A cytological characterization of genomes of *Alstroemeria*, the production of interspecific hybrids, and their performance during micropropagation

Een cytologische karakterisering van genomen van *Alstroemeria*, de productie van interspecifieke hybriden en hun gedrag tijdens *in vitro* vermeerdering

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Alstroemeria spp., Alstroemeriaceae, evolution, flow cytometry, genome size, Giemsa C-banding, Inca lily, interspecific hybridization, karyotypes, micropropagation, ovule culture, rhizome

Stellingen

1. Het verdient de voorkeur om tijdens verzamelexpedities van *Alstroemeria* soorten slechts (enkele) zaden mee te nemen en geen opgegraven plantmateriaal.

2. Zowel Giemsa C-bandering als *in situ* hybridisatie van *Alstroemeria* genomen bevestigen dat hoog repetitief DNA bij voorkeur accumuleert in specifieke patronen op de chromosomen.

   Dit proefschrift

   Kamstra SA, Kuipers AGJ, De Jeu MJ, Ramanna MS, Jacobsen E. 1997. Physical localization of repetitive DNA sequences in *Alstroemeria*: kayotyping of two species with species-specific and ribosomal DNA. *Genome* 40: 652-658

   Schweizer D, Loidl J. 1987. A model for heterochromatin dispersion and the evolution of C-band patterns. In: Stahl A, Luciani Jm, Vagner-Capodano AM, (eds.): *Chromosomes today*, vol 9. London: Allen and Unwin, 61-74

3. De huidige methoden voor het bepalen van de DNA hoeveelheid in celkernen van planten zijn nog steeds subjectief.

   Dit proefschrift

   Doležel J, Sgorbati S, Lucretti S. 1992. Comparison of three DNA fluorochromes for flow cytometric estimation of nuclear DNA content in plants. *Physiologia Plantarum* 85: 625-631

   Bennett MD, Leitch IJ. 1995. Nuclear DNA amounts in angiosperms. *Annals of Botany* 76: 113-176

4. Het geslacht *Alstroemeria* leent zich uitstekend voor een ecologische studie naar de samenhang tussen geografische verspreiding, de habitat, de grootte van het genoom, chromosoompolymorfismen en de aanwezigheid van bepaalde repetitieve DNA sequenties.

   Dit proefschrift

5. Het verdient aanbeveling om de vermeerderingssnelheid van plantmateriaal in weefselkweek op te nemen als selectiecriterium in veredelingsprogramma's van *Alstroemeria*.

   Dit proefschrift

6. Bewust consumeren leidt tot consuminderen.

7. "Je kunt respect niet kopen."

   José Ramos Horta, Oost Timorese verzetsleider en winnaar van de Nobelprijs voor de vrede.

   Je kunt het evenmin afdwingen. Je kunt respect alleen verdienen door je gedrag.

8. Het woord biodiversiteit wordt zwaar geëxploiteerd in de meest uiteenlopende onderzoeksvoorstellen.

9. Wie zijn nek uitsteekt, krijgt sterke nekspieren.

10. Het 'downshiften', een trend die in de jaren negentig is begonnen, en waarbij een pas op de plaats wordt gemaakt op de carrière ladder (of een stapje terug) ten behoeve van meer rust en ontspanning, zal ook in de 21e eeuw doorgaan.

11. Fusies en opsplitsingen zijn opeenvolgende processen die steeds worden voltrokken onder het mom van meer efficiëntie, flexibiliteit en winst, echter het zijn ook eenvoudigweg reorganisaties die noodzakelijk lijken in een steeds veranderend klimaat.

Stellingen behorend bij het proefschrift "A cytological characterization of genomes of *Alstroemeria*, the production of interspecific hybrids, and their performance during micropropagation" door Joska H. Buitendijk, 12 mei 1998.

Abstract

The genus *Alstroemeria* comprises more than 60 species that thrive in extremely diverse habitats in South America. Due to its ornamental value *Alstroemeria* has gained popularity as a cut flower, a bedding plant and a potted plant. In the present thesis basic information was provided on the rhizomes, the chromosomes and the genomes of species and interspecific hybrids. The diversity within the genus was reflected in the morphological features of the rhizomes, which differed considerably in size and branching pattern. The karyotypes of the species were asymmetrical, and were composed of eight pairs of large chromosomes. Giemsa C-banding revealed a characteristic banding pattern for each of eight species, that could be used to identify chromosomes in species and hybrids. The differentiation of the genomes was also demonstrated through a flow cytometric assessment of the amounts of nuclear DNA, the 2C-values, which varied from 36.5 to 78.9 pg. among twelve species. The results with respect to 2C-values and the fluorescence of nuclei that were stained with propidium iodide and 4',6-diamidino-2-phenylindole allowed a subdivision of these species into four groups. Interspecific hybridization was hampered by poor development of the endosperm in the young seeds. This hybridization barrier was overcome by culturing young seeds *in vitro*. The rate of rhizome multiplication during micropropagation was shown to be a heritable trait, which offers scope for selection in breeding programmes of *Alstroemeria*.

Contents

Chapter 1

General introduction

The family of Alstroemeriaceae

Taxonomic background
Family Alstroemeriaceae, Order Liliales, Superorder Liliiflorae, Class Monocotyledons (Dahlgren *et al.*, 1985).
The genus *Alstroemeria* was named in 1762 by Linnaeus after the Swedish envoy Clas Alströmer, who had sent him seeds of *Alstroemeria pelegrina* that were collected in Peru. Thus, the taxonomic type for the genus was *Alstroemeria pelegrina*. Initially, *Alstroemeria* was included in the family of Liliaceae, and later it was placed in the Amaryllidaceae (Herbert, 1837). In 1959, Hutchinson proposed to separate the genus *Alstroemeria* from the Amaryllidaceae into a new family of Alstroemeriaceae, comprising the four genera *Alstroemeria*, *Bomarea*, *Schickendantzia* and *Leontochir* (Hutchinson, 1959). There has also been discussion about the question in which order the family Alstroemeriaceae should be included. Hutchinson proposed that the Alstroemeriaceae together with the Philesiaceae and the Petermanniaceae should compose a new order, the Alstroemeriales, but this was not generally accepted. Dahlgren *et al.*, (1985) agreed that there were similarities between the Philesiaceae and the Alstroemeriaceae, but they pointed at some notable differences between these two families and decided to retain the families in separate orders, being the Asparagales for the family of the Philesiaceae, and the Liliales for the family of the Alstroemeriaceae. Dahlgren *et al.*, (1985), however, supported the view that the division of the orders Asparagales and Liliales is unclear, and considered the families of the Alstroemeriaceae and the Philesiaceae to bridge the gap between these two orders. Dahlgren *et al.*, (1985) also recognized similarities with the families of the Liliaceae, Uvulariaceae, Calahortaceae and Orchidaceae. The classification of the genus *Alstroemeria* as described in Dahlgren *et al.*, (1985) is generally accepted, although occasionally *Alstroemeria* is still considered as member of the Amaryllidaceae.

Botanical features of Alstroemeriaceae
The family of Alstroemeriaceae comprises erect or twining herbs of variable size, which are generally perennial. Whereas the genera *Alstroemeria* and *Schickendantzia* consist of erect herbs, *Bomarea* and *Leontochir* are scandent vines. Some of the features, that characterize the family, are presented in Table 1:

TABLE 1. Botanical features of the Alstroemeriaceae (Dahlgren *et al., 1985)*

- fusiform to cylindrical storage roots, that contain starch and water,
- a sympodial underground rhizome,
- scaliform perforation plates in the vessels of the stems and roots,
- linear to lanceolate leaves scattered on the stem, not sheathing the stem completely; leaves are narrowing at their base and leaf blades are generally twisted at their base, so that the morphologically lower side is turned upwards,
- terminal inflorescences consist of helicoid cymes, generally umbel-like, occasionally pendulous and rarely unifloral,
- trimerous flowers that are epigynous, bisexual, and actinomorphic or slightly zygomorphic, and of variable size; tepals are mostly free and vary from similar to conspicuously different in the two whorls, the outer whorl often being shorter, of different colour and less variegated than the inner whorl, which often bears a dotted or striate-dotted colour pattern; colours vary from green to yellow, orange, rose, red or purple; nectaries at the base of two or all inner tepals,
- six stamens (3+3), with narrow filaments, and elongate pseudo-basifixed anthers, longitudinal and introrse dehiscence; trilocular or unilocular pistil with a single style, with three apical stigmatic branches, that produce an exudate on its surface,
- a trilocular or unilocular ovary, with distinct decurrent borders of the outer tepals and a circular 'scar' at the position of attachment of the perigone; it contains numerous anatropous ovules on axile or parietal placentae; the fruit is a capsule which is generally loculicidal, occasionally it is indehiscent or opens explosively,
- globose or rounded-ellipsoidal seeds; the outer integument has sparse subepidermal 'bladder cells' that in *Alstroemeria* contain oxalate raphides; the endosperm contains aleuron and fatty oils, but no starch; the embryo is cylindrical and about two thirds the length of the endosperm; after germination seedlings have no coleoptile-like cotyledon,
- both oxalate raphides and tuliposides occur in most parts of the plants; alkaloids are lacking, chelidonic acid and probably steroidal saponins are produced (Dahlgren *et al.*, 1985).

Dahlgren *et al.*, (1985) made no remark on the following features:

- flowers are often protandrous, i.e. the anthers shed their pollen before the stigma is receptive. Anthers dehisce in a temporal sequence. Flowers are temporally unisexual. This mechanism limits the possibilities of self-pollination within and among flowers in the same umbel (Traub, 1943; Aizen and Basilio, 1995; Snow and Grove, 1995),
- flowers are visited, and most probably pollinated, by hummingbirds and bees (Stinson, 1942), bumble bees (Aker and Healy, 1990), butterflies (Bayer, 1988), and moths (Buitendijk, pers. observation).

The species of *Alstroemeria*

The species of Alstroemeria *and their distribution*

At present there is no consensus about the number of species that exist within the genus *Alstroemeria*, but it probably exceeds 60 (Uphof, 1952). The first descriptions and illustrations date back to 1714, although at that time the author, Feuillée, considered the material to belong to the genus *Hemerocallus*. Linnaeus combined the information of Feuillée and Alströmer, gave the genus its name and described three species. In 1802 Ruiz and Pavon recognized 23 species of which 17 are now transferred to *Bomarea*, in 1837 Herbert described 29 species, and in 1850 Kunth reported on 40 species (Uphof, 1952). In 1888 Baker considered a total of 44 species, and, based on geographical distribution, divided them into two groups, the Chilean

species and the Brazilian species, with 24 and 20 species, respectively. Uphof, who reviewed the genus in 1952, did not continue this division, which he considered artificial and incorrect, and mentioned a total of 62 species. This author stressed the need of a new well balanced monograph of the whole genus, with more detailed species descriptions (Uphof, 1952).

A next attempt to elucidate the species of *Alstroemeria* was made by Garaventa (1971), who had studied the species in their own habitat and in herbaria and described 19 Chilean species in great detail. A new monograph was written by Bayer in 1987, although it did not comprise the genus as a whole, but only the species that occur in Chile. In this monograph 31 species have been described, of which five were new and several others have been renamed. New descriptions were also given for six subspecies. Bayer concluded that only few species can be considered as clearly separate from each other, i.e. *A. graminea*, *A. patagonica* and *A. pelegrina*, and many intermediate forms hamper the classification of species and subspecies, for instance within *A. ligtu* or within the complex *A. pulchra* - *A. magnifica* (Bayer, 1987).

