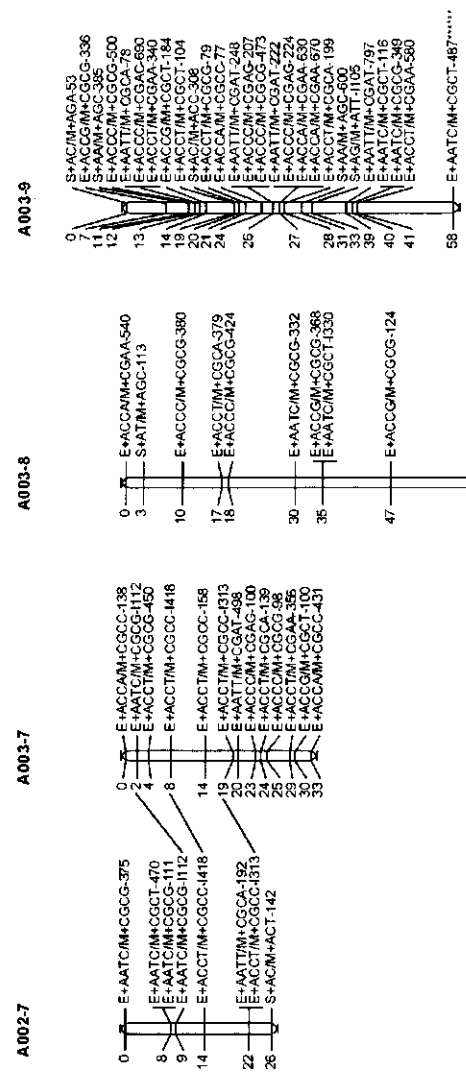
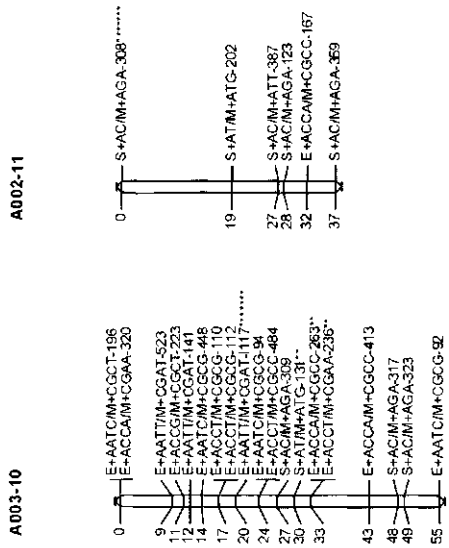


Figure 1, continued



Discussion

Marker detection

Large numbers of segregating AFLP markers could be found with the selected primer combinations the two enzyme combinations, used in this study: *Eco*RI+4/*Mse*I+4 and *Sse*+2/*Mse*I+3. To our knowledge this is the first application of *Sse*8387I in any AFLP mapping project. *Eco*RI+4/*Mse*I+4 and *Sse*+2/*Mse*I+3 primer combinations yielded on average 16 and 9 scorable and segregating polymorphic markers per fingerprint. The number of polymorphic markers generated with the *Eco*RI+4/*Mse*I+4 enzyme combination was approximately two times more than the number of polymorphic markers with the *Sse*+2/*Mse*I+3 enzyme combination. However considering the total number of bands per fingerprint, the level of polymorphism of *Sse*+2/*Mse*I+3 primer

combinations (30 %) was slightly higher than of *Eco*RI+4/*Mse*I+4 primer combinations (20 %). This higher level of polymorphism could be the result of the more clear fingerprints obtained by *Sse*+2/*Mse*I+3 primer combinations, where artifacts band due to mismatch amplification do not contribute to the fingerprint complexity. This higher level of polymorphism can not be explained on the basis of the number of nucleotides involved in the restriction enzyme recognition site and selective nucleotides from which polymorphisms can arise. For *Sse*+2/*Mse*I+3 this number is 8+2+4+3=17 whereas for *Eco*RI+4/*Mse*I+4 this number is 6+4+4+4=18. The chance that none of 17 nucleotides is different, is smaller than the chance that none of 18 nucleotides is different. Alternatively, the higher level of polymorphism in *Sse*+2/*Mse*I+3 fingerprints might be explained by the GC motif in the restriction enzyme recognition site. This motif is highly under-represented in plant genomes because of its sensitivity to mutation that might be due to structural constraints (Karlin and Mrázek, 1997), and mutation results in polymorphisms.

Another striking observation is that AFLP markers from both combinations are evenly distributed over the linkage groups, reflecting that the AFLP markers are truly random markers. The obvious clustering of *Eco*RI/*Mse*I markers as observed for many other species, due to centromeric suppression of recombination was not found. When the clustering is not obvious for *Eco*RI/*Mse*I markers, then it should be expected that *Sse*/*Mse*I markers are also well distributed. The recognition site of the eight-cutter restriction enzyme *Sse*8387I (5'-CCTGCA$^{\downarrow}$GG-3') includes the recognition site of *Pst*I which is well known for its uniform distribution.

Various numbers of selective nucleotides (*Sse*+2/*Mse*I+2, *Sse*+2/*Mse*I+3, *Sse*+2/*Mse*I+4, *Sse*+2/*Mse*I+5, *Sse*+3/*Mse*I+4 and *Sse*+4/*Mse*I+4) were tested with parents and ten progenies to reach fingerprints of moderate complexity (i.e. 70-100 band per lane). The first two primer combinations were tested on +1/+1 pre-amps, and the remaining primer combinations were tested on +2/+2 pre-amplified templates to avoid mismatches during PCR amplifications (Vos *et al.* 1995; Chapter 2). AFLP fingerprints with random patterns were obtained in latter four cases above, something which was only expected for *Sse*+2/*Mse*I+5 because of three selective nucleotides jump. Van Heusden *et al.* (2000) reported similar situation in *Allium* that has equally large genome size. In *Allium Eco*RI+4/*Mse*I+4 primer combination produced the fingerprints with rather random patterns, while *Eco*RI+4/*Mse*I+4 primer combination was used in *Alstroemeria*, as long as +2/+2 pre-amplified templates were used (Chapter 2). These two cases indicate that the AFLP system always needs some tests for optimization to determine the choice of the number of selective nucleotides for pre-amplification and fingerprinting within a certain species and with certain restriction enzymes.

The *Sse*+2/*Mse*I+3 enzyme combination has its own benefits as compared to the *Eco*RI+4/*Mse*I+4 enzyme combination. Firstly the simple fingerprints with around 30 clear bands per lane and less background rendered as easy scoring for the mapping population. Less background on the AFLP fingerprints might be due to less competition on the level of primary template. Theoretically, the fingerprint complexity with *Sse*+2/*Mse*I+3 should be four folds as compared to the fingerprint with *Eo*RI+4/*Mse*I+4. However, *Sse*+2/*Mse*I+3 resulted in better fingerprint patterns in terms of clarity and complexity, possibly because of the low frequency of the GC

motif mentioned before. Unfortunately the *Sse*+2/*Mse*I+3 fingerprints have nearly half of the polymorphisms as compared to *Eco*RI+4/*Mse*I+4 AFLP fingerprints.

In general the AFLP technique has claimed to be suitable for molecular discrimination at the species level because of its extraordinary capacity to generate polymorphisms within individuals having narrow genetic distances (Chapter 3). The result in this study indicated that the new enzyme combination, *Sse*8387I/*Mse*I, might be highly suitable for taxonomic classification in case of species with highly complex fingerprints, such as *Alstroemeria*, because of the more simple fingerprints, the clarity of the bands, less background and fewer polymorphisms. Secondly the window for the primer combinations in final PCR with *Sse*+1/*Mse*I+1 preamplification is more flexible than with *Eco*RI+2/*Mse*I+2 preamplication, and labour involved in one extra step of pre-amplification can be saved.

Thick bands as repeat and its use

Excessively thick bands were observed in 55 *Eco*RI/*Mse*I primer combinations out of 288 primer combinations. It disturbed the fingerprints by absorbing the radioactively labeled primer and never segregated. Several authors suggested that high copy sequences were over represented in the template competing with the single copy templates during PCR (Vos *et al.* 1995; Van Eck *et al.* 1995; Qi and Lindhout 1997). This pilot study testing parental genotypes with 288 *Eco*RI+4/*Mse*I+4 primer combinations confirmed the abundance of the repeats in *A. aurea* genome by observing twenty percent of primer combinations with excessively thick bands. In the study of barley Qi and Lindhout (1997) reported also the abundance of repeats with seven *Eco*RI+3/*Mse*I+3 primer combinations of the 96 *Eco*RI+3/*Mse*I+3 primer

combinations having the thick bands. These putative repeats shown as thick bands on AFLP fingerprints might be a good source to convert them as repetitive sequence probes, such as FISH probes (De Jeu *et al.* 1997). The absence of such thick bands in *Sse*/*Mse*I primer combinations might be due to the 8-cutter that has less chance to recognize sequences in repetitive DNA.

Map construction

This is to our knowledge the first report on the construction of an *Alstroemeria* map. The AFLP markers on this map have been named according to the primer combination and the mobility of the band. This allows other research groups to align future studies with this map, because of the locus specificity of AFLP markers (Rouppe van der Voort *et al.* 1997; Waugh *et al.* 1997).

The mapping effort resulted in eight A002 linkage groups and ten A003 linkage groups with over 300 markers including one morphological trait, pollen color. These maps were generated in an F_1 population descending from a cross between two accessions of *A. aurea*. The separate A002 and A003 parental maps were aligned by using bridge markers, except for four linkage groups. The use of F_1 populations immediately for mapping assumes that a high level of heterozygosity is already present in the parental genotypes. This assumption is generally true for outbreeders. However, on the basis of the results obtained here, we seriously doubt if this assumption is also valid for the specific genotype A002. We have observed a fifty percent reduction of the total map length of A002, as well as a fifty percent lower number of markers, as compared to A003. This difference is easily explained when the *A. aurea* accession A002 has been maintained for one generation by selfing before it was received by us. Usually the

context of *Alstroemeria*, the term "accession" refers to a single genotype which is maintained vegetatively. Selfing results in a fixation of 50 % of the genome, which corresponds with the reduced number of polymorphisms. Fixation of markers is not randomly distributed along the chromosomes, but is confined to certain (intervals on) chromosome arms or pericentric intervals. As a consequence the map will lack information of those arms and the length is shortened. Alternatively, in the case of a pericentric fixation the gap between the remaining heterozygosity on the chromosome arms may result into two short, independently behaving linkage groups. In the case that certain parts of the map are lacking, then the partial identification of homologous linkage groups via 3:1 segregating bridge markers is also explained. Because the latter is mostly the case for accession A003 (i.e. A003-8 A003-9 and A003-10), this genotype may as well have been maintained sexually. Addition of many more markers will not be a solution to bridge those gaps or to fill in the lacking arms, and therefor this map with its current marker density is presented as a final result.

Allelic bridges

For most of 'ab × ab' type markers, which are heterozygous in both parents, we scored the absence 'aa' or presence 'b-', because we were not able to distinguish between heterozygous 'ab' and homozygous 'bb' band phenotypes. Because of the conversion in ''b-', which turns a co-dominant marker into a dominant marker, the data are less informative. On the basis of the statistical properties of the estimators, large under- or overestimation of the pair-wise recombination frequencies (Maliepaard *et al.* 1997) should be expected, and JoinMap will easily assign a map position to a 3:1 segregating marker which is very different from its true position. For that reason it is stated that the method which is used in this paper, although it involves some hand work and

intelligent guesses, will result in an improvement of the marker order. Simulation studies on BC_1 populations have demonstrated the utility and power of the method of minimizing recombination events (Van Os *et al.* 2000). In this stage there is no reason to interpret the difference in marker order for 3:1 markers on the parental maps as a lack of co-linearity between the chromosomes of these *A. aurea* accessions.

Others have shown that AFLP markers could be scored co-dominantly (Van Eck *et al.* 1995), but feasibility depends on the average quality of the fingerprint in the laboratories. There is no guarantee that all laboratories will be able to produce the high quality fingerprints that allow scoring codominantly all the time. Therefore 3:1 segregating markers are generally given up as dominantly segregating markers and scored most of the times as 3:1 genetic ratio markers ignoring double intensity bands even after they were distinguished. We recommend converting the bands with clear double intensity from 'b-' to 'bb' within the acceptable fingerprint quality. This will add more statistical information to the 3:1 markers, and JoinMap easily handles mixed data of both 'ab' and 'b-' types (P. Stam, personal communication).

Comparison of AFLP templates

In this study two different restriction enzyme combinations were used (*Eco*RI/*Mse*I and *Sse*/*Mse*I) to generate AFLP templates. Evaluation of these different AFLP templates in terms of fingerprint clarity and ability to score the data unambiguously results in a recommendation of the use of the eight-cutter *Sse*8387I to control template complexity in large genome species, although we have optimised the protocol for *Eco*RI/*Mse*I in an earlier paper (Chapter 2). Evaluation of these different templates in terms of uniform distribution over the maps does not result in a clear difference

between *Eco*RI/*Mse*I and *Sse*/*Mse*I markers as both were uniformly distributed over the maps without severe clustering of markers except for A003-9. It suggests that neither of the enzyme recognition sites is restricted to particular chromosome regions.

In conclusion the present work provides a starting point for further molecular study of *Alstroemeria* genetics, leading to a genetic analysis of ornamental characters (i.e. pollen color) and the localization of quantitative trait loci. The potential utility of *A. aurea* map can be considerable because of the fact that the majority of *Alstroemeria* cultivars contain a genetic background of *A. aurea* species.

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5 Mapping of quantitative trait loci (QTL) involved in ornamental traits in *Alstroemeria*

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Abstract

An F_1 population, derived from an intra specific cross between two *Alstroemeria aurea* accessions, was used to identify and to map quantitative trait loci (QTL) involved in important ornamental and morphological characteristics. These traits comprise leaf morphology, the colour, size and shape of the flower, tepal stripe width, and productivity in terms of number of flowering stems and flowering period. Separate maternal and paternal linkage maps, well covered with AFLP markers, were used for QTL analysis using an interval mapping approach, unless when the trait value did not show a normal distribution. In the latter case the non-parametric Kruskal-Wallis test was used. Appropriate threshold values for declaring significant QTL effects were empirically obtained via a permutation.

Keywords: AFLP, *Alstroemeriaceae*, cut flower, interval mapping, leaf morphology, non-parametric Kruskal-Wallis test

Introduction

Alstroemeria is a cut flower, mainly originating from Chile and Brazil. It has very colourful flowers with a long vase life, and it is, therefore, a commercially important ornamental crop in the Netherlands. Like in all ornamentals, the morphological traits of flower and leaf are very important characteristics in *Alstroemeria* breeding programs. *Alstroemeria* accessions and cultivars are non-inbred and are maintained vegetatively. The large genome size of *Alstroemeria aurea* of 4C = 107 pg (Buitendijk *et al.* 1997), which equals to 25,000 Mbase, provides a limitation to several molecular requires. In an earlier study, we adapted the AFLP technique for *Alstroemeria* by increasing the number of selective nucleotides added to the core primers. This resulted in accurate and reproducible fingerprints despite the complex template (Chapter 2). By using a rare cutter (8-cutter: *Sse*8387I) for the preparation of primary template, a more simple template could be obtained, and reproducible fingerprints could be generated with the usual number of selective nucleotides (Chapter 4). Similarly, the AFLP technique was also applied in onion for the construction of linkage maps (Van Heusden *et al.* 2000) that has an equivalently 'large' genome size (30,850 - 33,500 Mbase) (Bennett *et al.* 1998). Recently, the AFLP marker technique was used for a biodiversity study of *Alstroemeria* species (Chapter 3) and for the construction of an *A. aurea* linkage map (Chapter 4).

Alstroemeria aurea is commonly used in cultivar development as one of the parents. If prior knowledge of the linkage relationships between marker loci and important ornamental characteristics of *A. aurea* were available, marker-assisted selection could be performed in seedling stages and used to eliminate undesirable individuals from progeny populations. Linkage relationships between molecular markers and

morphological traits have been studied in many crop plants such as potato (Van Eck *et al.* 1993; Van Eck *et al.* 1994a), wheat (Kato *et al.* 1999), sunflower (Gentzbittel *et al.* 1999), tomato (Grandillo *et al.* 1999), barley (Zhu *et al.* 1999) and cotton (Jiang *et al.* 2000). In ornamentals, flower traits such as petal number and flower colour have recently been mapped for the first time in rose by using RAPD and AFLP markers (Debener and Mattiesch, 1999). Till today no similar study has been reported in *Alstroemeria*.

The recent construction of the first molecular linkage map in *A. aurea* (accession A002 × accession A003) using AFLP markers (Chapter 4) facilitated the first QTL analysis in *A. aurea*. In this paper, data are presented on fourteen traits that are important in ornamentals. Subsequently, the inheritance of these traits has been selected by QTL analysis, to identify genetic loci responsible for the morphological variation in *A. aurea* accessions.

Material and methods

Plant material

A population of 134 F_1 individuals was obtained from an intra-specific cross of the *A. aurea* accessions; A002 × A003 (Chapter 4). The genotypes A002 and A003 showed distinctive morphological differences such as in flower colour; red and yellow respectively. Parents and the F_1 descendants were grown under the same greenhouse conditions.

Phenotypic analysis

134 individual F_1 plants were scored for fourteen morphological and ornamental traits as described in Table 1. The majority of these traits were chosen on the basis of the UPOV list of cultivar descriptors (Anonymous, 1987). UPOV characteristics that did not segregate were not studied, and characters that did segregate were included, although they were not part of the UPOV list. Obviously, the UPOV list includes traits that are very stable, highly heritable and not much affected by environmental factors. The genotypes that did not flower were treated as missing values unless it was specified. From each plant five mature leaves were taken from five individual vegetative stems to measure lengths (UPOV characteristic No. 4) and widths (UPOV characteristic No. 5) of the leaves, to calculate the length/width ratio of the leaves (UPOV characteristic No. 6) and to measure the length of the petiole (UPOV characteristic No. 7). Flower colour was dissected into three traits: (1) the main colour of complete flowers (UPOV characteristic No. 11), (2) the colour of the inner side of the outer lateral tepal (UPOV characteristic No. 16), and (3) the colour of the tip of the inner lateral tepal. Each of these traits was scored into five classes; yellow (A003), yellow-orange, orange, orange-red and red (A002). Flower size was recorded by measurements of the length of the outer lateral tepal (UPOV characteristic No. 14). Flower openness was measured into three classes, open (A002), intermediate and close (A003) (UPOV characteristic No. 13). This trait was defined as "open" when the distance between the tips of the tepals was large, "close", when the tepals were compact together without any space. Productivity was measured by counting the number of flowering stems. Plants that did not flower were scored as missing value. The flowering period was measured in terms of (1) date of appearance of the first and (2) last flowering stem, and (3) flowering period in days. Flowering period in days was

transformed by square root to achieve the normal distribution. The width of the stripes shown on the inner side of the inner lateral tepal was measured in two different flowers using a calliper rule when the flower was mature (UPOV characteristic No. 22).

Table 1. List of morphological and/or ornamental characters studied, the parental trait values and the trait number according to the UPOV guidelines.

Trait code	Trait name	Trait values of parental genotypes A002; A003	UPOV character No.
L1	Leaf length	9.99 ; 12.84 cm	4
L2	Leaf width	1.77 ; 1.65 cm	5
L3	Ratio of leaf length and width	5.67 ; 7.87	6
L4	Length of leaf petiole	2.05 ; 2.26 cm	7
C1	Main colour of complete flowers	Red ; Yellow	11
C2	Colour of inner side of outer lateral tepal	Red ; Yellow	16
C3	Tip colour of inner lateral tepal	Red ; Yellow	20**
F1	Flower size	3.92 ; 4.52 cm	12
F2	Flower openness*	Open ; Close	13
F3	Number of flowering stems	Not determined for the parents	--
F4	First date of flowering	June 4th ; April 10th	--
F5	Last date of flowering	June 28th ; June 15th	--
F6	Flowering period in days	2 days ; 268 days	--
S1	Stripe width of inner lateral tepal	0.83 ; 0.73 mm	22

* Trait analysed only by Kruskal-Wallis test.
** Modified UPOV character. Not middle zone, but tip colour of the inner lateral tepal was studied.

Genotyping and data analysis

AFLP segregation data and marker order on *A. aurea* linkage maps, used in this study for QTL identification, have been described previously (Chapter 4). QTL detection was based on separate parental data sets and non-integrated maps of A002 and A003. Each of the parental data sets does include some allelic bridge markers, which segregate in a 3:1 ratio. However, the position of these 3:1 markers on the A002 and

A003 linkage groups cannot be accurately determined (Maliepaard *et al.* 1997). As a consequence, the marker order on the integrated map will be also highly ambiguous, even if the marker order within each parental map would be fixed. Moreover, not all linkage groups could be integrated with a homologous group from the other parent. A more accurate integration of the A002 and A003 linkage groups would require at least co-dominant markers, and preferably allowing full classification such as SSR or RFLP markers. These are not available in *Alstroemeria*.

Average trait values and correlation coefficients between traits were calculated to detect putative relationships between various characters. Correlations were tested using the parameter free Spearman's rank correlation test (two sided, $P>0.01$).

Two analytical approaches were used to identify putative QTL and to estimate their phenotypic effects: the Kruskal-Wallis test (Kruglyak and Lander, 1995) and interval mapping (Lander and Botstein, 1989). For both analyses, the software package MapQTL® Version 4.0 (Van Ooijen, 2000) was used. Both interval mapping and Kruskal-Wallis test was used to analyse the metric traits, where the trait values represented a normal distribution. Trait values that represent an ordinal distribution, such as the five shades of colour, ranging from yellow to red, were also analysed with both interval mapping and Kruskal-Wallis test. Although these traits were ordinal, analysis of variance with marker class as explanatory factor showed that the residuals, and thus the trait, behaved more or less in accordance with standard analysis of variance assumptions. Therefore, the results of QTL analysis on these traits, as if it were truly quantitative, should allow sufficiently reliable conclusions. Flower openness was a discrete trait, and therefor analysed only by Kruskal-Wallis test.

In order to keep the probability of claiming one false-positive QTL below 5% in the *Alstroemeria* genome (8 chromosomes, approximately 305–605 cM), a probability level of $P<0.005$ was chosen for the rank sum test of Kruskal-Wallis, to declare a QTL significant (Van Ooijen, 2000). For interval mapping, a permutation test was used to empirically determine the significance threshold of the LOD score for each linkage group (Churchill and Doerge, 1994). The frequency distribution of the maximum LOD score was determined by 1000 permutations of the quantitative trait data over individuals while the marker data remained fixed. LOD scores that exceeded the 99% (or 95%) percentile of this distribution were declared significant.