The studies of Bayer more or less coincided with those of Ravenna, a Chilean herbalist, who also attempted to revise the genus of *Alstroemeria*. Somehow, the publication of Ravenna's revisional work was hindered, and finally only short Latin descriptions of 17 new species and some information on three other species were published (Ravenna, 1988), just after the publication of Bayer's work. It is very unfortunate that the specimen collected by Ravenna were not studied by Bayer.

A large part of the genus is to date still unclear. Especially the species that have their centre of distribution in eastern Brazil need further clarification. Recently, Meerow and Tombolato (1996) reported on the occurrence of four species on Itatiaia, a mountain in south-eastern Brazil, and announced to report on other surveys of *Alstroemeria* diversity in Brazil. Aker and Healy (1990) compiled a list of 103 species in a paper that aimed at revealing the geographical distribution of the genus. The names of the species and the geographical data derive from earlier publications, including those of Bayer and Ravenna, and several of these names are probably synonymous. The compilation of Aker and Healy (1990) included 64 species from Chile, 30 from Brazil, and few from Argentina, Bolivia, Paraguay, Peru and Venezuela. These authors concluded that there are two centres of distribution, one in central Chile, and another, referred to as a 'satellite distribution centre', in eastern Brazil. The so-called Chilean species occur in Chile, coastal Peru, and across the Andes in Bolivia and Southern Argentina. Most Brazilian species occur in the Brazilian highlands and one in Venezuela. The habitats of *Alstroemeria* are extremely diverse, and range from 'the Mediterranean climate of central Chile, marked by extreme seasonality of precipitation and the infrequency of freezing weather, with cool, moist winters and hot, dry summers' to the 'much harsher conditions on the steppes of Patagonia', the extremely dry conditions of the Atacama Desert, the alpine conditions at high elevations, and the 'marshy' conditions in the tropics. Aker and Healy (1990) concluded that the genus *Alstroemeria* probably evolved in the persistent arid region between the temperate *Northofagus* forest of southern South America and the tropical rain forest of the

Amazon basin. Undoubtedly the rise of the Andes played a major role in the distribution of species (Aker and Healy, 1990).

Vernacular names of *Alstroemeria* are Peruvian lily, lily of Lima, or Inca lily (Stinson, 1942).

Botanical features that were used in the taxonomic classification of Alstroemeria *species*

In the taxonomic classification of *Alstroemeria* frequent use was made of characteristics of the flowers, such as size, shape, colour and striae, besides features of the stems, leaves and fruit. Because the vegetative shoots are often different from the generative shoots, Bayer (1987) described them separately. Illustrations of vegetative and/or generative shoots, flowers, leaves individual petals, stamen, fruit accompanied Bayer's work. Descriptions of rhizomes and tuberous roots were given when underground material was available. Unfortunately, only four illustrations of the rhizome were presented (see also Chapter 2).

The chromosomes and the genomes of taxa of the *Alstroemeriaceae*

A compilation of the literature on the chromosomes and the genomes of taxa of the Alstroemeriaceae, is presented in Table 2. The species of *Alstroemeria*, *Bomarea* and *Leontochir* that have been investigated are all diploid, with a basic chromosome number of n=8 for the *Alstroemeria* species, and n=9 for *Bomarea* (Strasburger 1882 in Taylor, 1938; Whyte, 1929) and for *Leontochir* (Bayer, 1988). The chromosome number of *Schickendantzia* is, to my knowledge unknown. The karyotypes of *Alstroemeria*, *Bomarea* and *Leontochir* displayed many similarities. All karyotypes were asymmetrical, with one, two or three large metacentric chromosomes and four to six acrocentric chromosomes of variable size. Most *Alstroemeria* species had two small (sub)metacentric chromosomes, which were absent in the genomes of *Bomarea* and *Leontochir*. In the past 25 years, valuable cytological work was carried out by Koornneef, Koolstra and Van Eijk-Bos (unpublished theses under supervision of Dr. Arends at the Department of Plant Taxonomy of Wageninningen Agricultural University) and by Tsuchiya and co-workers. Recently, Giemsa C-banding, flow cytometry, and *in situ* hybridization of labelled DNA or RNA probes provided powerful instruments for the characterization of *Alstroemeria* genomes (for references see Table 2).

Karyotypes of species can provide useful cytotaxonomic markers, especially when they are being used together with morphological markers. However, both morphological and cytological studies are laborious. Therefore, the collaboration of research groups would be most valuable.

Interspecific hybridization and the production of cultivars

Although spontaneous interspecific hybridization probably occurs in nature (Garaventa, 1971), no specific examples have been reported so far. Several attempts to synthetically hybridize species were successful (Foster, 1948; Duncan, 1977 and 1982), but other attempts failed (Stinson, 1942; Traub, 1943). Some hybrids, such as the *A. ligtu* hybrids (Comber, 1946) and the 'Van Houtte's hybrids' (Sahin, 1973) were fertile, but most other hybrids were sterile. The hybridization barriers restricted the progress in breeding, but nevertheless during the 1960's the hybrid 'Walter Fleming' (synonymous to 'Orchid flowered') was raised, and it proved to be a commercial success (Goemans, 1962). Although this diploid (2n=2x=16) cultivar was highly sterile, it was used as a parent, and the 'Parigo hybrids' were developed from it. The exact parentage of the 'Parigo hybrids' has either been unknown or it has not been revealed, but they derived predominantly from the Chilean species. A characteristic feature of these early hybrids is, that they possess large open flowers that resemble orchids, and for this reason they became generally known as 'Orchid type' cultivars. Most of these hybrids were highly sterile.

A remarkable fact is that these early hybrids consisted of diploids, triploids (2n=3x=24) and occasionally tetraploids (2n=4x=32), and thus probably originated through the functioning of unreduced gametes (Ramanna, 1991, 1992a). Spontaneous polyploidization has played a significant role in the development of many other horticultural crops as well (Ramanna, 1992b), such as daffodil, freesia, tulip, chrysanthemum and rose. An extensive survey of the diploid interspecific hybrids between *Alstroemeria* species that have been used for the development of cultivars, has indicated the occurrence of unreduced gametes (Ramanna and Buitendijk, unpublished). It was found that the interspecific hybrids between Chilean species produced hardly any unreduced gametes, whereas those between Chilean and Brazilian species produced high frequencies of both unreduced pollen and unreduced eggs. From the latter interspecific hybrids, large numbers of sexual polyploids have been produced experimentally.

The application of mutation breeding offered breeders the possibility of inducing favourable mutations in already existing cultivars (Broertjes and Verboom, 1974). After irradiation of young actively growing rhizomes with X-rays, usually a range of new forms was created, of which some proved to be improvements. Hence, the irradiation of cultivars with X-rays became a standard procedure in breeding programmes.

Another significant development was that, besides inter Chilean species hybrids, also Chilean-Brazilian species hybrids were produced (Tsuchiya *et al.*, 1987). Probably because the flowers of such hybrids resembled butterflies, they were referred to as 'Butterfly type' cultivars. Also in these hybrids spontaneous polyploidization led to triploid and tetraploid cultivars. When more different species were used to produce new cultivars, with intermediate flower shapes, the type indication became useless,

TABLE 2. Compilation of literature on chromosomes and genomes of taxa in the Alstroemeriaceae.

Technique	Result	Taxon	Reference
chromosome staining (paraffin sectioning)	chromosome number	*A. chilensis*	Strasburger, 1882 (in Taylor 1926)
chromosome staining (paraffin sectioning)	chromosome number	*A. psittacina, A. pelegrina, A. versicolor*	Guignard, 1884, 1889, 1891 (in Taylor 1926)
chromosome staining*	chromosome number	*A. psittacina*	Svenson-Stenar, 1925 (in Satô 1938)
chromosome staining*	karyotypes	*A. brasiliensis*	Taylor, 1926
chromosome staining*	karyotypes	*A. aurantiaca, A. pulchella, A. haemantha, A. pulchella, Bomarea*	Whyte, 1929
chromosome staining*	karyotypes	*A. chilensis, A. pulchella, Bomarea salsilla*	Satô, 1938
chromosome staining*	chromosome number	*A. ligtu*	Goodspeed, 1940
chromosome staining*	karyotypes	*A. psittacina, A. inodora, A. pelegrina, A. aurantiaca , A. gayana,* unidentified *A.* sp., *A. ligtu* hybrids, hybrid 'Walter Fleming'	Koornneef, 1972 (unpublished)
chromosome staining*	meiotic configurations	*A. psittacina,* unidentified *A.* sp., diploid interspecific *Alstroemeria* hybrids, hybrid 'Walter Fleming'	Koolstra, 1973 (unpublished)
chromosome staining*	karyotypes	*A. psittacina, A. pulchra* f. *maxima, A. haemantha,* unidentified *A.* sp.	Koolstra, 1973 (unpublished)
chromosome staining*	karyotypes	*A. haemantha, A. chilensis, A. ligtu, A. recurvata, A. spathulata, A. pulchra, A. violacea, A. gayana* var. *humilis, A. sierrae,* unidentified *A.* sp., *Bomarea* sp., tetraploid interspecific *Alstroemeria* hybrid	Van Eijk-Bos, 1974 (unpublished)
chromosome staining*	karyotypes	*A. pulchella, A. japonica*	Lakshmi, 1976
chromosome staining*	chromosome numbers and some karyotypes	ten *Alstroemeria* cultivars	Tsuchiya *et al.*, 1987
chromosome staining*	chromosome numbers and some karyotypes	eleven *Alstroemeria* cultivars	Hang and Tsuchiya, 1988
chromosome staining*	karyotype	*Leontochir ovallei*	Bayer, 1988
chromosome staining*	chromosome numbers, meiotic configurations, pollen fertility	*A. aurantiaca, A. caryophyllaea, A. chilensis, A. haemantha, A. hookeri, A. ligtu, A. pelegrina, A. psittacina, A. pulchella, A. pulchra, A. versicolor, A. violacea,* di-, tri- and tetraploid *Alstroemeria* hybrids	Tsuchiya and Hang, 1989

TABLE 2. Continued

Technique	Result	Taxon	Reference
chromosome staining*	karyotypes	*A. ligtu* hybrids	Rustanius *et al.*, 1991
chromosome staining*	karyotype	*A. pelegrina*	Stephens *et al.*, 1993
hybridization with repetitive probes	repetitive species-specific probes	*A. aurea, A. ligtu* ssp. *ligtu, A. psittacina, A. inodora*	De Jeu *et al.*, 1995
'genetic fingerprinting'	RAPD** profiles	*A. pelegrina, A. psittacina*, cultivars and breeding lines of *Alstroemeria*	Anastassopoulos and Keil, 1996
chromosome staining*	karyotypes	diploid interspecific *Alstroemeria* hybrids, *A. philippii*	Buitendijk and Ramanna, 1996
Giemsa C-banding*	karyotypes with C-bands	*A. angustifolia* ssp. *angustifolia, A. aurea, A. inodora, A. ligtu* ssp. *ligtu, A. magnifica* ssp. *magnifica, A. pelegrina, A. philippii, A. psittacina*, diploid interspecific *Alstroemeria* hybrid	Buitendijk and Ramanna, 1996
flow cytometry	genome sizes	*A. angustifolia* ssp. *angustifolia, A. aurea, A. brasiliensis, A. caryophyllaea, A. hookeri* ssp. *hookeri, A. inodora, A. ligtu* ssp. *ligtu, A. ligtu* ssp. *simsii, A. magnifica* ssp. *magnifica, A. pelegrina, A. philippii, A. psittacina, A. pulchra* ssp. *pulchra*, diploid and tetraploid interspecific *Alstroemeria* hybrids	Buitendijk *et al.*, 1997
chromosome staining*	karyotypes	tetraploid interspecific *Alstroemeria* hybrids	Buitendijk *et al.*, 1997
GISH**	karyotypes with GISH-bands	diploid interspecific *Alstroemeria* hybrids	Kuipers *et al.*, 1997
genomic slot blot analysis	characterization of repetitive DNA sequences	*A. aurea, A. ligtu* ssp. *ligtu, A. pelegrina, A. magnifica* ssp. *magnifica, A. inodora, A. psittacina*	Kuipers *et al.*, 1997
FISH**	karyotypes with FISH-bands	*A. aurea, A. inodora*	Kamstra *et al.*, 1997
genomic slot blot analysis, FISH	characterization of repetitive DNA sequences	*A. aurea*	De Jeu *et al.*, 1997
'genetic fingerprinting'	RAPD profiles	*Alstroemeria* cultivars	Dubouzet *et al.*, 1997
Giemsa C-banding* flow cytometry	karyotypes with C-bands, genome sizes	*A. aurea, A. ligtu* ssp. *ligtu, A. ligtu* ssp. *simsii, A. ligtu* ssp. *incarnata, A. magnifica* ssp. *magnifica*, diploid interspecific Alstroemeria hybrids	Buitendijk *et al.*, in press

* Hydrolysed tissue was squashed

** GISH = genomic *in situ* hybridization, FISH = fluorescence *in situ* hybridization, RAPD = random amplified polymorphic DNA

because a clear distinction could no longer be made. The development of *in vitro* methods to overcome hybridization barriers has recently opened the possibility to produce hybrids on a relatively large scale. Due to these techniques many new interspecific combinations are expected to be produced.

Breeders continued to broaden the assortment, with respect to the flower colour, the size of the flower, the size of the stem, and the length of the flowering period (Verdegaal, 1989). Winter flowering or year-round flowering has remained a very important selection criterion. Additional important criteria for *Alstroemeria* cut flowers are: the flower colour and its ornamental value, the production of high quality stems (> 200 stems per m^2 per year), the size and colour of the flower buds and the quality of the leaves. For pot plant cultivars breeders concentrate on plant characters such as short stems, simultaneous and uniform flowering, good leaf quality and performance of the whole plant. The fusion of breeding companies and the collaboration in research projects in The Netherlands has led to a concentration of expertise. At present there are a few major breeding companies, and several small ones in The Netherlands that are concerned with the breeding of *Alstroemeria* (Anonymous, 1997a).

To protect their cultivars against illegal distribution, breeders can apply for plant breeders' rights in each country separately. For protection, cultivars must be thoroughly checked with respect to distinctness, uniformity, stability and novelty. At present more than 150 *Alstroemeria* cultivars are registered in the Netherlands Register of Varieties, a three-fold increase as compared to 1980 (Figure 1). Since 1995 it is possible to apply for European Union Community plant variety rights, which gives protection in whole Europe. In 1995, 1996 and 1997 it was possible to convert registration in the Netherlands Register into registration in the Community Register. No new *Alstroemeria* cultivars have yet been registered in the Community Register, but since 1995 the rights of 37 cultivars were converted into European Union Community plant variety rights, and there have been 20 new applications (Figure 1).

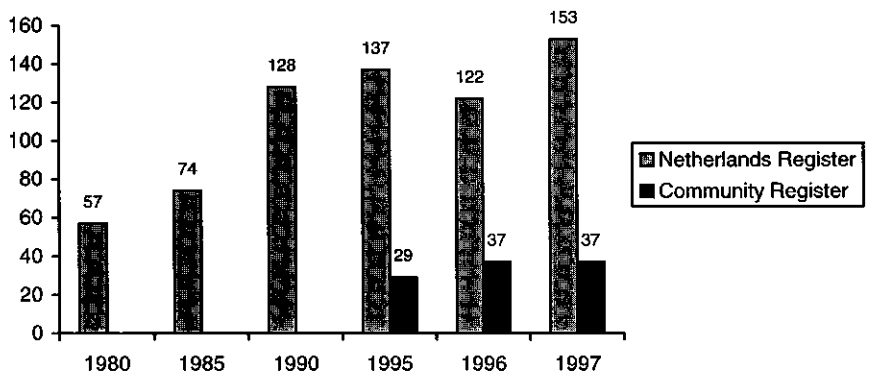

FIGURE 1. The number of registered cultivars for plant breeder's rights in the Netherlands and in the European Union in 1980, 1985, 1990, 1995, 1996 en 1997 (Anonymous, 1981- 1997a; pers. comm. European Union Community Plant Variety Office, Angers, December 1997).

The economic importance of the crop

Until 1960 *Alstroemeria* was a relatively unimportant crop. It was primarily grown as a garden plant. The introduction of new cultivars that were suitable as cut flower (Goemans, 1962) and the research input for the improvement of cultivation methods (e.g. Heins and Wilkins 1979; Verboom, 1980; Vonk Noordegraaf, 1981), enabled *Alstroemeria* to become more attractive for growers. Due to its low energy requirement for cultivation, its popularity increased rapidly during the energy crisis of the early seventies. *Alstroemeria* has also gained popularity due to its long vase life and the attractiveness of its flowers which are highly suitable for the use in mixed bouquets. Since 1980, the crop *Alstroemeria* is mainly cultivated for the production of cut flowers, but during the last five years, there has been an increasing demand for *Alstroemeria* pot plants and garden plants as well. The market for *Alstroemeria* as a garden plant is small, and focuses on The United Kingdom, The United States, Canada and Australia. Most pot plants of *Alstroemeria* are directly exported to Japan, The United States, Canada, Finland and other countries, that have a good market for *Alstroemeria* pot plants. It is difficult to assess the extent of the production of pot plants. The production of cut flowers of *Alstroemeria* in The Netherlands is well documented (Table 3). *Alstroemeria* occupies the ninth place in the top ten of cut flowers where it concerns auction turnover, and is preceded by rose (1), chrysanthemum (2), tulip (3), lily (4), carnation (5), gerbera (6), freesia (7) and cymbidium (8), and followed by gypsophyla (10) (Anonymous, 1997b). The greenhouse acreage of *Alstroemeria* in the Netherlands has increased from 7 ha in 1972 (Broertjes and Verboom, 1974) to 113 ha in 1997. Auction supply and turnover has more than doubled since 1985. There are seasonal fluctuations in the production, with a peak in May / June and a decrease in January / February. For the production of cut flowers, growers obtain planting material directly from the breeders, to whom they pay licence fee. These plants are grown to produce flowers for a period of three to seven years, after which period new planting material is bought.

TABLE 3. Production area and auction turnover of *Alstroemeria* in The Netherlands.

	1985	1990	1995	1996	1997
Greenhouse acreage[1] (hectare)	n.a.[3]	83	118	115	113
Auction supply[2] (number of stems x million)	104	185	230	261	243
Auction turnover[2] (hfl x million)	38	67	79	84	85
Price per stem[2] (hfl)	0.37	0.37	0.35	0.33	0.35

[1] Source: Centraal Bureau voor de Statistiek, Voorburg, counts made each year in May.
[2] Source: Statistiekboeken, Vereniging van Bloemveilingen in Nederland, Leiden.
[3] n.a.= data not available.

Growers tend to delay the replacement of plants when the net income from this crop is low, as was the case during the last three years. Within Europe *Alstroemeria* is grown on a large scale in The Netherlands, and on a smaller scale in The United Kingdom, Germany, France, Italy, Spain, Portugal, Poland and Finland, amongst others. Outside

Europe *Alstroemeria* is predominantly grown in Japan, Colombia, Bolivia, Equador, The United States, Canada, Kenya, South Africa, Australia and New Zealand.

Aseptic cultures of *Alstroemeria*

Table 4 presents a compilation of the literature on aseptic cultures in *Alstroemeria*. The first publications on *in vitro* cultures of *Alstroemeria* date back to 1973 and 1974, when attempts were made to initiate rhizome cultures from greenhouse grown plants. In later years, aseptic cultures were used to eliminate viruses, to micropropagate rhizomes, to produce hybrid plants, and to regenerate plants through a phase of callus or somatic embryos for genetic transformation purposes. A recent paper reported on the application of a cold treatment to *in vitro* plantlets in order to induce uniform flowering of *Alstroemeria* pot plants (for references see Table 4).

Through the efforts of Ziv (1974), Hussey (1980), Pierik (1988) and co-workers micropropagation has become a common practice for the mass propagation of *Alstroemeria* plant material. From an inquiry on the numbers of plants that are micropropagated in The Netherlands, Pierik (1996) estimated that in 1995 nearly two million *Alstroemeria* plants were produced, a three-fold increase as compared to 1990. This increase in production is remarkable, because for most crops there has been a decrease in production of micropropagated plants in The Netherlands, and an increase of the import. A large part of the micropropagation of bulbous and cormous plants, cut flowers and pot plants is now practised abroad, where the cost of labour is lower. One of the reasons that the production of micropropagated *Alstroemeria* plants still takes place in The Netherlands, is probably that the number of micropropagated plants of this crop does not justify a transfer of the production. Together with the fear of illegal distribution and cultivation, breeders tend to micropropagate their cultivars in their own laboratories, or in other Dutch laboratories. Another reason is probably that *Alstroemeria* can be difficult to propagate *in vitro* (Pierik, 1996) and that it needs close attention in order to obtain high quality propagules. The difficulties with *in vitro* propagation of *Alstroemeria* concern the low multiplication rate of some cultivars (Pierik, 1988), the different requirements of cultivars with respect to temperature, culture medium, dissectioning (Bond and Alderson, 1993a and b) and the rapid decline of cultures when they are not treated properly. The crop is very sensitive to any changes in culture conditions and plants are physiologically disturbed easily. Some cultivars display abnormalities of the plant material *ex vitro* (e.g. multiple shoots with retarded or reduced flowering, fasciation of shoots, leaf colour or flower shape changes).

TABLE 4. Compilation of literature on aseptic cultures of *Alstroemeria*.