Results

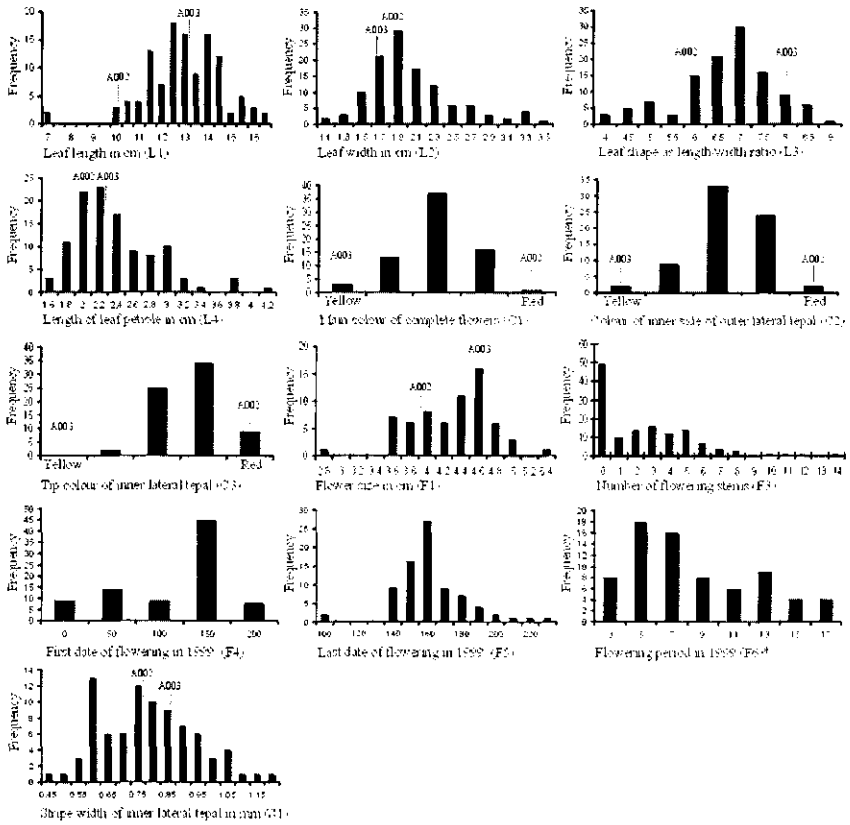
The distribution for the morphological and ornamental traits in the F_1 population is shown in Fig. 1, together with an indication of the parental trait values. These parental trait values are also listed in Table 1. For all traits, except for flower openness, the offspring trait values displayed a continuous distribution, not deviating from normality. None of the traits were correlated (data not shown) except for those cases where correlation was obvious. An obvious correlation should be expected between colour of the different parts of the flowers (e.g. C1, C2 and C3 were correlated), and correlation between the different aspects of leaf shape (L1, L2, L3 and L4).

Marker-trait associations are shown in Table 2 and Fig. 2. Interval mapping allowed the detection of three QTL exceeding the 99% significant threshold obtained by permutation. When allowing a 95% significant threshold, another 14 QTL were detected, but we prefer to call these putative QTL. The Kruskal-Wallis test allowed the

detection of four significant QTL at $P \leq 0.001$, and another four putative QTL at a significance threshold of $P \leq 0.005$.

Leaf traits QTL

Fig. 1 Histograms of genotype estimates for traits relating to morphological traits scored on individuals from the segregating population derived from 'A002 × A003'. Mean parental values are indicated with lines.



* The phenotypic values are transformed by square root

The parents of the F_1 population showed differences in leaf length and leaf width; A002 was shorter and wider (9.99 cm and 1.77 cm), whereas A003 was longer and narrow (12.84 cm and 1.65 cm) (Table 1). One QTL, involved in leaf length (L1) was mapped on linkage group three of both parent A002-3 and A003-3 near marker E+ACCT/M+CGCA-I165 which also segregates from both parents (Table 2 and Fig. 2). At the QTL peak position, near marker E+ACCT/M+CGCA-I165, 20.0% of the phenotypic variance could be explained using markers from linkage group A002-3.

Table 2. Position and nearest markers of QTL (bold) and putative QTL detected for morphological and/or ornamental traits, as detected by interval mapping (A) or Kruskal-Wallis test (B).

A (interval mapping)

Trait code	Linkage group[a]	LOD peak position (cM)	Nearest Marker	R^2(%)[b]	LOD	95%[c]	99%[d]
L1	**A002-3**	28.8	E+ACCT/M+CGCA-I165	20.0	**4.81**	2.9	3.7
L1	A003-3	39.0	E+ACCT/M+CGCA-I165	14.8	3.63	3.0	3.7
L2	A002-3	28.8	E+ACCT/M+CGCA-I165	13.8	3.42	3.5	4.9
L2	A002-6	37.7	S+AT/M+AGA-164	24.7	3.57	3.0	4.4
L3	A002-4	1.3	S+AG/M+ATT-113	12.8	2.59	2.4	3.2
L3	A002-4	15.9	S+AC/M+ACT-I162	11.2	2.79	2.4	3.2
L3	A003-4	39.7	S+AC/M+AGA-I465	13.8	3.45	3.3	3.8
C1	**A003-1**	47.1	E+AATC/M+CGCG-I168	27.8	**4.18**	3.4	4.1
C1	A003-2	62.6	E+ACCA/M+CGAA-150	32.2	3.57	3.1	4.5
C2	A003-9	26.5	E+AATT/M+CGAT-222	26.5	2.70	2.7	3.7
C3	A003-1	47.1	E+AATC/M+CGCG-I168	24.5	3.35	3.3	4.1
F1	**A002-5**	9.1	E+ACCG/M+CGCT-I193	31.6	**3.85**	3.0	3.8
F1	A003-2	38.1	E+ACCG/M+CGCG-197	31.9	3.06	3.0	3.8
S1	A002-2	47.1	S+AG/M+ATT-I79	15.5	2.97	2.8	3.6

B (kruskal wallis test)

Trait code	Linkage group	Marker position (cM)	Marker	K[e]	*P*-value
L3	**A003-5**	39.6	E+AATC/M+CGCG-123	**11.570**	0.001
L4	A002-1	6.9	E+ACCC/M+CGAG-900	10.414	0.005
C3	**A002-1**	42.5	E+ACCC/M+CGCG-93	**12.455**	0.0005
C3	**A003-9**	26.0	E+AATT/M+CGAT-248	**10.989**	0.001
S1	A003-4	54.8	E+AATT/M+CGCA-65	10.330	0.005
F2	A003-9	0.0	S+AC/M+AGA-53	10.514	0.005
F4	A002-7	8.9	E+AATC/M+CGCG-I112	8.714	0.005
F5	**A003-1**	58.7	E+ACCA/M+CGCC-222	**11.265**	0.001

[a]: Linkage groups as assigned in Chapter 4.
[b]: Percentage of variance explained.
[c]: LOD threshold values at 95% confidence level obtained by permutation test.
[d]: LOD threshold values at 99% confidence level obtained by permutation test.
[e]: Kruskal-Wallis test statistic (K).

The same estimate but based on markers from linkage group A003-3 explained 14.8% of the phenotypic variance. A marker that would allow full classification at this locus is desired to allow a more accurate estimate. This QTL had the effect to increase the leaf length when the marker alleles were homozygous absent.

Two putative QTL, near marker E+ACCT/M+CGCA-I165 and S+AT/M+AGA-164, were detected on leaf width (L2) on two different linkage groups, A002-3 and A002-6. These putative QTL explained 13.8% and 24.7% of the phenotypic variance, respectively. For both putative QTL the leaf width was smaller when the marker band was present.

The ratio of the leaf length and width (L3) was used to describe the shape of leaf. Three putative QTL, involved in the leaf length/width ratio were identified with interval mapping and one significant QTL, exceeding the $P \leq 0.001$ significance threshold of the Kruskal-Wallis test, was identified on linkage group 5 of parent A003. This Kruskal-Wallis detected QTL was not observed by interval mapping because it did not reach the 95% significance threshold. The three putative QTLs obtained by interval mapping were all positioned on linkage group 4; two QTL peaks were observed in parent A002, and a QTL on the homologous linkage groups of the other parent (Table 2). All four QTL together accounted in total for 46.7% of the phenotypic variance. No interaction was detected between the QTL of linkage groups 4 and 5 (data not shown). However, significant interaction was observed between markers S+AC/M+ACT-I162 and S+AC/M+AGA-I465 in a two-way ANOVA (data not shown). Full classification of this QTL locus by 1:1:1:1 segregating markers (or a well

integrated map of A002 and A003) is required to describe this QTL locus in greater detail.

For the length of the leaf petiole (L4) one putative QTL, near marker E+ACCC/M+CGAG-900 was detected on A002-1 only by Kruskal-Wallis test with 8.8% of the phenotypic variance. A short leaf petiole was associated with the presence of the band.

Flower traits QTL

The parents, A002 and A003, differed in their main flower colour, red and yellow, respectively (Table 1). A continuous range of different shades of colours between yellow and red was observed in the offspring. For the main colour of the flower (C1) we identified one QTL (exceeding 99% significance threshold) near marker E+AATC/M+CGCG-I168 on linkage group A003-1. In addition, one putative QTL (95% threshold) was identified near marker E+ACCA/M+CGAA-150, on the linkage group A003-2. The total variance explained by these two QTL sums up to 60%. This suggests that flower colour is under simple genetic control, because a major proportion of the phenotypic variance is explained by these two loci. Statistical analysis by a two-way ANOVA of the joint action of these two QTL showed a significant interaction component (F=5.56, P=0.0221) indicating epistatic interaction. This may also explain the discrepancy between the high amounts of explained variance, in contrast with marginally significant LOD scores.

A QTL near marker E+AATT/M+CGAT-222 was involved in colour of the inner side of outer lateral tepal (C2) with 26.5% of the phenotypic variance explained. The colour

of these outer tepals constitutes a major aspect of the main colour of the flower. Although there is no overlap between the QTL of the traits C1 and C2, marker E+AATT/M+CGAT-222 was also associated with main colour of the flower at a LOD of 2.65, which is just below the 95% threshold value (LOD=2.9).

Two QTL, near marker E+ACCC/M+CGCG-93 and E+AATT/M+CGAT-248, were detected with the Kruskal-Wallis test for the tip colour of inner lateral tepal (C3). The QTL detected by E+ACCC/M+CGCG-93 on linkage group A002-1 is possibly the same QTL as the one that was detected by interval mapping near marker E+AATC/M+CGCG-I168 on linkage group A003-1. This could suggest a single gene that is heterozygous in both parents. The QTL near marker E+AATC/M+CGCG-I168 on linkage group 1 is involved in two related traits, the main colour of complete flowers and tip colour of the inner lateral tepal. The QTL, near marker E+AATT/M+CGAT-248, for the tip colour of the inner lateral tepal was mapped very close to (0.5 cM) marker E+AATT/M+CGAT-222, associated with the colour of the inner side of the outer lateral tepal. Consequently flower colour traits were significantly correlated (data not shown).

A QTL for the length of the outer lateral tepal, representing flower size (F1), was mapped near marker E+ACCG/M+CGCT-I193 on linkage group A002-5 and a putative QTL near marker E+ACCG/M+CGCG-197 on linkage group A003-2. Each QTL explained 32% of the phenotypic variance (Table 2).

Flower openness segregated in the offspring, but the amount of variation in this offspring is rather subtle because the parents differ only slightly. The range of

variation is ranging between the values 5 and 7 of the UPOV list, which is typical for Chilean species such as *A. aurea*. The more cylindrical flower shapes, typical for Brazilian species should not be expected in this gene pool. In our case we did not attempt to classify the variation in the offspring in more than three classes: "close", "intermediate" and "open" resembling UPOV value 5, 6 and 7 respectively. A putative QTL, at the end of the linkage group marked by S+AC/M+AGA-53, involved in flower openness (F2), was detected on linkage group A003-9 by the Kruskal-Wallis test.

Several aspects of productivity have been studied. Productivity of cut flowers can be represented by the number of flowering stems. Therefore, the number of flowering stems was counted for during the flowering season. However, out of the 134 genotypes 49 did not produce any flowering stem. The trait value of a genotype that did not flower could be indicated with a 'zero' or it could be a missing value. Both options have been tested in QTL analysis and both options resulted in putative QTL. However, the putative QTL observed by interval analysis were not significant with the Kruskal-Wallis test, and vice versa. Moreover, none of these putative QTL was detected for both options. A third option to analyse this trait is to treat it as a binary character: Yes or No flowering. This did not result in the detection of any genetic locus. In view of these contradictory results, we do not feel confident to mention any of the putative QTL.