Culture	Result	Reference
Inflorescence	*Initiation of rhizome culture*	Ziv *et al.*, 1973
Rhizome tip	Initiation of rhizome culture	Quak, 1974
Vegetative shoot, inflorescence, rhizome tip, *in vitro* rhizome	Initiation of rhizome culture micropropagation	Hussey *et al.*, 1980
Rhizome segment	Organogenisis	Gabryszewska and Hempel, 1985
Rhizome tip, meristem	Virus elimination, initiation of rhizome culture	Hakkaart and Versluijs, 1985
Inflorescence, shoot tip, ovule	Callus, somatic embryos, hybrid plants, plant regeneration	Bridgen, 1986
Inflorescence, rhizome tip	Initiation of rhizome cultures, micropropagation	King and Bridgen, 1987
Rhizome tip	Initiation of rhizome culture	Lin and Monette, 1987
Rhizome tip, *in vitro* rhizome	Initiation of rhizome culture micropropagation	Pierik *et al.*, 1988
Rhizome tip, meristem	Virus elimination, initiation of rhizome culture	Hakkaart and Versluijs, 1988
Ovule	Hybrid plants	Winski and Bridgen, 1988
Ovule	Hybrid plants	Miyake, 1989
Ovule, *in vitro* rhizome	Hybrid plants micropropagation	Bridgen *et al.*, 1989
Rhizome tip	Initiation of rhizome culture, rooting	Monette and Lin, 1991
Rhizome tip, *in vitro* rhizome	Review, initiation of rhizome culture, micropropagation	Monette, 1992
Ovule	Hybrid plants	Buitendijk *et al.*, 1992
Rhizome tip	Disinfection	Pedersen and Brandt, 1992
Rhizome tip, meristem	Virus elimination, initiation of rhizome culture	Van Zaayen *et al.*, 1992
Zygotic embryo	Callus, somatic embryos, pant regeneration	Gonzalez-Benito and Alderson, 1993
In vitro rhizome	Micropropagation	Bond and Alderson, 1993a
In vitro rhizome	Micropropagation	Bond and Alderson, 1993b
Ovary with ovules	Callus, hybrid plants	Tombolato *et al.*, 1993
Rhizome tip	Optimization of phosphate in culture medium	Elliott *et al.*, 1993
Zygotic embryo	Callus, somatic embryos, plant regeneration	Hutchinson *et al.*, 1994
Half-ovule	Hybrid plants	Buitendijk *et al.*, 1995
Ovule	Hybrid plants	Kristiansen, 1995
Ovule	Hybrid plants	De Jeu and Jacobsen, 1995
Rhizome, stem, pedicel, ovary	Organogenisis, rooting	Gabryszewska, 1995
In vitro rhizome	Flower induction	Pedersen *et al.*, 1996
Zygotic embryo	Callus, somatic embryos, plant regeneration	Van Schaik *et al.*, 1996
Ovule	Hybrid plants	Lu and Bridgen, 1996
Zygotic embryo	Somatic embryos, plant regeneration	Hutchinson *et al.*, 1997
Ovule	Hybrid plants	Ishikawa *et al.*, 1997
Leaf with stem node of *in vitro* seedling	Initiation of rhizome culture, plant regeneration	Lin *et al.*, 1997
In vitro rhizome	Chromosome doubling	Lu and Bridgen, 1997

Problems with breeding, propagation and cultivation of *Alstroemeria*.

The tremendous variation that is present in the genus *Alstroemeria* offers many opportunities for the improvement and renewal of the cultivar assortment. There are however also limitations in the breeding of *Alstroemeria*. One of these limitations is the poor knowledge of the species, especially the Brazilian ones. Hybridization barriers and sterility of interspecific hybrids impose further restrictions. Breeders, however, have learned how to cope with these restrictions, and produced successful polyploid cultivars. An understanding of the functioning and application of unreduced gametes may in the future improve the efficiency of breeding methods. Another concern is that the genetics of traits is unknown. Improvement of the crop through efficient genetic transformation is as yet not possible, due to the rather low frequency of simultaneous regeneration and transformation events, in the presently available protocols.

Although much improved by the application of micropropagation, the propagation of plant material of *Alstroemeria* is hampered by the low multiplication rate of groups of cultivars, and, in general, by the limited knowledge of the influence of parental species on the performance of hybrids. Other problems in micropropagation are the phenotypic alterations that occur *in vitro* and *ex vitro*, and the limited flexibility in the handling of *Alstroemeria* cultures.

The cultivation of *Alstroemeria* has undergone many improvements, for example with respect to the application of light, soil temperature and nutrition and its effect on the control of flowering (Heins and Wilkins, 1979; Vonk Noordegraaf, 1981; Healy *et al.*, 1982; Healy and Wilkins, 1986). Infection of plant material with viruses (Bouwen and Van der Vlugt, 1996), phytoplasmas (Bertaccini *et al.*, 1996) or nematodes (Barker and Hooper, 1995; Amsing, 1996) can cause severe damage. Although meristem culture has helped to eliminate the viruses, the threat of re-infection remains a possibility, and a constant monitoring of the plant material is necessary (Van Zaayen *et al.*, 1992).

Aims and outline of the thesis

For the breeding of *Alstroemeria* it is important to have a good knowledge of the species and of methods to hybridize them. It is also important to have an insight into the genetics of traits. With only a short history of breeding of *Alstroemeria* there are still many unanswered questions. The available biosystematic information on *Alstroemeria* species is rather restricted and little is known on the differentiation of genomes within the genus. In *Alstroemeria* as well as in other crops, interspecific hybridization is hampered by a poor seed set in interspecific crosses. The causes of this phenomenon are in most cases unknown, and although some publications reported methods to overcome hybridization barriers in *Alstroemeria*, further investigation of these matters is required. Because cultivars of *Alstroemeria* are mostly interspecific hybrids that cannot reproduce sexually, and the conventional method of vegetative propagation allows only slow multiplication of planting material, it is attractive to use micropropagation techniques. For the breeding of cultivars it is therefore important to gain an insight into the genetic aspects of micropropagation. This matter had thus far not received proper attention in any of the vegetatively propagated horticultural crops.

The research that is described in this thesis aimed to increase the knowledge regarding the species and interspecific hybrids of *Alstroemeria* in order to contribute for the development of cultivars as well as to provide a basis for fundamental research in the genus. The specific aims of this research were to provide information on the biosystematic status of the family of Alstroemeriaceae, and the genus *Alstroemeria*, to summarize the literature on chromosome studies and on aseptic cultures of *Alstroemeria*, and to give an overview of the breeding activities and the economic importance of the crop, and to note some of the problems that are encountered in breeding, propagation and cultivation (Chapter 1). It was also aimed to analyse the morphological structure of the rhizome of *Alstroemeria*, and characterize the underground organs of a number of species and interspecific hybrids (Chapter 2). With respect to the chromosomes and genomes it was aimed to analyse the karyotypes of species and interspecific hybrids, through Feulgen-staining and through Giemsa C-banding (Chapter 3), to determine genome sizes of species and interspecific hybrids through flow cytometry (Chapter 4), and to assess the extent of intraspecific variation concerning Giemsa C-banding pattern and genome size in some of the species (Chapter 5). Furthermore, it was aimed to develop an embryo rescue method, to produce a series of hybrid plants between species, that have frequently been used in the development of the modern cultivars, and to confirm the hybridity of the obtained plants (Chapter 6), and to assess the *in vitro* multiplication rate of rhizomes of species accessions and interspecific hybrids, and to determine the influence of the genetic back-ground of hybrids on their performance during micropropagation (Chapter 7). Finally, the implications of the results for applied and fundamental research on *Alstroemeria* were evaluated and some new questions that have been raised, were highlighted (Chapter 8).

Chapter 2

The morphology of the underground organs of *Alstroemeria*

Abstract

The rhizomes and the storage roots of *Alstroemeria* L. are morphologically diverse and can be useful as taxonomic markers. The general morphological structure of the rhizome shows sympodial growth, in which new shoots arise from underground basal buds. The rhizomes of species and hybrids vary considerably in length, diameter, internodal length and branching pattern. These factors are assumed to affect the multiplication of plantlets *in vitro*.

Introduction

Monocotyledons display a striking uniformity in growth habits, characterized by an unlimited growth, and in most cases by a lack of cambium (Holttum, 1955; Rees, 1989). According to Holttum (1955) this continuous sympodial growth pattern evolved in the moist tropics, but proved to be peculiarly adaptable to the production of resting organs, thus allowing the spread to seasonal climates. The geophytic growth habit is one of such adaptations, in which new growth arises from underground buds. In the unfavourable (dry or cold) season the leafy shoots die, leaving the underground parts of the stem to rest until the next growing season (Holttum, 1995; Rees, 1989).

In geophytic taxa, the underground organs can be useful as taxonomic markers, because they are highly specialized structures and morphologically diverse. Biosystematic work, however, often lacks detailed information on the underground organs. This is not in the last place because it is a difficult task to excavate the root system of plants, without damaging it. Another difficulty is the preservation of underground organs as dried specimen in herbaria. The morphology of these organs, in most cases, cannot be preserved properly. Drawings, photographs, and preservation of the material in alcohol are useful alternatives.

Within the genus *Alstroemeria* the diversity of the above ground organs is tremendous. Together with the fact that more than 60 species occupy a range of extreme habitats, it may be expected that the morphology of the underground organs is highly variable as well. The early taxonomic work in *Alstroemeria* was occasionally illustrated with drawings of the whole plant, including the underground organs, as for example in the work of Feuillée (1714, in Uphof 1952; *A. pelegrina*) and Jacquin (1797; *A. caryophyllaea*). Several researchers paid attention to the rhizome and the root system for different purposes, and drawings and photographs have been presented by Comber (1946, *A. ligtu* hybrids), Buxbaum (1951, *A. aurea, A. brasiliensis, A. psittacina*), Broertjes and Verboom (1974, unspecified), Dahlgren *et al.*, (1985, *A. aurea*), Wilkins (1985, unspecified), Bayer (1987, *A. aurea, A. pulchra* ssp. *pulchra*,

A. ligtu ssp. *incarnata*, *A. exserens*) and Monette (1992, *A. pulchella*, cultivar 'Regina').

The aim of our investigations of the underground organs of *Alstroemeria* was to analyse the general morphological structure of the rhizome, and to determine the specific morphological features of the rhizomes of some species and hybrids. Special attention was given to the size and the branching capacity of the rhizomes, because it was suspected that these features might play a role in micropropagation. During our studies, the rhizomes and root systems of species and hybrids were studied. These observations, and the study of the literature on the rhizomes of *Alstroemeria* enabled us to outline the general structure of the rhizome, and its manifestation in some species and hybrids.

Materials and Methods

The species that were used are the Chilean species *A. aurea*, *A. hookeri* ssp. *hookeri*, *A. ligtu* ssp. *simsii*, *A. magnifica* ssp. *magnifica*, *A. pelegrina*, *A. philippii* and *A. werdermannii*, and the Brazilian species *A. inodora* and *A. psittacina*. The interspecific hybrids consisted of the diploid hybrid cultivar 'Orchid' (*A. violacea* × *A. aurea*), the triploid hybrid cultivar 'Eleanor' (with two genomes of *A. aurea* and one unspecified genome), and a tetraploid hybrid between *A. pelegrina* and *A. psittacina*. The plants had grown in greenhouses at Wageningen and Rijswijk, in pots or directly in the soil, for two years, and were dug out in October. The rhizomes and the root systems of two plants of each taxon were excavated, washed, and their features were recorded in drawings and photographs, and described. These features concerned the shape of the rhizome, the direction of growth of the main rhizome and the lateral rhizomes, its colour, length, diameter, internodal length, the features of the scaly leaves, the mode of branching, the breakability of lateral rhizomes from the main rhizome, and the numbers of living shoots, nodes, rhizomes (> 5 mm), swollen (visible) axillary buds (< 5 mm). The storage roots were described through their total number on the plant, their shape, whether the tuberous roots reduced in width gradually or suddenly, the position of the storage roots (distance from the rhizome), their length, and diameter.

Results and discussion

The morphological structure of the rhizome

Bayer (1987, p. 9) described the general appearance of the rhizomes of *Alstroemeria*. Translation from German into English leads to the following description:

- The rhizomes are sympodial, with several stems, and fleshy. They can be elongated and cylindrical, with a diameter from 0.7 to 1.2 cm, orthotrop to plagiotrop, or compact and gnarled, yellowish brown or white, apically generally white, with loose or occasionally dense (*A. polyphylla*, *A. andina*) triangle-shaped scales, which can be

fleshy or membranous. Older parts are often more slender than younger parts.
The older parts are 'woody', brown or darkly red. The rhizomes are often stoloniferous (*A. ligtu*, *A. magnifica* ssp. *maxima*) or ramified (*A. aurea*). Generally, the rhizomes are found at considerable depths. -

Buxbaum (1951) studied the morphological structure of the rhizome of *A. aurea* in great detail and described it, as follows:

"The thickened apex of the rhizome develops a very large bud so that the rhizome appears monopodial "....." however, the large bud is really the axillary bud of the first scale leaf of the aerial sprout. Its first internode is greatly enlarged and the axillary bud is forwarded to such an extent that sometimes the further internodes are suppressed. Thus a chain of basal internodes is developed, imitating a long monopodial rootstock, although really the rhizome is sympodial. As even the first scale of the thin part of the stem bears an axillary bud, "....." there is a beginning of ramification of the rhizome".

One of the detailed drawings that accompanied this work is presented in Figure 1.

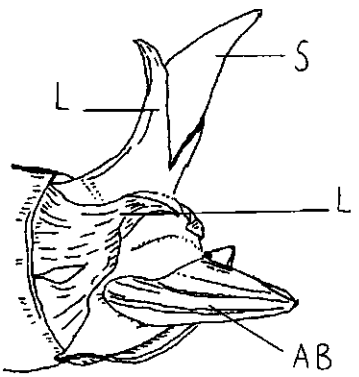

FIGURE 1. Drawing of rhizome tip of *A. aurea* (from Buxbaum, 1951). AB= axillary bud, L= leaf, S= shoot.

After studying the literature and an inspection of the rhizomes of several species, an attempt was made to describe and schematically illustrate the sympodial structure of the rhizome, starting from a seedling of which the first shoot had just emerged:
The rhizome of *Alstroemeria* has a sympodial growth habit, i.e. the first shoot of a seedling terminates with a leaf and the apical meristem stops to grow (Figure 2a). Growth is continued by the axillary bud, which is positioned in the axil of the first (scaly)

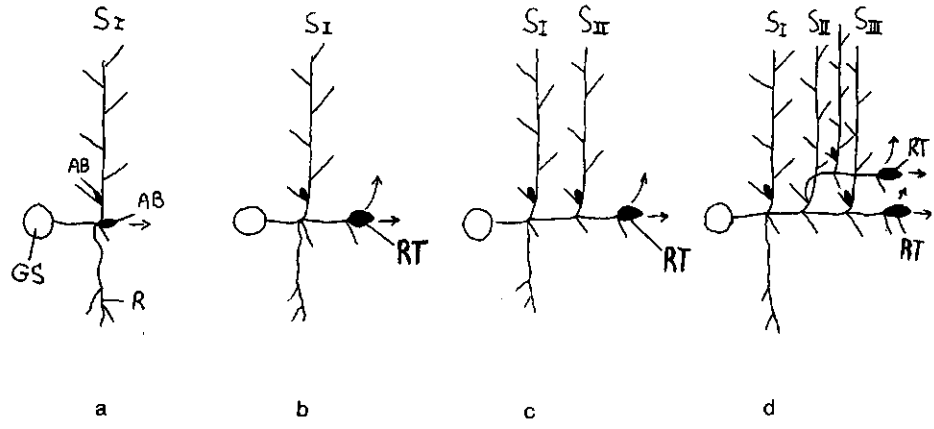

FIGURE 2a-d. The structure of the rhizome of *Alstroemeria* at four stages of its development. AB= axillary bud, GS= germinated seed, L= leaf, N= node, R= root, RT= rhizome tip, S= shoot. The numbers 1,2,3,4,5,6,7 indicate the subsequence of leaves. The numbers I, II and III indicate the subsequence of shoots.

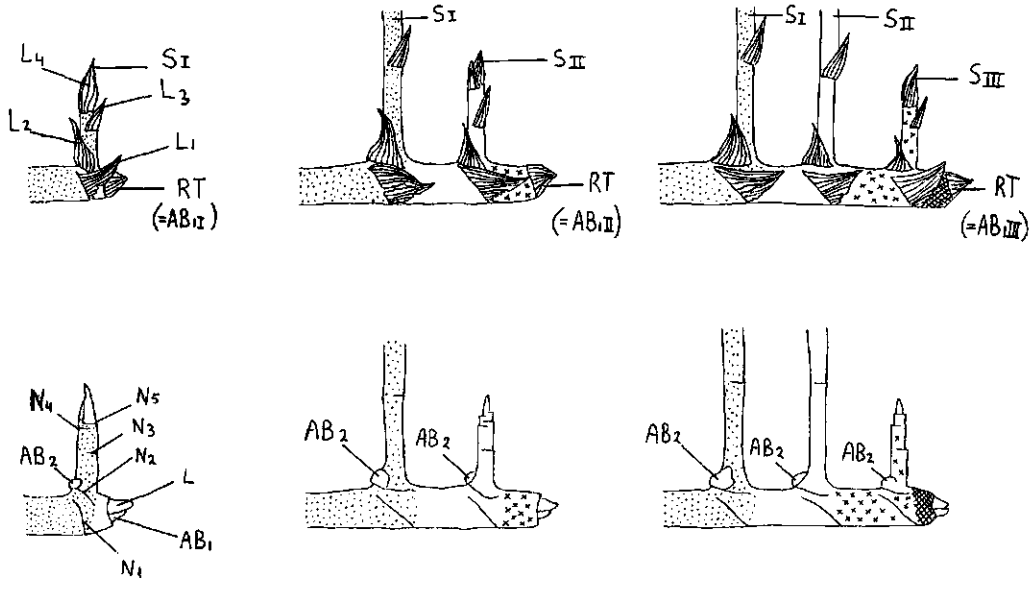

FIGURE 3a-c. Three stages in the development of the rhizome, before and after the removal of the scaly leaves at the nodes. For an explanation of codes see Figure 2.

leaf. The bud develops into the rhizome tip and then gives rise to the second shoot (Figure 2b-c). Again an axillary bud in the axil of the first leaf of this shoot is pushed forward and the process repeats itself. Thus a rhizome is formed by the chain of basal stem internodes of successively developed shoots (Figure 2c). Each shoot also has a bud in the axil of the second leaf. The higher leaves have no visible axillary buds. The axillary bud of the second leaf can either remain dormant or it can develop into a lateral rhizome. In the latter case, the rhizome starts to branch (Figure 2d).

A schematic drawing of the first rhizome segments of a seedling further illustrates the structure of the rhizome (Figure 3). The different types of shading that have been used indicate the subsequence of shoots and of rhizome segments. The triangle-shaped scales on the rhizome are in fact the halves of the scaly leaves that are torn by the expansion of the axillary buds (AB1; see Figures 1 and 3). The axillary buds, which are positioned in the axils of the second leaf of each shoot,
may recover from their dormancy and develop into lateral rhizomes. Figure 4 shows details of the rhizome of *A. psittacina*, with newly grown lateral rhizomes.

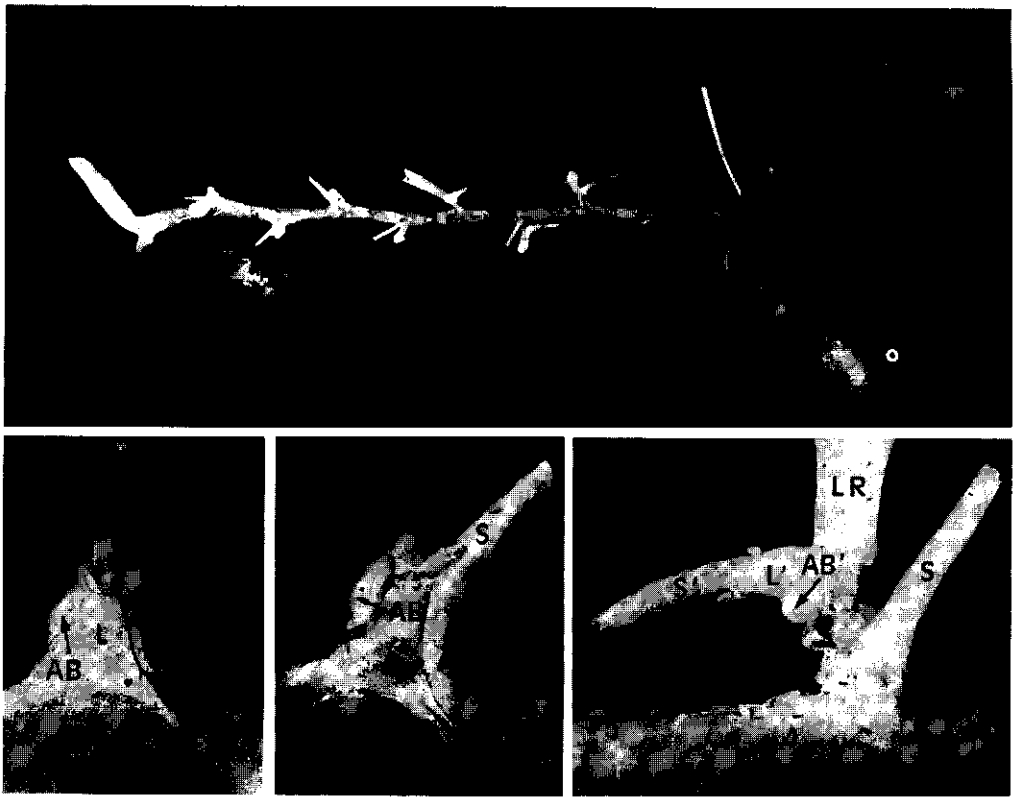

FIGURE 4. The rhizome of *A. psittacina*, with lateral rhizome formation.
AB= axillary bud, L= leaf, LR= lateral rhizome, MR= main rhizome, S= shoot,
AB'= axillary bud of lateral rhizome, L'= leaf of lateral rhizome, S'= shoot of lateral rhizome.

About the storage roots Bayer (1987, p. 9, translated) writes:

-There are several storage roots on a rhizome. They are tuberous, 2 to 10 cm long and up to 12 mm in diameter. They are rarely ending indistinctly (*A. ligtu*) or very long (up to 60 cm) and cylindrical (*A. revoluta*); Exceptionally no storage roots are found (*A. graminea*). The storage roots are always fleshy, with dense, woolly hairs at the surface, and very brittle.-

In the species descriptions Bayer describes that the storage roots can be tuberous, cylindrical, club-shaped or spindle-shaped, and that the thickened part of the root can start closely near the rhizome or at a larger distance. The storage roots contain water and starch (Dahlgren *et al.*, 1985). In Chile, the starch of the storage roots, mostly of *A. ligtu*, has been used to prepare 'chuño', which served as food for small children and persons suffering from digestive troubles (Bullock, 1952).

The underground organs of nine species and three hybrids

In our studies we observed the rhizomes and tuberous roots of nine species and three hybrids (Table 1). The rhizomes in two to four years old plants of five of these species (*A. aurea*, *A. hookeri*, *A. ligtu*, *A. pelegrina* and *A. werdermannii*) were also described by Bayer (1987). Our observations are summarized in Table 1. Line drawings of the rhizomes and storage roots of each of the species and hybrids, including a side view and a bottom view of the rhizomes, are presented in the figures 5-16. Our descriptions and drawings on *A. aurea*, *A. ligtu* and *A. psittacina* are in most cases concurrent with those of Bayer (1987), Buxbaum (1951) and Comber (1946), except for the fact that Bayer (1987) described the lateral rhizomes of *A. aurea* as orthotrop stolons which were in our observations plagiotrop lateral rhizomes. Other discrepancies are the classification of the rhizomes of *A. pelegrina* and *A. werdermannii* as elongated and cylindrical ("langgestreckt, walzlich", Bayer, 1987), instead of compact and gnarled rhizomes in *A. pelegrina* and compact and cylindrical rhizomes in *A. werdermannii*. The rhizomes of *A. magnifica* ssp. *magnifica* and *A. inodora* have, to our knowledge, not been described or drawn before. Drawings of the rhizomes of *A. hookeri* ssp. *hookeri*, *A. pelegrina*, *A. philippii* and *A. werdermannii* have not been published before.