Another aspect of productivity, which is highly confounded with the number of flowering stems, is the flowering period. When more stems are produced, the period will also be longer. Three aspects of flowering period were studied: (1) first date of

flowering, representing earliness of the crop, (2) last date of flowering, representing the joint effect of number of stems and lateness of the crop, and (3) duration of flowering period, which is the interval between the first two traits. A putative QTL for first date of flowering (F4) was detected with marker E+AATC/M+CGCG-I112 on A002-7 with 7.7% of the phenotypic variance explained. A putative QTL marker for last date of flowering (F5) was marker E+ACCA/M+CGCC-222 on A003-1 with 13.3% of the phenotypic variance explained. No QTL were detected for duration of flowering period.

Stripe trait QTL

One putative QTL marker S+AG/M+ATT-I79 was detected for the stripe width of the inner lateral tepal (S1) on linkage group A002-2. This QTL accounted for 15% of the phenotypic variance, and wider stripes were associated with the presence of the band.

Discussion

In the offspring of the cross between *Alstroemeria* accessions A002 × A003 a large number of morphologically and/or ornamentally important traits segregated, resulting in highly diverse offspring genotypes. This rich genetic variation was studied by QTL analysis using AFLP markers mapped on separate parental linkage groups of A002 and A003. The range of phenotypes observed in the offspring is described by the frequency distributions of the trait values in Fig. 1., together with the parental trait values. For the morphological traits of the leaves: length, width, shape (length/width ratio) and leaf petiole length, flower size, stripe width and three aspects of flowering period, phenotypic values have been observed in the progeny that go beyond the parental values. These transgressive segregations are typical for the offspring of non-inbred

species, and result from heterozygosity in the parents for QTL alleles with positive as well as negative effects. In the case of flower colour traits (C1, C2 and C3) the parents showed extreme phenotypes, with offspring phenotypes ranging between the parental extremes. Nevertheless, extreme parental phenotypes did not result from fixation (homozygosity) at genes involved in yellow and red flower pigments, because homozygosity at these loci would have resulted in a uniform F_1. On the contrary, heterozygosity was present both in parent A002 and A003, as QTL involved in flower colour have been mapped on A002 and A003. Although the number of loci involved in flower colour does not seem large, a simple genetic model could not be deduced. On the basis of our results dominance relations between red and yellow could not be inferred. Some of the QTL were involved in more than one trait. For example the QTL at E+ACCT/M+CGCA-I165 on linkage group A002-3 was involved in L1 and L2, which suggests a pleiotropic effect of a single QTL. Similarly, on linkage group A003-9 two closely linked markers E+AATT/M+CGAT-222 and E+AATT/M+CGAT-248 each map a QTL involved in colour traits C2 and C3 respectively, also suggesting the presence of a single QTL with pleiotropic effect. The presence of two different QTL with close linkage is not very likely in these cases, because of the highly related leaf and colour phenotypes, respectively.

In this study, quantitative trait loci (QTL) for ornamental characters have been mapped on *Alstroemeria* linkage maps using AFLP markers mapped on non-integrated parental linkage groups of A002 and A003. It was observed that many of the QTL did not reach high LOD values, or did not reach highly significant K-values in the Kruskal-Wallis test. It was also observed that QTL detected by interval mapping were not often confirmed by the Kruskal-Wallis test and vice versa. One of the obvious explanations

is that our approach is not good. The phenotype is the joint result of the allele(s) derived from A002 and A003, and therefore, QTL should be mapped with the integrated map instead of the separate parental maps. Possibly some QTL would have reached much higher LOD scores when data from an integrated map were available, and possibly more QTL would have been detected. Although this is in principle true, unfortunately in practice this is not easily achieved. A large amount of co-dominant and multiallelic markers such as SSR or RFLP, resulting in a 1:1:1:1 segregation is required to obtain full classification of the offspring, and to obtain a good integrated map. In *Alstroemeria* such markers have not been developed, and moreover, it will not be easy to develop such single locus markers in view of the limitations posed by the very large genome size of *Alstroemeria*. The signal detection threshold for RFLP is at picogram amounts of DNA on a Southern Blot, but before reaching such amounts of target sequence, diluted in the huge genome, then probably the binding capacity of such a blot has been exceeded. The reproducibility of single locus PCR based markers such as SSR has to be demonstrated first in large genome species. Moreover, the development of SSR via the identification or enrichment of simple sequence repeat in DNA sequences is also hampered by the large genome.

The only markers that could be used for the integration of homologous A002 and A003 linkage groups were the 3:1 segregating AFLP markers. Maliepaard *et al.* (1997) have described the poor accuracy of the position of these 3:1 markers relative to the position of the 1:1 markers. The MapQTL option for cross-pollinators will use the information of the nearest flanking 1:1 markers to estimate the full genotypes at a certain position on the linkage group. In this way information of several 1:1 and/or 3:1 markers are used to compensate for the missing information, and allow to reach the power of a

1:1:1:1 segregating marker for QTL analysis. We assume that ambiguities in marker order in the integrated map will have such a negative effect on these estimated genotypes at a certain map position, that the power of interval mapping and the Kruskal-Wallis test will be affected severely. In that case the gain of power by integration of the map is lost by the inaccuracies in marker order.

Another explanation for the poor significance of the QTL could be found in the (effective) population size. Although 134 offspring genotypes have been genotyped and phenotyped, this number has not always been accomplished. The amount of missing values in the AFLP data is approx. 14 %, mainly caused by *Sse*8387I /*Mse*I markers. The *Sse*8387I /*Mse*I markers were applied on a subset of 90 genotypes only (one micro titre plate). For morphological / ornamental traits that were studied in the flower, a large number of missing values were caused by 49 genotypes that did not flower. On the other hand, the remaining 85 genotypes that did flower provided a population size that is not much lower than often seen in literature.

There are not many examples of QTL mapping in F_1 populations derived from non-inbreds. In many papers QTL are published where the majority of the QTL are just above the threshold. Grattapaglia *et al.* (1995) published QTL with LOD scores between 1.3 and 2.7 (one QTL at LOD = 5.8) in a 122 offspring. Byrne *et al.* (1997) published leaf area QTL detected with the *F*-test at significance levels of $0.045<P<0.009$ using a 118 population size. King and Maliepaard *et al.* (2000) found in an offspring of 152 genotypes LOD scores up to 14.8, but the majority of QTL had LOD values ranging between 3 and 4.5. From this perspective it seems that high LOD values are not easily reached in mapping populations derived from non-inbred parents.

This could be based on the high number of possible allele combination resulting from heterozygosity in both the male and female parent. Up to four different alleles can be accommodated on a single locus in non-inbred mapping populations, where alleles of intermediate genotypic value will not add to the power of detection of such a QTL. The common existence of series of multiple alleles at QTL loci was discussed by Groover *et al.* (1994) in *Eucalyptus* and by Van Eck *et al.* (1994b) in potato. Although the LOD scores were not very high, we have not been overly greedy to mention QTLs of questionable significance. Two analytical approaches have been used in this paper: The Kruskal-Wallis test and interval mapping. It is generally assumed that the Kruskal-Wallis test is a conservative test, not easily resulting in false positives. Because the test was performed on 10 and 12 linkage groups of A002 and A003 respectively, a stringent significance level for the individual tests of P=0.005 was used in order to obtain an overall significance level of about 0.05. To control the significance level for interval mapping we used the permutation test (Churchill and Doerge, 1994), implemented in MapQTL. Due to the massive amount of calculation, however, Van Ooijen (1999) described an alternative method of getting the significance threshold without permutation test. However, current computer capacity allowed us to calculate significance thresholds for each trait, taking into account the quality of the map and the trait values. In this study significance LOD thresholds ranging between LOD = 3.2 – LOD = 4.9 were obtained at the 99% confidence level. At this level we detected a total of 3 QTL on 3 linkage groups, and additionally 11 QTL on 9 linkage groups at the 95% confidence level.

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Fig. 2 QTL (solid bars or **) and putative QTL (hatched bars or *) associated with the traits are depicted along the linkage groups of the *A. aurea* map. QTL detected by interval mapping are indicated by a bar. The length of the bar equals the 1 LOD support interval. QTL detected by Kruskal-Wallis are indicated by one or two asterisk attached to the marker position.

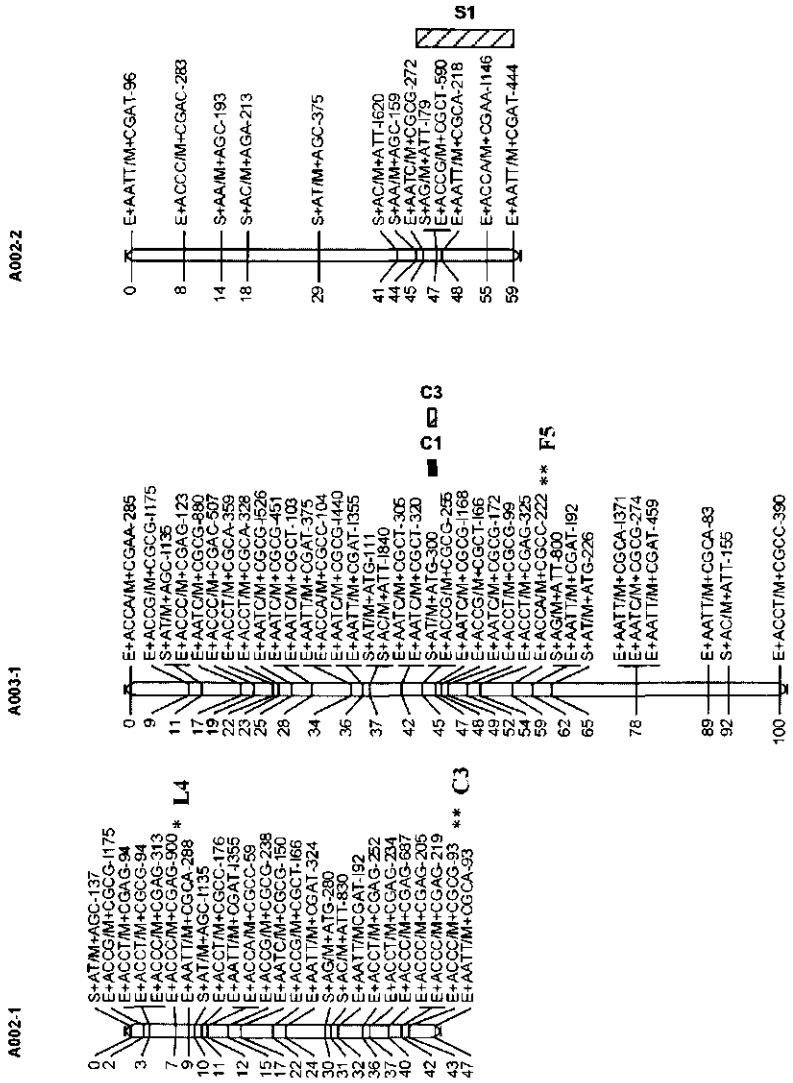


Figure 2, continued

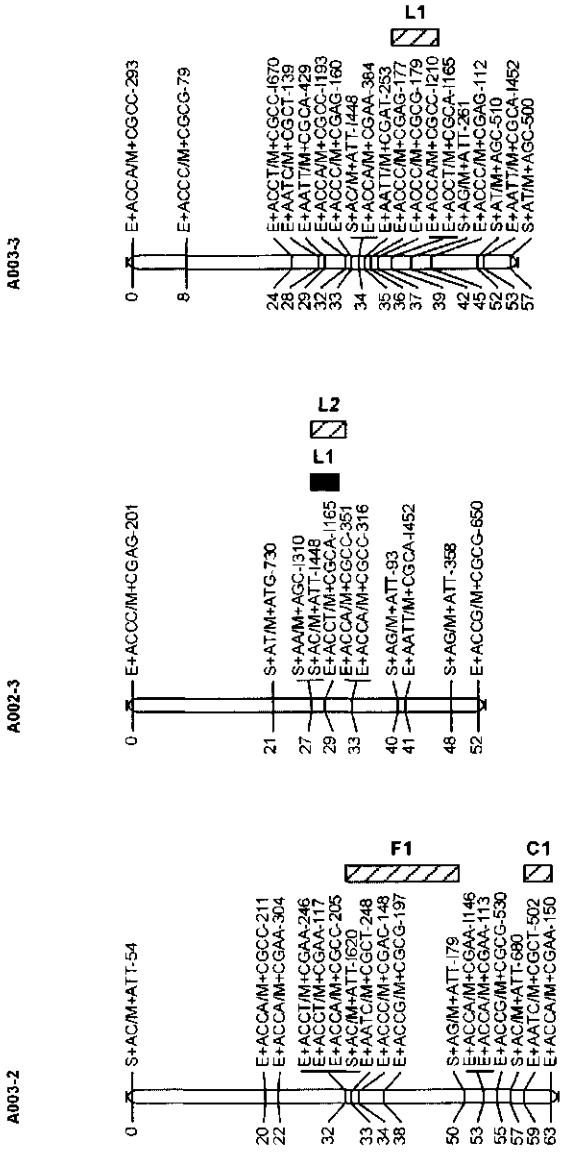


Figure 2, continued

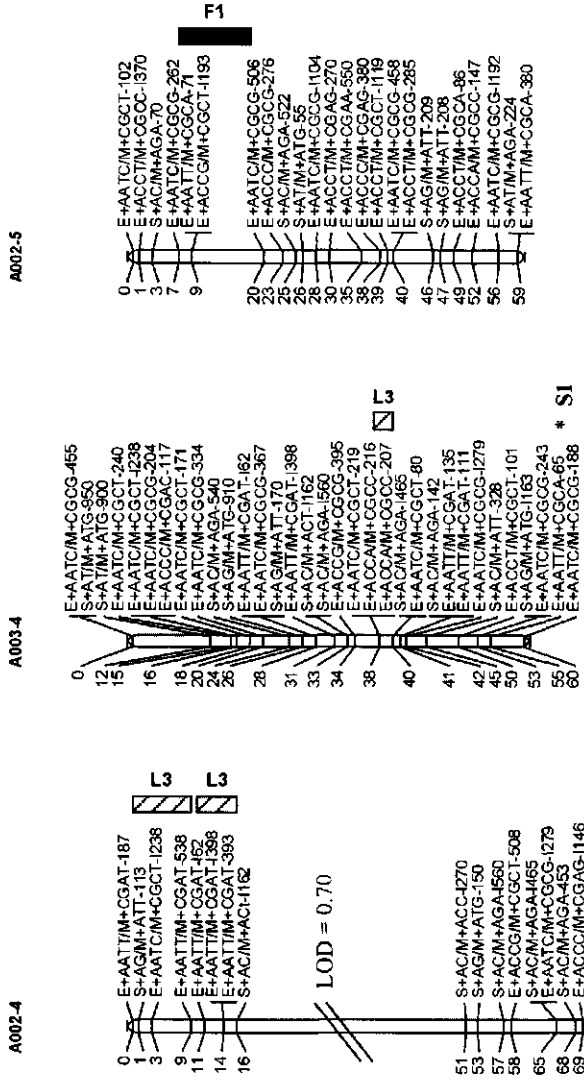


Figure 2, continued

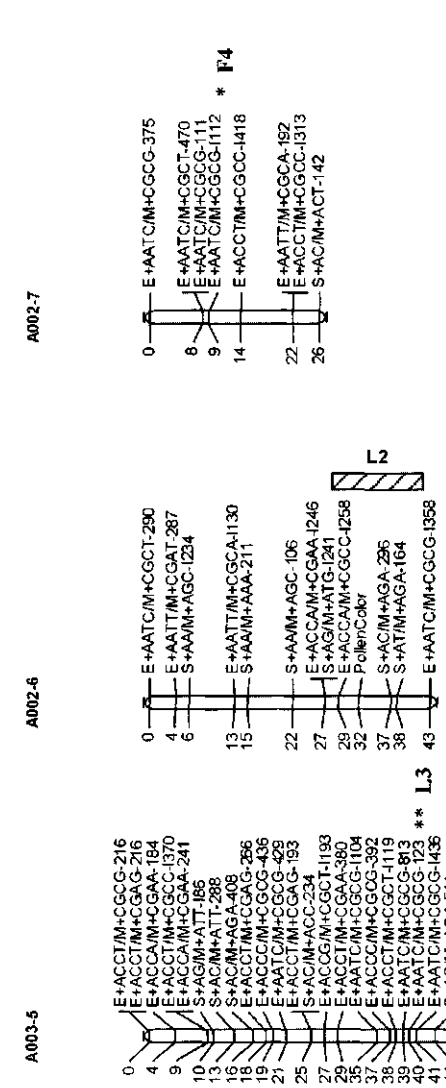
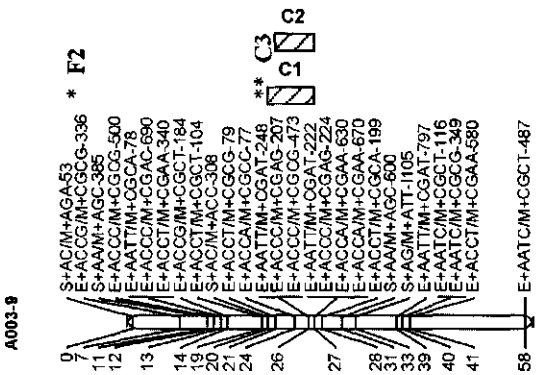


Figure 2, continued



6 General discussion

Genetic research in *Alstroemeria spp.*

This thesis describes the application of genetic marker techniques in *Alstoemeria spp*, and belongs to a series of research projects performed by the laboratory of Plant Breeding. Other thesis projects have focussed on the development of interspecific hybrids using in vitro techniques such as embryo rescue and ovule culture (Buitendijk 1998), studies on flowering biology (De Jeu *et al.* 1995), cytogenetic studies of *Alstroemeria* (Kamstra 1999), and the development of a protocol for genetic modification (Kim *et al.* in preparation). The general message of these research projects is not only to present results on the various issues mentioned above. In many cases the major obstacles in *Alstroemeria* research were related to adaptation of technology used on model species, to make it suitable for *Alstroemeria*.

Alstroemeria can be regarded as a recalcitrant crop, not only in the sense of difficulties in transformation. In this thesis we focussed on the application of genetic markers. The major obstacles to overcome were: (1) The non-inbred nature of the *Alstroemeria* accessions, (2) the long generation cycle, which hampers the rapid development of mapping populations, and (3) the large genome size, which pose limitations to the applications of various molecular genetic techniques.

Optimisation of AFLP for *Alstroemeria* spp.

Generally, the *Alstroemeria* species are diploid (2x=2n=16) and the nuclear DNA content (2C-value) is ranging from 37,000 to 79,000 Mbase (Buitendijk *et al.* 1997). The large genome size is a complicating factor for the development and use of molecular markers, either using DNA-DNA hybridisation or PCR amplification of specific DNA fragments. Subsequently, for the application of the molecular marker techniques in *Alstroemeria* this technical barrier had to be removed.

AFLP, a PCR-based marker technique (Vos *et al.* 1995), has been used for various studies like, the construction of linkage maps in rose (Debener and Mattiesch 1999) and *Rhododendron* (Dunemann *et al.* 1999), marker saturation at specific genomic regions in barley (Schwarz *et al.* 1999) and rice (Xu *et al.* 2000), molecular phylogeny in *Allium* (Van Raamsdonk *et al.* 2000) and cultivar identification in celery (Li and Quiros 2000). Although the previous studies were performed in organisms with a moderate genome size, Vos *et al.* (1995) indicated that the AFLP fingerprinting can be optimised for organisms with a larger genome size. This can be done in two directions: (1) by increasing the number of selective nucleotides during pre-amplification and/or during the final PCR step and (2) by using a rare cutting restriction enzyme (i.e. 8-cutter: *Sse*8387I) for the production of a more simple primary template mixture. In this thesis both methods were tested, optimised and confirmed (Chapter 2 and Chapter 4, respectively). The AFLP markers were subsequently used for the biodiversity study of *Alstroemeria* species (Chapter 3), for the construction of a genetic linkage map using an intra-species F_1 population of *A. aurea* (Chapter 4) and for QTL mapping of 14 ornamentally important traits (Chapter 5), using the population and maps mentioned in Chapter 4.

The AFLP technique had never been earlier published for an organism with a large genome size with exception of the work of Van Heusden *et al.* (2000) in onion. Two *Allium* species, *A. roylei* and *A. cepa*, were used in order to produce a mapping population. The genome size of the onion species used ranged from a 2C-value of 30,850 to 33,500 Mbase, respectively (Bennett *et al.* 1998). In onion, two enzyme combinations, *Pst*I+3/*Mse*I+3 and *Eco*RI+3/*Mse*I+4 were employed with a preamplification step, whereas *Eco*RI+4/*Mse*I+4 and *Sse*8387I+2/*Mse*I+3 were used in *Alstroemeria* (Chapter 4). Notably the complexity of the fingerprints with *Eco*RI+3/*Mse*I+4 for both species, *Allium* and *Alstroemeria* was analogous (Van Heusden, personal communication). Moreover, in onion *Eco*RI+4/*Mse*I+4 could not reduce the complexity of the fingerprints. Similar puzzlement was observed when *Sse*8387I+3/*Mse*I+3 was tested in *Alstroemeria*. Similar limitation in increasing the selective nucleotides was observed when *Sse*8387I+3/*Mse*I+3 was tested in *Alstroemeria*.

Two different reasons can be inferred for using less selective nucleotides in *Pst*I+3/*Mse*I+3 and *Sse*8387I+2/*Mse*I+3 compared to *Eco*RI+3/*Mse*I+4 and *Eco*RI+4/*Mse*I+4 in *Allium* and *Alstroemeria*, respectively. The *Pst*I restriction enzyme is methylation sensitive, which cuts only a subset of the genome. Therefore, it was expected that a lower complexity of the primary template would be obtained by using *Pst*I, the 6-cutter restriction enzyme, in comparison to *Eco*RI, also a 6-cutter restriction enzyme (Haanstra *et al.* 1999). It promises that less number of selective nucleotides than *Eco*RI+4/*Mse*I+4 can be used when *Pst*I would be employed for *Alstroemeria*. The *Sse*8387I restriction enzyme is an 8-cutter enzyme, which rendered

three less selective nucleotides than *Eco*RI, 6-cutter enzyme. Theoretically the enzyme combination of *Sse*8387I and *Mse*I with 6 selective nucleotides (8+4+6=18) in total should obtain clear and workable fingerprints, when compared by the 8 selective nucleotides (6+4+8=18) in *Eco*RI+4/*Mse*I+4. However, in practice the *Sse*8387I and *Mse*I combination used five selective nucleotides, and produced even simpler fingerprints than *Eco*RI+4/*Mse*I+4. In retrospect, we conclude that the properties of *Sse*8387I based AFLP template are superior than the properties of *Eco*RI+4/*Mse*I+4 derived template. Although fewer polymorphisms could be obtained, the clarity of the fingerprint image allows a greater accuracy during data collection. We are very much aware, that data accuracy is the most important criterion in scientific research.

Based on observations of various fingerprints with different selective nucleotides in the preamplification and final PCR steps, we concluded that none of the relations between (1) the number of selective nucleotides, (2) the enzyme combination, and (3) the genome size of an organism are linear. There is still an empirical fist-rule that AFLP fingerprints with less than 50 or more than 125 fragments are loosing accuracy. There is also a poorly understood process of competition between bands during the PCR reaction. There are some questions left about the signal capture system, where the non-linear dynamic range of autoradiography does not always to show the weaker bands. This is illustrated by pictures of a +3/+4 fingerprint in comparison with the single and the mix of the fingerprints of the same primer combination +A, +C, +G, +T. Consequently, investigators should perform a pilot study in other species before choosing the number of selective nucleotides and restriction enzymes.