From Table 1 and the figures 5-16 it can be concluded that the underground organs, and especially the rhizomes of species, vary considerably in size and overall appearance. Especially their length (cf. *A. ligtu* ssp. *simsii* and *A. aurea*), diameter (cf. *A. philippii* and *A. aurea*), and internodal length (cf. *A. philippii* and *A. aurea*) can be highly diverse. It is not easy to characterize the mode of branching of a rhizome, because a description does not give a complete image of how and to what extent the rhizome is branched. For instance, it is not clear what is exactly meant by Bayer, when she uses the terms stoloniforous and ramified. However, the terms linear branching, or radial branching may also be insufficient, because there are also rhizomes with more or less intermediate modes of branching, e.g. *A. inodora*. In our descriptions an attempt was made to further characterize the mode of branching based on the number of

rhizomes, and the number of swollen axillary bud. From these observations it is evident that the rhizome of *A. inodora* had by far the most rhizomes and axillary buds. The bottom view of the rhizomes provides a further characterization of the mode of branching in each of the species. Nevertheless, it should be considered that these plants had not grown in their natural environment, and several of the morphological traits of the rhizome might have been influenced by the conditions under which the plants had grown.

TABLE 1. Observations on the rhizomes and storage roots of nine species and three cultivars of *Alstroemeria*. For an explanation of the features that were observed, see Material and Methods.

Observations	*A. aurea*	*A. hookeri* ssp. *hookeri*	*A. ligtu* ssp. *simsii*	*A. pelegrina*	*A. philippii*	*A. werdermannii*
Plants grown in:	ground bed	ground bed	ground bed	ground bed	pot	ground bed
Rhizome	(drawing p. 24)	(drawing p. 25)	(drawing p. 26)	(drawing p. 28)	(drawing p. 29)	(drawing p. 30)
shape	elongated, cylindrical	compact, cylindrical	short-compact, gnarled	compact, gnarled	short-compact, gnarled	compact, cylindrical
direction main/lat	plagiotrop/plagiotrop	plagiotrop/plagiotrop	plagiotrop/orthotrop	plagiotrop/plagiotrop	plagiotrop	plagiotrop/plagiotrop
colour	white	white	white to brownish	brown, apically white	white to brownish	brown,apically white
length	3-14cm	4-5 cm	1-1.5cm	6-7 cm	1-2 cm	3-4 cm
diameter	10-12 mm	9-10 mm	8 mm	8-9 mm	3 mm	6-8 mm
internodal length	15-25 mm	3-5 mm	2-3 mm	1-3 mm	1-1.5 mm	2 mm
scaly leaves	small, fleshy	small, fleshy	fleshy	mostly membranous	mostly membranous	many, large, fleshy
branching mode	lineair	radially	radially	radially	radially	radially
breakability	not	not, see remark below	not very	very	very	very
n living shoots	5-10	± 40	2-5	15-20	10-15	10-20
n nodes	± 35-40	± 100	± 30	± 100-150?	± 50	± 100
n rhizomes	5-6	6-8	3-4	8-10	4-5	9
n axillary buds	0-2	4-5	3-4	10-13	3	12
remarks	solid rhizome with a smooth surface	plant cespitose, shoots initially horizontal, keeping rhiz. together	first internodes of lateral rhizomes are extremely long	old rhizome is corky	old rhizome is corky	-
Storage roots						
number	30-35	20-25	3-5	40-50	100-150	2
shape	cylindrical, irregular	cyl. to spindle-shaped	cyl. to club-shaped	cylindrical, irregular	spindle-shaped	spindle-shaped
tip red. in width	gradually	gradually	suddenly	gradually	± suddenly	gradually
position	directly at rhizome	1-20 cm from rhiz.	1-1.5 cm from rhiz.	3-10 cm from rhiz.	0.5-20 cm from rhiz	18-21 cm from rhiz.
length	8-10	5-15 cm	4-6 cm	3-18 cm	2-8 cm	3-3.5 cm
diameter	4-5 mm	6-10 mm	8-9 mm	7-9 mm	5-8 mm	8 mm

Table 1 continued.

Observations	*A. magnifica* ssp. *magnifica*	*A. inodora*	*A. psittacina*	diploid hybrid cv. 'Orchid'	triploid hybrid cv. 'Eleanor'	tetraploid hybrid (*A. pelegr.* ´ *A. psitt.*)
Plants grown in :	ground bed	pot	pot and ground bed	ground bed	ground bed	ground bed
Rhizome	(drawing p. 27)	(drawing p. 31)	(drawing p. 32)	(drawing p. 33)	(drawing p. 34)	(drawing p. 35)
shape	compact, gnarled-cyl.	elongated, cylindrical	elongated, cylindrical	elongated, cylindrical	elongated, cylindrical	compact, cylindrical
direction main/lat	plagiotr. to orth./orth.	pagiotrop/plagiotrop	plagiotrop/plagiotrop	plagiotrop/plagiotrop	plagiotrop/plagiotrop	plagiotrop/plagiotrop
colour	brown, apically white	white, apically pink	white, apically pink	white	white, apically pink	white, apically pink
length	1.5-6 cm	1-17 cm	9-11 cm	5-8 cm	12-14 cm	?-8 cm
diameter	6-8 mm (old: 4-5 mm)	5-7 mm	2-5 mm	4-6 mm	3-6 mm	9 mm
internodal length	1.5-2 mm	13-15 mm	5-15 mm	4 -5 mm	4-7 mm (old: 3-5 mm)	3-4 mm
scaly leaves	large, fleshy	small, fleshy	fleshy, (old membran.)	fleshy	fleshy	fleshy
branching mode	radially	lineair to radially	lineair	lineair	lineair	radially
breakability	very	not	not very	very	not very	not
n living shoots	0-1	± 50	5-10	5-10	5-10	20-25
n nodes	± 70	80-100	60-80	50-60	20-25	± 100
n rhizomes	4-5	50	6	3	1	15
n axillary buds	6-8	0-5	2	0	3	5
remarks	old rhizome is corky	continuously growing, solid, highly ramified rhizome with a smooth surface	smooth surface, first five to ten internodes of lateral rhizomes slender without roots or shoots	long rhizomes with smooth surface	long rhizomes with smooth surface	continuously growing, solid, higly ramified rhizome with smooth surface
Storage roots						
number	12-15	50	30-35 (clusters of ± 8)	35-40	10-15	50-60
shape	cyl. to club-shaped	spindle-shaped	spindle-shaped	cyl. to club-shaped	irregular, cylindrical	spindle to club-shaped
tip red. in width	± suddenly	± suddenly	suddenly	suddenly	gradually	± suddenly
position	2-25 cm from rhiz.	1.5-15 cm from rhiz.	0-1cm from rhiz.	1-2 cm from rhiz.	4-10 cm from rhiz.	5-16 cm from rhizome
length	2-7 cm	6 cm	3-3.5 cm	12 cm	10-15 cm	6-7 cm
diameter	5-8 mm	9 mm	10 mm	9 mm	5-6 mm	10-12 mm
remarks	-	appear along the rhizome	appear in clusters on rhizome	end in a small tip		appaer along the rhizome

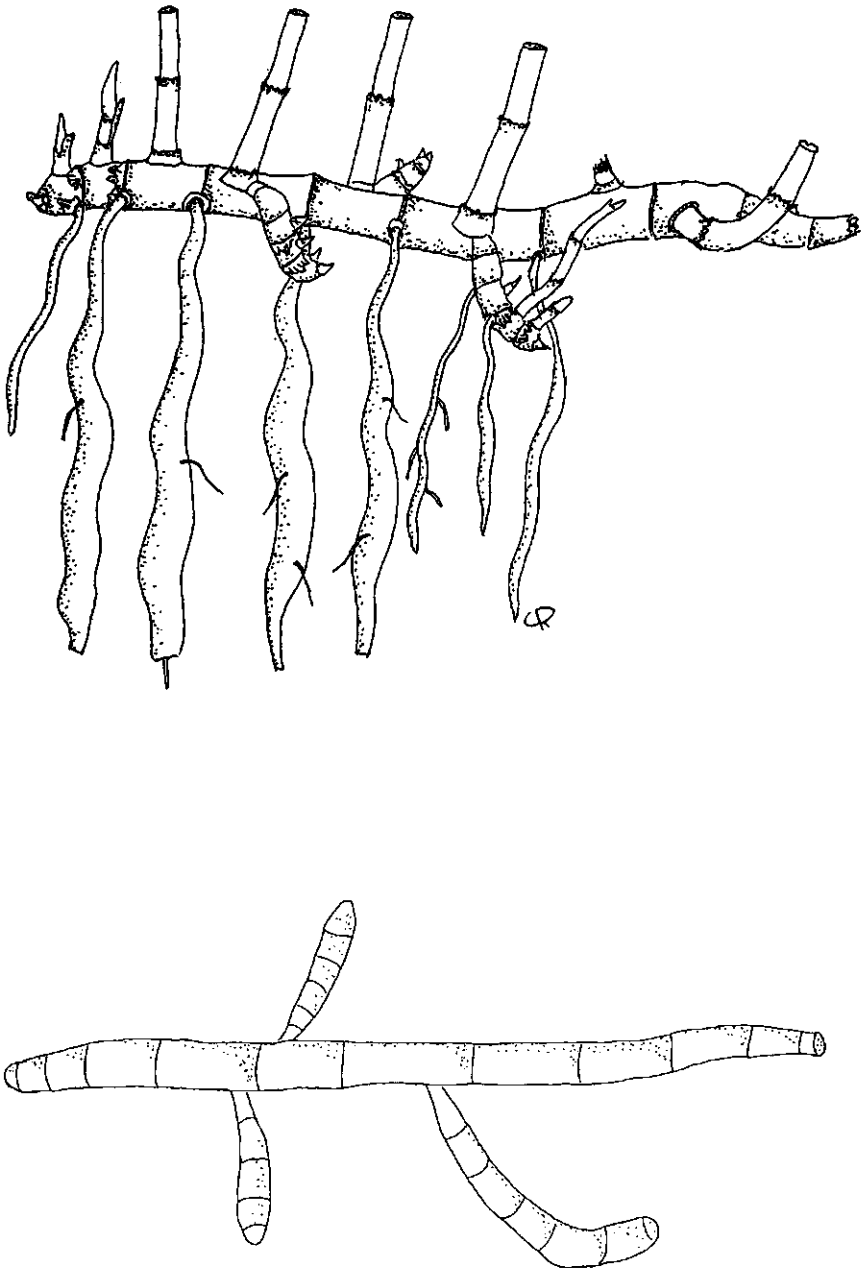

FIGURE 5. Side and bottom view of the rhizomes of *A. aurea*.

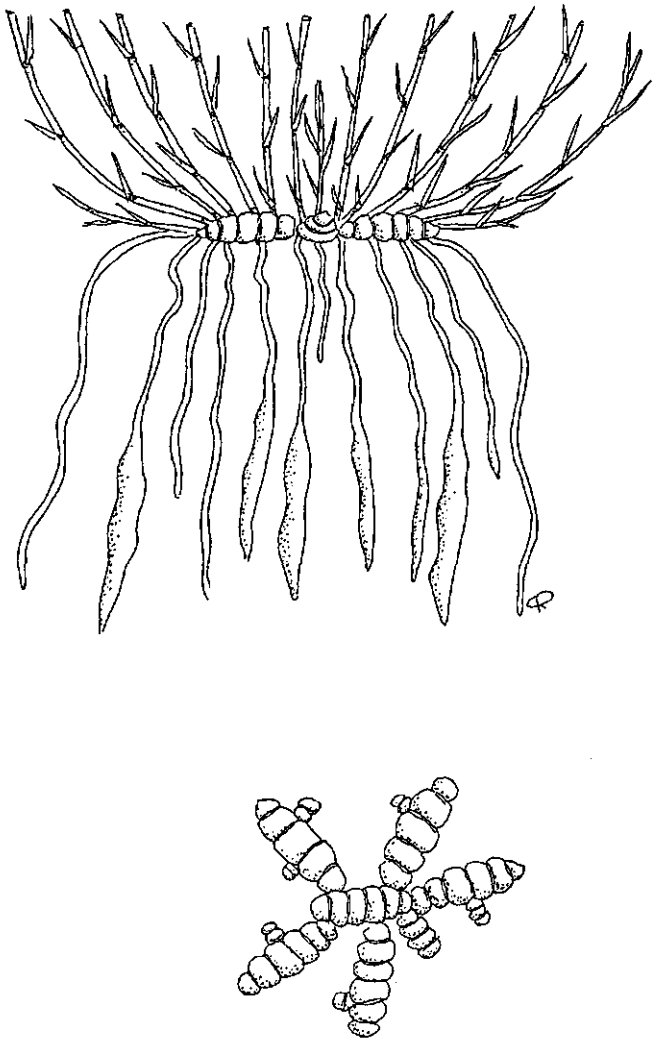

FIGURE 6. Side and bottom view of the rhizomes of *A. hookeri* ssp. *hookeri*.

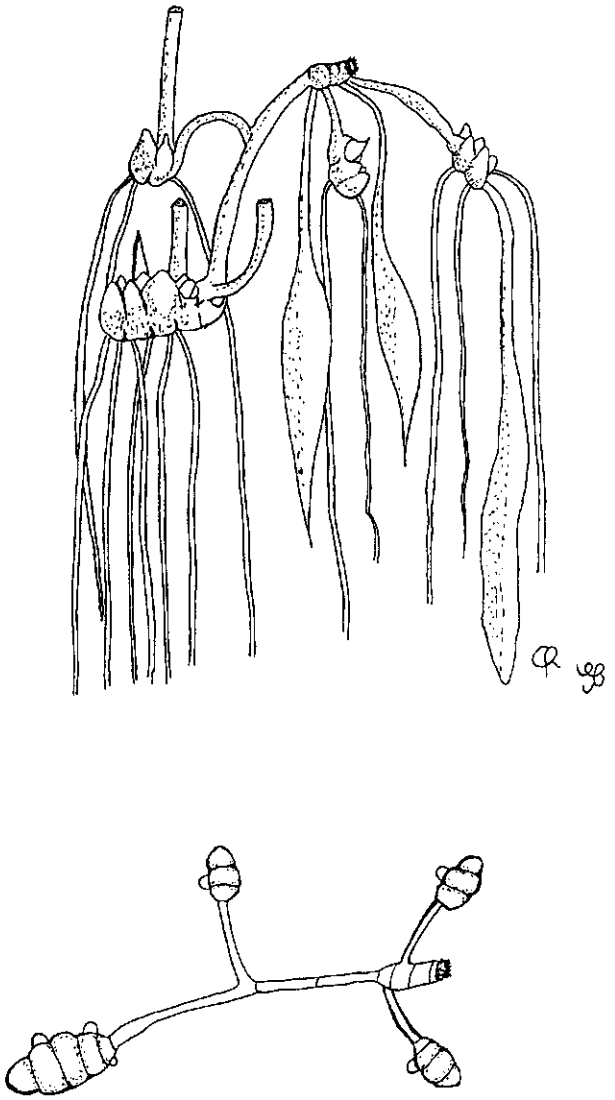

FIGURE 7. Side and bottom view of the rhizomes of *A. ligtu* ssp. *simsii.*

FIGURE 8. Side and bottom view of the rhizomes of *A. magnifica* ssp. *magnifica*.

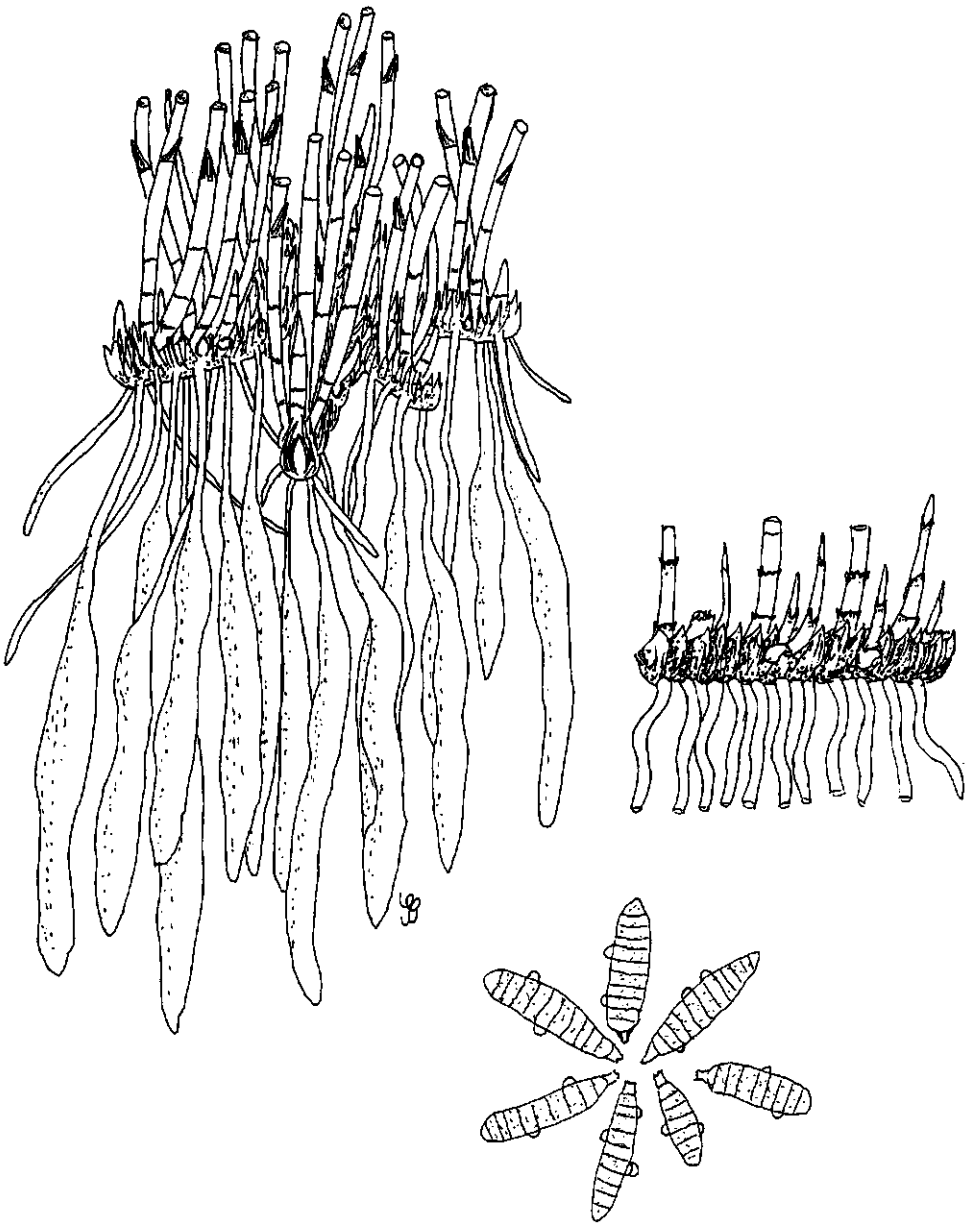

FIGURE 9. Side and bottom view of the rhizomes of *A. pelegrina*.

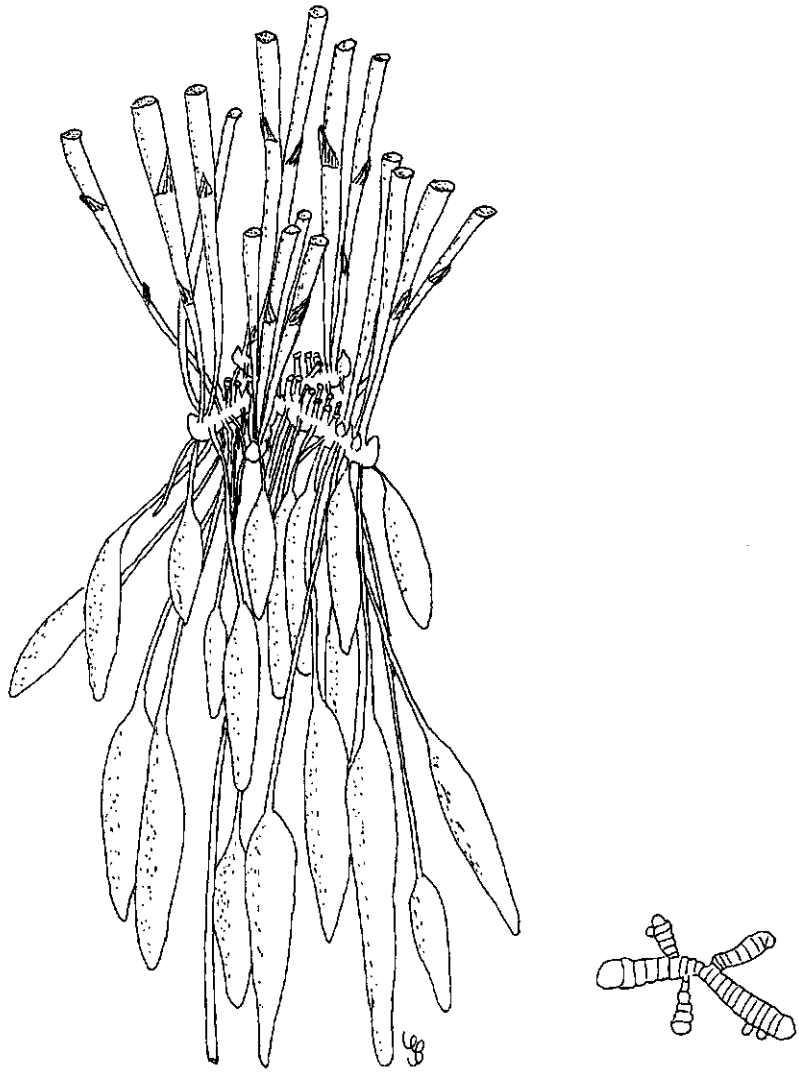

FIGURE 10. Side and bottom view of the rhizomes of *A. philippii*.