Investigation of *Alstroemeria* biodiversity by AFLP

In the biodiversity study of *Alstroemeria* species (Chapter 3), three *Eco*RI+4/*Mse*I+4 primer combinations resulted in overall 716 markers. These autoradiograms contained highly complex fingerprints, and were thus extremely laborious to score manually. In chapter 4, *Sse*8387I+2/*Mse*I+3 and *Eco*RI+4/*Mse*I+4 produced around 30 and 80 bands, and on average 9 and 16 polymorphic bands in an *A. aurea* F_1 population, respectively. It demonstrated that *Sse*8387I+2/*Mse*I+3 produced considerably less complex fingerprints than *Eco*RI+4/*Mse*I+4. It is, therefore, conceivable that employment of *Sse*8387I and *Mse*I enzyme combination allows easier and less laborious scorings in the biodiversity study of *Alstroemeria* species.

In order to study biodiversity using molecular markers, ample number of polymorphic bands is prerequisite. The fingerprints of intra-species by using *Eco*RI+4/*Mse*I+4 showed relatively low amount of polymorphic bands, which resulted in narrow genetic distances. Although that *Sse*8387I+2/*Mse*I+3 produced fewer polymorphism compared to *Eco*RI+4/*Mse*I+4 (Chapter 4), it is expected that *Sse*8387I+2/*Mse*I+3 is still sufficiently polymorphic to study genetic diversity at or below the species level of *Alstroemeria*.

Construction of a genetic linkage map of *Alstroemeria* and QTL mapping

The mapping population has to be fertile for the future crossings in the breeding program. In case of inter-specific combinations, severe hybridisation barriers have been reported (De Jeu and Jacobsen 1995) and the majority of such progenies was sterile (Kamstra *et al.* 1999). To be able to create desirable and informative breeding lines, an intra-specific cross was performed between *A. aurea* accessions.

A. aurea has been commonly used in the breeding program as one of the parents, partly because of the resistance for *Alstroemeria* Mosaic Virus. For that reason, an *A. aurea* accession that is susceptible for *Alstroemeria* Mosaic Virus were searched, so that it can be used as one of the parental genotype in the mapping population. If one of the parents is heterozygous for the resistance alleles and the other parent is a homozygous susceptible plant, the resistance for the virus is expected to segregate in the F_1 population. In this way we were expecting to map the virus resistance gene, and find a molecular marker. However unfortunately an *A. aurea* accession, known to be susceptible for AMV could not be obtained. Under this circumstance, we selected *A. aurea* accessions, A002 and A003, which showed morphologically distinctive differences according to the UPOV list and from an ornamental point of view (Table 1 in Chapter 5).

The linkage map based on AFLP markers consisted of eight A002 and ten A003 linkage groups with 122 and 214 markers covering 306.3 and 605.6 cM, respectively (Chapter 4). The population size was 134 F_1 individual plants that allowed QTL mapping. Originally 38 traits were measured. Trait values that could be classified into discrete groups, should allow the mapping of the relevant loci via linkage analysis or via the Kruskal-Wallis test. The latter test was also used for ordinal traits, or trait without a normal distribution. QTL analyses were performed when the traits were continuous. The attempt to incorporate the traits as qualitative factors into the map was only successful in the locus for pollen colour on linkage group A002-6 (Chapter 4).

In this thesis, Kruskal-Wallis test and interval mapping has been applied to find QTL for ornamental and morphological traits. However results of QTL analysis showed that it is not easy to find major QTL. This can be ascribed to several reasons. (1) The lack of full classification and the lack of an integrated map will have reduced the detection power of QTL. In a cross between non-inbred parents, up to four different alleles can segregate and be responsible for the trait variation in *Alstroemeria*. The number of alleles that can be explained by using AFLP markers are only two. In the case of 3:1 segregating markers the information is even more reduced. In Chapter 4, the majority of marker types are consisted of 1:1 and 3:1 markers. A large amount of co-dominant and multiallelic markers such as RFLP or SSR are required to obtain full classification of four alleles. However in reality it will be extremely difficult to produce such markers due to the large genome size. As far as authors' knowledge, no such markers have been developed for organisms with a large genome size. (2) Non-integrated map, used in the current QTL analysis, only contains information of gametes, instead of zygotes. Therefore only two alleles at most can be explained even if co-dominant and multiallelic markers are applied. In our case only one allele could be explained based on dominant type markers. In order to integrate two parental maps, large amounts of informative markers such as RFLP and SSR are required, which was not the case as mentioned above. Consequently, we did not dare to integrate two parental maps based on 3:1 markers that have a poor statistical power (Maliepaard *et al.* 1997). (3) The population size is also a major factor to allow the detection of QTL. The current study included 134 individual plants. In comparison to other studies, our population size was fair. In other QTL studies based on non-inbreds, population sizes of 112, 118 and 152 offspring individuals were used in species *Eucalyptus* Grattapaglia *et al.* (1995) and Byrne *et al.* (1997), and in apple King and Maliepaard *et al.* (2000), respectively. Even

though the population size of approximately 100 individuals might be enough to find major QTL, even 250 individuals might not be sufficient to detect minor QTL as well as major QTL. However, increasing the number of individual plants in order to have sufficient statistical power will not be feasible in practice when experimental costs are considered. (4) Several replications by years and locations will reduce the experimental noise, because phenotypic values are biased by environmental influences. Unfortunately, the trait measurements were conducted only for season in one replication. This may have causing an unknown amount of noise in the phenotypic data. (5) Hereditability of the traits we studied might be low, so then consequently the LOD of QTL will be low. Again, the estimation of the heritability would require replications. (6) A technical problem can be encountered to handle the integrated map data for MapQTL® Version 4.0 (Van Ooijen 2000). Determination of the phase type, coupling and repulsion, will be extremely ambiguous due to 3:1 type markers.

Many QTL mentioned in Chapter 5 reached just above the threshold of permutation test, and the many QTL found by interval mapping were not detected by Kruskal-Wallis test and vice versa. Similar findings were obtained in the study of apple (King and Maliepaard *et al.* 2000). The only way to confirm minor QTL with low LOD scores will be repeating the phenotypic measurements by years and locations, and the results should be compared.

In case of interval mapping, the permutation test could provide empirical LOD thresholds. The computational time of the permutation test with 1,000 iterations took approx. four hours per trait with a Pentium III 450Mhz. We expect that the current speed of a PC is no longer a limitation for QTL studies. The growing awareness of

applying stringent QTL thresholds will improve the quality of scientific publications on QTL, by a reduction of false positives. In Chapter 5 we have tried to avoid the publication of false positive QTL, but it is not possible at this stage to substantiate this aspect.

Future research: Comparative mapping between Chilean and Brazilian linkage maps

Brazilian species, *A. inodora* (P002) and *A. psittacina* (D032) have been crossed, and subsequently *A. inodora* (P002) was crossed back to one of the F_1 hybrids (PD7) resulting in the 122 BC_1 individuals. This backcross population resulted in on average 21 % of polymorphism based on 10 primer combinations that generated 228 AFLP markers in total. Preliminary mapping studies showed that P002 and PD7 resulted in 4 and 11 linkage groups with 3.8 and 7.7 LOD thresholds, spanning 177 and 423 cM with 26 and 141 AFLP markers, respectively. More than 30 ornamentally important traits are under investigation with three replications (Han *et al.* in preparation).

Two kinds of comparative genomics can be investigated based on the genetic studies of Chilean and Brazilian *Alstroemeria* species, (1) comparing the genetic maps and (2) comparing QTL positions for comparable traits (Paterson *et al.* 2000). The assigned markers in both Chilean and Brazilian maps will be compared on the linkage groups. This kind of study might elucidate the genomic evolution (Livingstone *et al.* 1999) as well as geographical evolution between genetically distant species. Common QTL between Chilean and Brazilian species will also be compared. In a similar way, Osborn *et al.* (1997) could compare flowering time genes in *Brassica rapa*, *B. napus* and

Arabidopsis thaliana, and Grube *et al.* (2000) could compare disease resistance within the Solanacease.

In chapter 3, the genetic distance between Chilean and Brazilian species was determined on a genome-wide level by AFLP markers. Another genome-wide approach to study genetic distance is by cytogenetics. In the study of Kamstra *et al.* (1999), there was an indication of common evolutionary roots at the chromosome level between the Chilean and Brazilian genetically distant species by observing the paring of homoeologous chromosomes. In contrast to these genome wide approaches, comparative mapping of QTL and finding orthologous genes is being used, as well as the construction of gene-trees by DNA sequencing of e.g. ITS sequences.

The prospect of molecular markers in ornamentals

The marker technology has gradually been accepted in major crop breeding programs, and is regarded nowadays as one of the most important breeding tools together with conventional breeding methods. One of the main reasons to be accepted by the breeders is the shortening of the breeding period needed for the introgression of particular resistance genes from a wild type by backcrossing. Such marker-assisted backcross breeding programs in sexually propagated selfers facilitates the conventional breeding programs considerably.

In contrast to crop and vegetable breeding, ornamental breeding does not require intensive backcross programs to produce a cultivar enriched with particular genes. There are three reasons to explain this difference. (1) Many ornamentals are vegetatively propagated. (2) Any new phenotype that appears to be attractive can be

marketable. In *Alstroemeria* cultivar selection is still relatively easy in comparison with cultivar development in other crops. This also explains why *Alstroemeria* breeding does not heavily rely on genetic knowledge, and it should be expected that genetic data will not yet change the *Alstroemeria* breeding approach in the next decade. (3) The ornamental markets follow the contemporary fashion that distinctively differs from crop markets. The following questions arise. Where can the marker technology find a position in ornamental breeding programs? Are there traits on which you can earn back the costs involved in marker analysis? Ornamental breeders would not promptly seek a solution for traits such as disease or virus resistances as in vegetables with the help of marker technology. The first reason for such disinclination would be that most ornamentals are grown in greenhouses in which the environmental conditions are controllable in order to prevent any type of infections, as compared to field crops exposed to infections in every respect. The second reason would be the use of herbicides and insecticides, that are still allowed, since flowers are only decorative, not for consumption. Lastly, moderate resistance against viruses and diseases is unfortunately a desirable trait for the breeding companies to impede illegal vegetative propagations. As a result, traits related with high yield in a condition of energy-saving greenhouses, or with post-harvest quality might be good candidates for marker-assisted breeding. In addition, as Young (1999) proposed in his prospective article, the projects related with marker-assisted breeding have to utilize better scoring methods, larger population sizes, multiple replications and environments, appropriate quantitative genetic analyses, various genetic backgrounds and independent verification through advanced generations or parallel populations.

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Summary

This thesis describes the results of various applications of the AFLP technique in *Alstroemeria*. The aim of this study was 1) to adapt the AFLP technique for *Alstroemeria* species which has a large genome size, 2) to study the genetic diversity of *Alstroemeria* species of Chilean and Brazilian origin, 3) to construct genetic linkage maps of the *A. aurea* genome and 4) to map in *A. aurea* QTLs involved in ornamentally important traits.

The AFLP technique was adapted to obtain a method that produces clear fingerprints in *Alstroemeria*. We used PCR primers with two selective nucleotides (=*Eco*RI+2/*Mse*I+2) during preamplification before PCR amplification with 33P labelled primers with four selective nucleotides (*Eco*RI+4/*Mse*I+4) in the final step (Chapter 2). It was noticed that increasing the number of intermediate pre-amplifications was not preferable, because of the increased bias due to competition between fragments and the extra labour. The GC contents of the selective nucleotides had a significant influence on the number of bands. The primer combinations with GC residues in the selective nucleotides showed fewer bands per lane (Chapter 2). This result confirmed earlier observations that the *Alstroemeria* genome is AT rich.

In our study, the reproducibility of the AFLP technique for genetic analysis was verified. All bands in the fingerprints of offspring genotypes could be explained from the parental genotypes (Chapter 2). No PCR artefacts, mismatching and random priming had been detected in general, and reliable fingerprints of a species with a large genome such as *Alstroemeria* can be obtained with *Eco*RI+2/*Mse*I+2 preamplification and *Eco*RI+4/*Mse*I+4 for final amplification.

AFLP fingerprints were produced of 22 *Alstroemeria* species, one interspecific hybrid (*A. aurea* × *A. inodora*) and the distantly related species *Bomarea salsilla* and *Leontochir ovallei* as outgroup (Chapter 3). AFLP template of three accessions per species was mixed to obtain a more generalised fingerprint to represent an *Alstroemeria* species. Three primer combinations (E+ACCA/M+CATG, E+ACCT/M+CATC and E+AGCC/M+CACC), selected on the basis of their fingerprint quality, resulted into a data set of 272, 211 and 233 markers per primer combination.

The UPGMA dendrogram revealed three main clusters: the Chilean species, the Brazilian species and the outgroup. The principal co-ordinate plot revealed the same three groups, but additionally, the *A. ligtu* group was separated from the Chilean group. *A. aurea* was positioned between Chilean and Brazilian groups. The unique position of *A. aurea* suggests that other Chilean and Brazilian species may have evolved from *A. aurea* ecotypes (Tombolato, A.F.C., pers. comm.). *A. haemantha* Ruiz and Pavon was grouped with *A. ligtu* subsp. *ligtu*, *A. ligtu* subsp. *incarnata* and *A. ligtu* subsp. *simsii*. This confirms previous studies which assigned *A. haemantha* to the *A. ligtu* group. In the monography of Bayer on taxonomy of Chilean species suggested that *A. haemantha* and *A. ligtu* were synonymous names. Two species, *A. umbellata* and *A. pelegrina*, showed a genetic distance of only 0.26 GD, which is in the range of within-species genetic distances. The interspecific hybrid (*A. aurea* × *A. inodora*) showed a genetic distance of 0.45 GD and 0.59 GD with *A. inodora* and *A. aurea*, respectively. In the matrix of pairwise genetic distances these values were the lowest observed between the hybrid and any of the putative parental species. This example demonstrates that it

seems to be feasible to identify the parental species of an interspecific hybrid on the basis of genetic distance values.

An F_1 hybrid mapping population (N = 134) was established between two diploid *A. aurea* genotypes (A002 × A003; 2n = 2x = 16) in order to construct linkage maps. Over 374 polymorphic AFLP markers have been produced with 28 primer combinations (Chapter 4). Around 70 % of these polymorphic markers have been assigned to the linkage maps in either of the A002 and A003 parental map. As a result, these maps consisted of 8 and 10 linkage groups with 122 and 214 markers covering 306.3 and 605.6 cM, respectively. These differences between the two maps in terms of number of markers and total map length, indicates a different level of heterozygosity. This could have been caused by self-pollination for sexual maintenance of the accession by the breeders, leading to fixation on 50% of the genome. The two maps were integrated by using the F_2 type of AFLP markers. The pollen colour locus was assigned on the A002-6 linkage group.

We also tested another method to handle the complexity of the large genome. Instead of adding selective nucleotides to 6-cutter template we generated AFLP template with an 8-cutter restriction enzyme *Sse*8387I. Fingerprints generated with *Eco*RI+4/*Mse*I+4 primers produced around 80 clear bands from which around 16 markers were polymorphic, whereas the fingerprints generated with *Sse*+2/*Mse*I+3 primers produced 30 clear bands from which 9 markers were polymorphic (Chapter 4). On the one hand the simpler *Sse*8387I /*Mse*I fingerprints were more easy to evaluate, on the other hand a higher number of useful markers could be obtained with the *Eco*RI/*Mse*I fingerprints.

The previously established *A. aurea* linkage maps were used in order to map and characterise QTL for important traits, such as leaf morphology, the colour, size and shape of the flower, tepal stripe width, and productivity in terms of number of flowering stems and flowering period (Chapter 5). The majority of these traits were chosen on the basis of the UPOV list of cultivar descriptors. For all traits, except for flower openness, the offspring trait values displayed a continuous distribution, not deviating from normality. The transgressive segregations that phenotypic values of the progeny go beyond the parental values have been observed for the morphological traits of the leaves due to heterozygosity in the parents. Interval mapping and the Kruskal-Wallis test was performed to detect and to localize QTL, using separate parental data sets and non-integrated maps of A002 and A003. For interval mapping, a permutation test was used to empirically determine the significance threshold of the LOD score for each linkage group. This resulted in putative QTL with 95 % confidence threshold values ranging between LOD = 2.6 and LOD = 4.9, or in QTL with 99% confidence threshold values ranging between LOD = 3.85 and LOD = 4.81. In total 22 QTL for the traits studied were located throughout the map. The phenotypic variance explained by the QTL ranged from 11.2% to 32.2%. It was observed that many of the QTL did not reach high LOD values, or did not reach highly significant K-values in the Kruskal-Wallis test. It was also observed that QTL detected by interval mapping were not often confirmed by the Kruskal-Wallis test and vice versa. Probable explanations were discussed in Chapter 5. The overall conclusion is that, despite its large genome, the AFLP technology can be applied relatively easily in *Alstroemeria* for genetic and biodiversity studies.

Samenvatting

In dit proefschrift worden de resultaten beschreven van verschillende toepassingen van de AFLP- techniek in de snijbloem *Alstroemeria*. Het doel van deze studie was om:

1) de AFLP-techniek aan te passen voor toepassing in *Alstroemeria*, een plantensoort met een groot genoom
2) met behulp van deze aangepaste techniek de genetische diversiteit van Chileense en Braziliaanse *Alstroemeria* soorten te bestuderen
3) een genetische kaart te maken van de soort *A. aurea*
4) de QTLs betrokken bij belangrijke sierwaarde eigenschappen te karteren op de genetische kaart van *A. aurea.*

De AFLP- techniek is gebruikt als methode om heldere en duidelijke DNA-fingerprints te verkrijgen in *Alstroemeria* met zo min mogelijk (lab)arbeid. Voor de preamplificatie is de restrictie-enzym combinatie van *Eco*RI+2/*Mse*I+2 gebruikt, gevolgd door de restrictie-enzym combinatie *Eco*RI+4/*Mse*I+4 voor de actieve PCR (Hoofdstuk 2). Opvallend is dat bij toename van het aantal PCR- tussenstappen eveneens een toenemend aantal afwijkingen werd gevonden door competitie tussen de amplificatie van verschillende fragmenten. Bovendien levert het aantal tussenstappen extra (lab)werk op. Het aantal bandjes wordt tevens beïnvloed door de samenstelling van de selectieve basen: het aantal GC (Guanine-Cytosine) basen heeft een significante invloed op het aantal bandjes. 'Primer' combinaties met GC als selectieve basen geven een kleiner aantal bandjes per laan te zien (Hoofdstuk 2). Dit resultaat

wordt ook bevestigd door ander onderzoek waaruit naar voren kwam dat het *Alstroemeria* genoom veel AT (Adenine-Thymine) bevat (De Jeu et al.1997).

Vervolgens werd de reproduceerbaarheid van de AFLP techniek voor genetisch onderzoek geverifieerd. Alle amplificatieprodukten van de genotypen van de nakomelingen kunnen verklaard worden uit die van de genotypen van de ouders (Hoofdstuk 2). In het algemeen zijn er geen PCR –artefacten of aspecifieke amplificatieproducten gevonden. Onze conclusie is dat we betrouwbare fingerprints kunnen verkrijgen van een soort met een zeer groot genoom zoals *Alstroemeria* met de restrictie-enzym combinatie van *Eco*RI+2/*Mse*I+2 als preamplificatie, gevolgd door de restrictie-enzym combinatie *Eco*RI+4/*Mse*I+4 voor de actieve PCR.

We hebben AFLP fingerprints gemaakt van 22 *Alstroemeria* soorten, één hybride (interspecifieke hybride) tussen twee soorten nl *A. aurea* x *A. inodora* afkomstig uit onderzoek van Buitendijk et al (1995) en de ver verwante soorten *Bomarea salsilla* en *Leontochir ovallei* als referentiegroep (Hoofdstuk 3). Het DNA van drie planten van verschillende herkomsten van één soort *Alstroemeria* is gemengd om een representatieve fingerprint voor die soort te verkrijgen. Drie primer combinaties (E+ACCA/M+CATG, E+ACCT/M+CATC en E+AGCC/M+CACC) zijn geselecteerd op basis van de kwaliteit van de fingerprints. Deze drie combinaties leveren een aantal van respectievelijk 272, 211 en 233 amplificatieproducten op.

Het UPGMA dendrogram is uit deze gegevens geanalyseerd en toont drie hoofdgroepen van clusters: de Chileense soorten, de Braziliaanse soorten en de ver verwante soorten. De principale coördinaten-analyse levert dezelfde groepsindeling op,

maar daarbij is de *A. ligtu* groep bovendien nog gescheiden van de Chileense groep. *A. aurea* is apart geplaatst tussen de Chileense en Braziliaanse groepen. Deze unieke positie van *A. aurea* doet vermoeden dat andere Chileense en Braziliaanse soorten ontstaan kunnen zijn uit *A. aurea* ecotypes (Tombolato, A.F.C., pers.comm.). De soort *A. haemantha* Ruiz and Pavon is ingedeeld bij *A. ligtu* subsp. *ligtu*, *A. ligtu* subsp. *incarnata* en *A. ligtu* subsp. *simsii*. Dit bevestigt het resultaat van eerdere studies waarin *A. haemantha* ook is ingedeeld bij de *A. ligtu* groep (Aker and Healy, 1990; Ishikawa et al. 1997). Bayer (1987) geeft zelfs aan dat de namen *A. haemantha* en *A. ligtu* synoniem kunnen zijn voor één en dezelfde soort. Twee soorten *A. umbellata* en *A. pelegrina* verschillen van elkaar in genetische afstand van slechts 0.26.Deze afstand valt in de reeks van waarden van de genetische afstand binnen een soort. Vervolgens hebben we vastgesteld dat de plant die we als *A. umbellata* hebben verkregen eigenlijk een *A. pelegrina* plant was die gedurende vele jaren niet gebloeid had. De hybride tussen de soorten *A. aurea* x *A. inodora* vertoont een genetische afstand van 0.59 van *A. aurea* en 0.45 van *A. inodora*. In de matrix van paarsgewijze genetische afstanden zijn deze waarden de laagst waargenomen waarden tussen de hybride en elk van de mogelijke oudersoorten. Dit betekent dat het mogelijk is om de oudersoorten van een interspecifieke hybride vast te stellen op basis van de waarden van de genetische afstand.

Voor het maken van een genetische kaart hebben we een F1-hybride populatie gemaakt (N=134) uit de kruising tussen twee diploide *A. aurea* genotypen (A002 x A003; 2n=2x=16). Met de toepassing van ongeveer 28 'primer' combinaties zijn in totaal meer dan 374 AFLP merkers verkregen (Hoofdstuk 4). Ongeveer 70 % van deze merkers hebben een plaats gekregen op de genetische kaart van de ene (A002) of de

andere (A003) ouder. De genetische kaart van A002 bevat 8 koppelingsgroepen met 122 merkers die een totale lengte van ongeveer 300 cM beslaan. De kaart van A003 bevat 10 koppelingsgroepen met 214 merkers die een totale lengte van ongeveer 600 cM beslaan. De verschillen tussen beide genetische kaarten in aantallen merkers en totale kaartlengte duiden op een verschillend niveau van heterozygotie tussen beide ouders. Dit kan het gevolg zijn van het jaren achtereen maken van zelfbevruchtingen door de veredelaars om het plantmateriaal in stand te houden, waardoor een hoge mate van homozygotie is opgetreden, bij de ene ouder meer dan bij de andere ouder. Integratie van beide kaarten kan gedaan worden op basis van de zogenaamde F_2-type AFLP merkers die bij beide ouders voorkomen. De merker voor de kleur van het stuifmeel is gevonden in de A002-6 koppelings groep.

Fingerprints die gemaakt zijn met *Eco*RI+4/*Mse*I+4 primer combinaties leveren ongeveer 80 duidelijke amplificatieproducten op, waarvan er 16 als merkers aangeduid kunnen worden. Fingerprints die gemaakt zijn met behulp van *Sse*+2/*Mse*I+3 primers leveren ongeveer 30 duidelijk scoorbare amplificatieproducten op, waarvan er 9 als merkers kunnen worden onderscheiden (Hoofdstuk 4). Het percentage merkers dat daadwerkelijk geplaatst kan worden op de genetische kaart is voor beide primer combinaties hetzelfde namelijk 70 %. De *Sse*+2/*Mse*I+3 primer combinaties leveren eenvoudigere fingerprints op die gemakkelijk gescoord kunnen worden. De *Eco*RI+4/*Mse*I+4 primer combinaties leveren echter meer bruikbare merkers op.

De genetische kaarten van *A. aurea* zijn eveneens gebruikt om de plaats van QTLs (Quantitative Trait Loci) voor belangrijke eigenschappen te bepalen en deze QTLs te karakteriseren. Belangrijke kwantitatieve kenmerken die gescoord zijn zijn: vorm van

het blad, bloemkleur, bloemgrootte, stand van de bloemblaadjes (open of gesloten bloemvorm), vroeg of laat (in het seizoen) bloeitijdstip en breedte van de strepen op de bloemblaadjes (Hoofdstuk 5). Al deze eigenschappen vertonen een continue en bijna normaalverdeling, waardoor het kwantitatieve karakter kon worden vastgesteld. Alleen één eigenschap nl. bloemvorm (open of dicht) vertoont deze normaalverdeling niet. Voor beide genetische kaarten van A002 en A003 is gekeken of QTLs van de bovengenoemde eigenschappen geidentificeerd konden worden met behulp van 'interval-mapping' en de Kruskal-Wallis test. Duizend herhalingen van de permutatietest zijn uitgevoerd om de drempelwaarde van het karteren van een QTL te bepalen. In totaal werden 26 QTLs geidentificeerd.De verklaarde fenotypische variantie (PVE) van de bestudeerde eigenschappen is bepaald en varieerde van 7.7 tot 93.9 %. Een paar QTLs waren niet goed onderscheidbaar. Met behulp van de F_2-type merkers zijn de genactiviteiten binnen een locus bestudeerd. Vijf QTLs vertonen partiële dominantie en vier overdominantie. Tussen drie QTLs, die bij bloemkleur betrokken zijn, is een interactie tussen de loci waargenomen, waarbij zowel dominante als recessieve epistasie is vastgesteld.

적요 (摘要)

本 論文은 AFLP 마커 테크닉을 *Alstroemeria* 에 처음으로 適用한 硏究內容을 技術하고있다. 따라서 이 論文은 4 가지의 主題로 간추려진다. 1) *Alstroemeria* 의 큰 게놈 사이즈로 인하여 AFLP 테크닉에 약간의 變更을 했고, 2) 칠레産과 브라질産의 *Alstroemeria* 種들 간의 遺傳的 關係를 밝히고, 3) *A. aurea* 게놈을 바탕으로 하여 遺傳子 地圖를 만들고, 4) *A. aurea* 에서 花卉 측면에서 중요한 특성들과 聯關이 있는 遺傳的 要素 즉 QTL 을 찾아냈다.

약간의 補完을 거친 AFLP 테크닉으로 *Alstroemeria* 를 材料로 使用하였을 경우 깨끗하고 정확한 遺傳子指紋을 얻을 수 있었다. Preamplification 段階에서는 각 primer 에 2 개씩의 selective nucleotides (=*Eco*RI+2/*Mse*I+2)을 適用하였으며, 最終 PCR 段階에서는 4 개씩 의 selective nucleotides (*Eco*RI+4/*Mse*I+4)을 使用하였다 (2 장). 여러 번의 preamplification 은 500bp 이상의 밴드들을 잃었으며, 더 많은 勞動力 또한 要하기 때문에 바람직하지 않았다. 이유는 selective nucleotides 의 GC 염기비율은 총 밴드 수에 影響을 끼쳐, 比率이 높을 경우 平均値보다 적은 밴드가 나왔기 때문이다. (2 장). 따라서 이 結果는 *Alstroemeria* 의 게놈의 더 높은 AT 염기비율이 證明되었다.

다음으로는 AFLP 테크닉이 큰 게놈에 適用되었을 때 再現性에 대하여 遺傳的으로 확인 하였다. 모든 子孫들에서 나온 밴드들은 父母들에서 모두 다시 檢出되었다 (2 장). PCR 上의 技術的인 問題는 일반적으로 解決이 되었으며,

이는 preamplification 은 *Eco*RI+2/*Mse*I+2 로 하고, 最終 PCR 은 *Eco*RI+4/*Mse*I+4 로 함으로써 얻어질 수 있었다.

AFLP 遺傳子 指紋을 使用하여 22 *Alstroemeria* 種들과, 種間 雜種 (*A. aurea* × *A. inodora*), 그리고 原種인 *Bomarea salsilla* 와 *Leontochir ovallei* 을 外集團으로 使用하여, 그들 간의 遺傳的 相互關係를 규명하였다 (3 장). 같은 種에서 3 植物을 先發하여 AFLP template 를 섞은 標本을 각 *Alstroemeria* 種들을 代表하도록 하였다. 이에 따라 3 개의 primer 조합 (E+ACCA/M+CATG, E+ACCT/M+CATC and E+AGCC/M+CACC)들이 쓰였으며, 각각 272, 211 and 233 밴드들을 生産하였다.

UPGMA 系統手相圖는 3 개로 크게 나뉘어졌다: 칠레産, 브라질産, 그리고 外集團으로 나뉘어졌다. Principal co-ordinate 系統圖는 위와 마찬가지로 3 개의 基本 集團들에 *A. ligtu* 集團이 칠레産 集團에서 분류되어졌다. 칠레産과 브라질産의 사이에 위치한 *A. aurea* 種은 *A. aurea* 種이 칠레産과 브라질産의 原從으로 推測 되었다 (Tombolato, A.F.C., pers. comm.). *A. haemantha* Ruiz and Pavon 種은 *A. ligtu* subsp. *ligtu*, *A. ligtu* subsp. *Incarnata* 와 *A. ligtu* subsp. *simsii* 種들과 함께 속하였으며, 이는 Bayer 의 分流學的 견해와 일치하였다. *A. umbellata* 와 *A. pelegrina* 의 遺傳距離는 0.26 으로 아주 가까워, 이는 같은 種에 속하는 듯 하였다. F_1 終刊 雜種 (*A. aurea* × *A. inodora*) 은 각 父母種 (*A. inodora*, *A. aurea*) 과 0.45 GD 그리고 0.59 GD 으로 다른 種들에 비해서 가장 근접한 유전거리를 보여, 父母種임을 입증하였다. 이런 예는 마커테크닉을 이용하여 계산된 유전거리가 父母種을 밝혀낼수있는 可能性을 시사하였다.

二倍體 *A. aurea* F_1 種內雜種集團 (개체수 = 134; A002 × A003; 2n = 2x = 16)을 遺傳子地圖와 遺傳的 考察을 위하여 만들었다. 28 개의 primer 조합을 통하여 374 이상의 AFLP 마커를 얻을 수 있었다 (4 장). 약 70 % 정도의 마커들은 A002 와 A003 遺傳子 地圖에 지정되었다. 각 A002 와 A003 에는 8 그리고 10 개의 遺傳子 그룹이 122 와 214 마커들로 이루워졌으며, 이는 306.3 와 605.6 cM 의 遺傳子地圖 距離를 나타냈다. 이러한 두 遺傳子 地圖들의 마커 갯수나 遺傳子 地圖 길이의 차이는 A002 와 A003 간의 雜種性에 차이가 있다고 보여진다. 이와 같은 현상은 育種會社에서 관리를 하는 동안에 라인維持側面에서 自家受精을 통한 50%정도의 게놈의 固定이 이루워진걸로 推定된다. 이 두 遺傳子 地圖는 F_2 타입의 AFLP 마커를 사용하여 統合되었다. 아울러 꽃가루 색깔에 관련된 게놈에서의 위치는 遺傳子 地圖에서 A002-6 그룹에 속해있었다.

AFLP 의 遺傳子指紋의 鮮明度나 簡便度를 조절할 수 있는 또 하나의 방법은 6 - 制限酵素 대신에 8- 制限酵素 (*Sse*8387I) 를 사용함으로써 얻을 수 있었다. *Eco*RI+4/*Mse*I+4 의 遺傳子指紋은 약 80 개의 밴드들과 16 개의 다형의 마커들이 나오는 반면에, *Sse*+2/*Mse*I+3 에서는 약 30 개의 밴드들과 9 개의 다형의 마커들로 구성되었다 (4 장). 한편으로는 간단한 *Sse*8387I /*Mse*I 遺傳子 指紋은 檢索, 判斷하기에 쉬운 반면, 좀 더 많은 양의 정보를 보여주는 *Eco*RI/*Mse*I 遺傳子 指紋은 나름대로 長點이 있다.

위에서 만들어진 遺傳子 地圖는 花卉에 중요한 形質에 관련된 QTL 을 찾는데 쓰였으며, 형질로는 잎 형태, 꽃 색깔, 크기, 모양, 꽃의 生産量 등이었다 (5 장).

대부분의 이러한 形質은 國際 育種協會에서 判斷基準으로 쓰이는 UPOV 목록을 참조하였다. 꽃 開閉程度를 나타내는 形質을 빼고는, 다른 形質들은 모두 標準分布에 가까웠다. 父母種의 雜種性에 기인하여 많은 形質들의 표준분포는 父母의 平均形質 값을 超過하는 경우가 발견되었다. 각 A002 와 A003 에서 QTL 을 찾기 위해서 Interval mapping 와 Kruskal-Wallis 테스트들이 쓰였다. Interval mapping 의 경우에는 LOD 의 有意性 값을 찾기 위해 permutation 테스트를 각 遺傳子 地圖 그룹에 실행을 하였다. 결과로 95 % permutation 테스트에서는 잠정적인 QTL 을 LOD = 2.6 와 LOD = 4.9 사이에 얻었으며, 99 % permutation 테스트 결과로는 LOD = 3.85 와 LOD = 4.81 사이에 얻었다. 전체 22 개의 QTL 을 遺傳子 地圖에서 찾을 수 있었다. 表現形은 찾아진 QTL 을 통해 11.2% 에서 32.2%을 설명할 수 있었다. 많은 Interval mapping 에서 발견된 QTL 들이 Kruskal-Wallis 테스트에서는 유의한 LOD 에 미치지 않고, 반대의 경우 또한 마찬가지였다.높은 有意性의 LOD 에 이르지 못하였다. 이에 따른 推論은 5 장에서 언급하였다. 結論的으로 본 論文은 *Alstroemeria* 의 큰 게놈 크기에도 불구하고 AFLP 테크닉을 이용하여 여러 用度로 遺傳 硏究가 가능하다는 걸 보여주었다.

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CURRICULUM VITAE

Tae-Ho Han was born on 6 December, 1969 in Kwang-Ju, Republic of Korea. He finished the primary, junior and high school education in Kwang-Ju from 1979 to 1987. In 1988, he started the academic study in the Department of Horticulture, Chonnan National University (Kwang-Ju city), and received a bachelor degree in 1991. At the same year, he was accepted in the MSc program of the Department of Horticulture at University of Illinois at Urbana-Chapmaign (U.S.A.). From 1992 till graduation, he performed as a research assistant participating in maize marker-assisted-breeding program and received a scholarship. In 1994 he obtained an MSc degree under Dr. J.A. Juvik. After a two-year compulsory military service, he was recommended by his professor, Dr. Soon-Ju Jung at his home university and presented to Prof. dr. ir. H. Challa at the Department of Horticulture in Wageningen University. Since Tae-Ho Han was searching for an ornamental breeding program, Prof. dr. ir. H. Challa redirected and recommended Prof. dr. ir. E. Jacobsen and Mrs. Dr. ir. M.J. De Jeu at the Laboratory of Plant Breeding in Wageningen University. From January 1997 on he performed his Ph.D. programme in *Alstroemeria* at the Laboratory of Plant Breeding. The results of this research are described in this thesis.