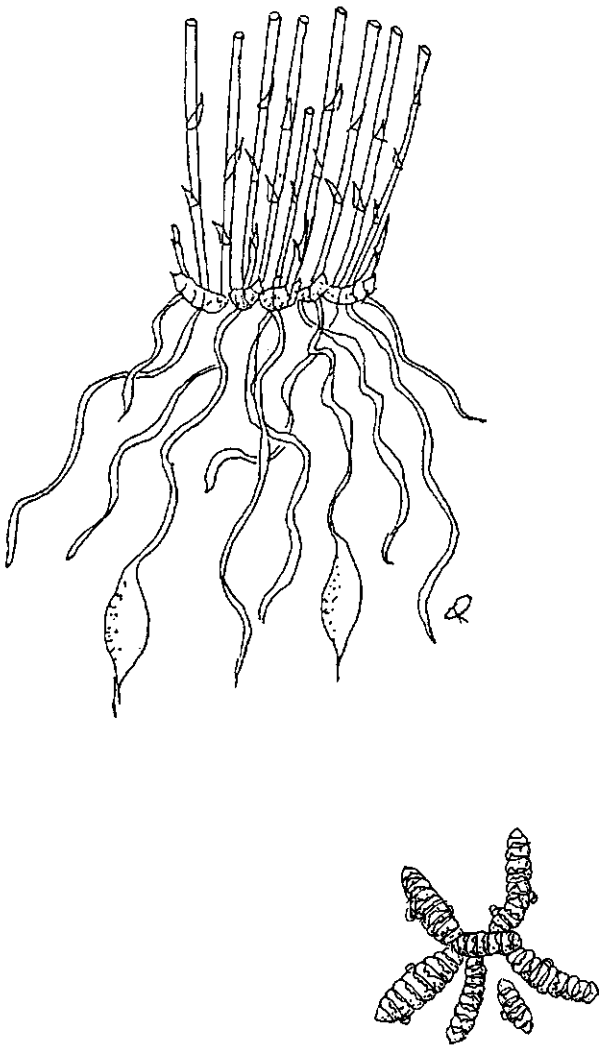

FIGURE 11. Side and bottom view of the rhizomes of *A. werdermannii*.

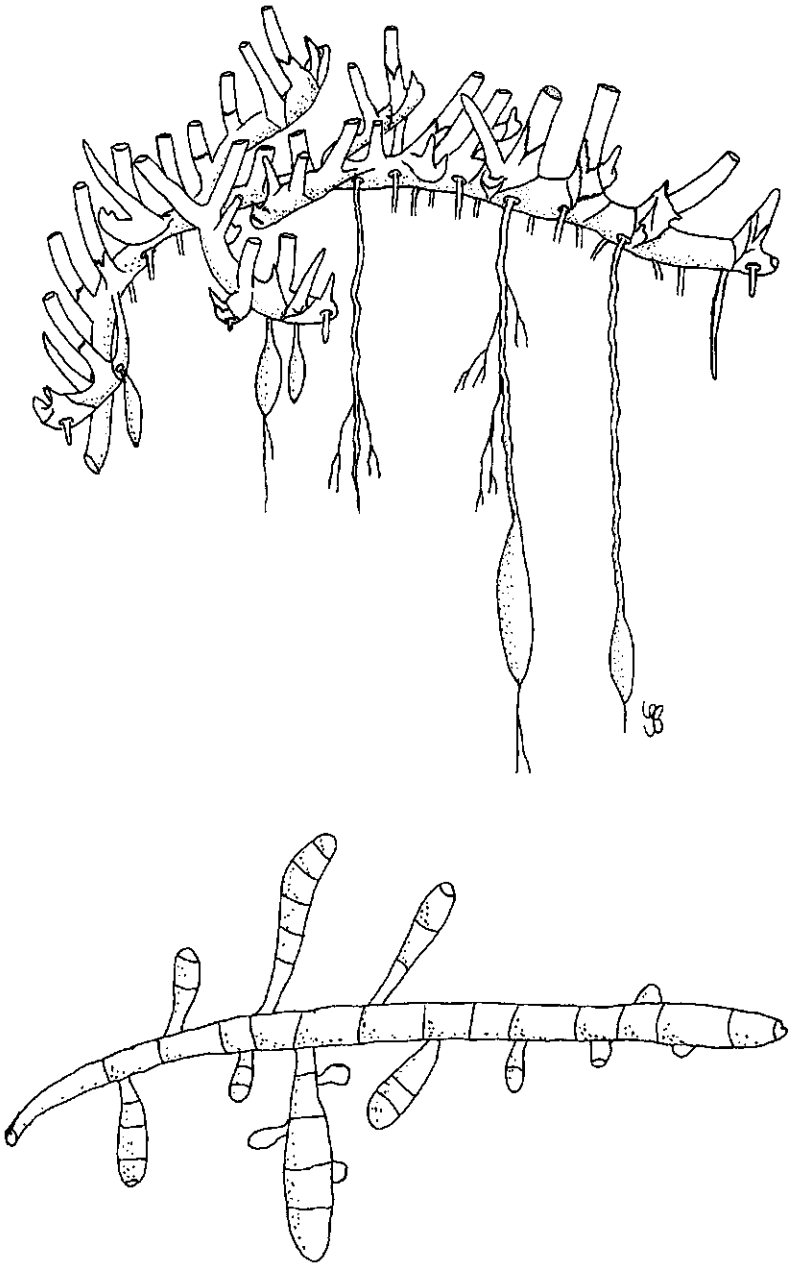

FIGURE 12. Side and bottom view of the rhizomes of *A. inodora*.

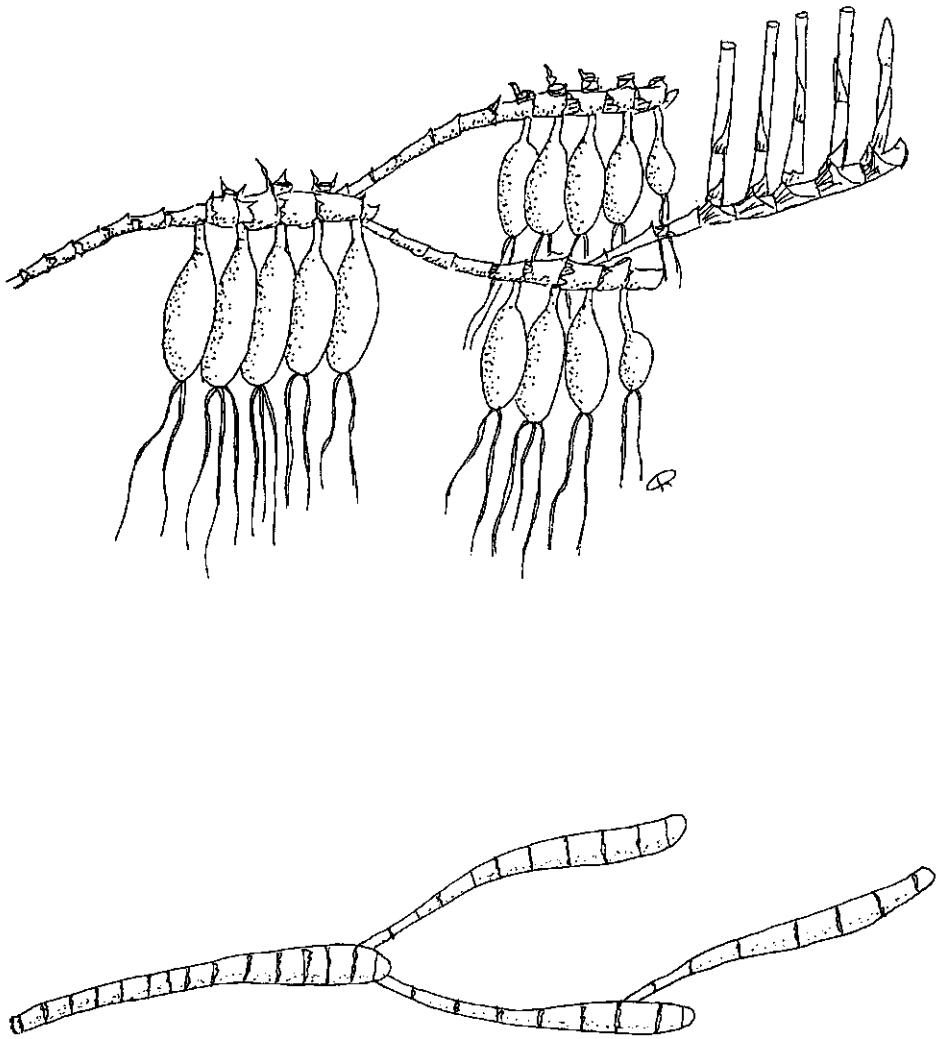

FIGURE 13. Side and bottom view of the rhizomes of *A. psittacina*.

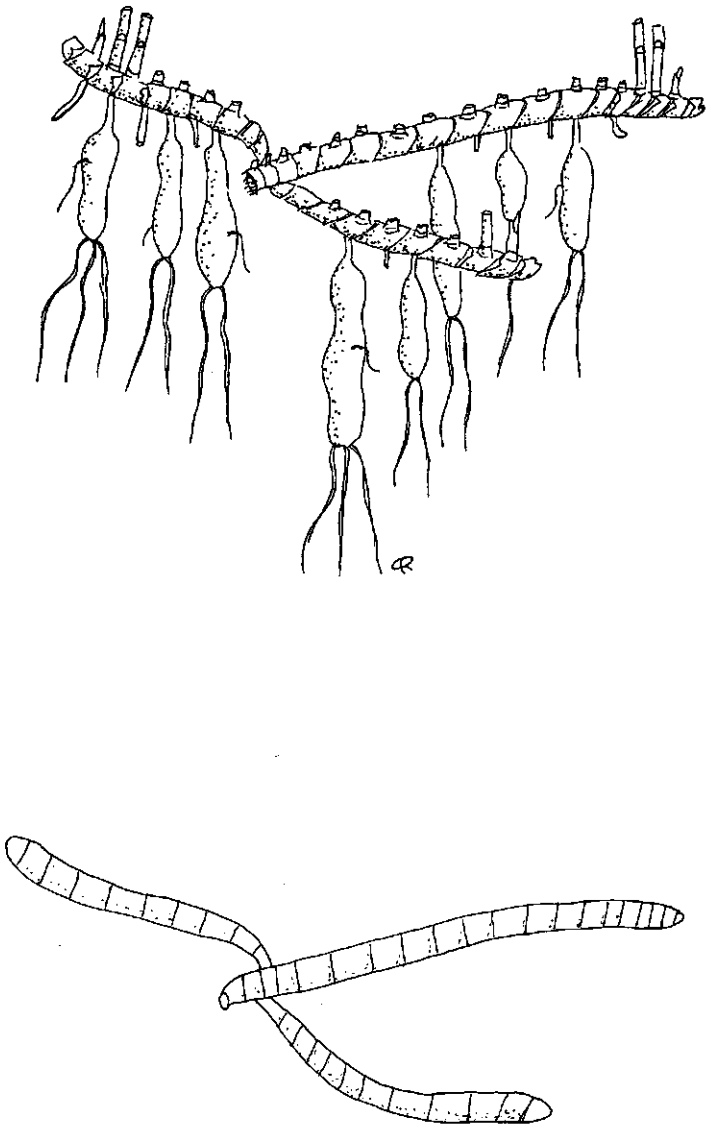

FIGURE 14. Side and bottom view of the rhizomes of the diploid hybrid cv. 'Orchid'.

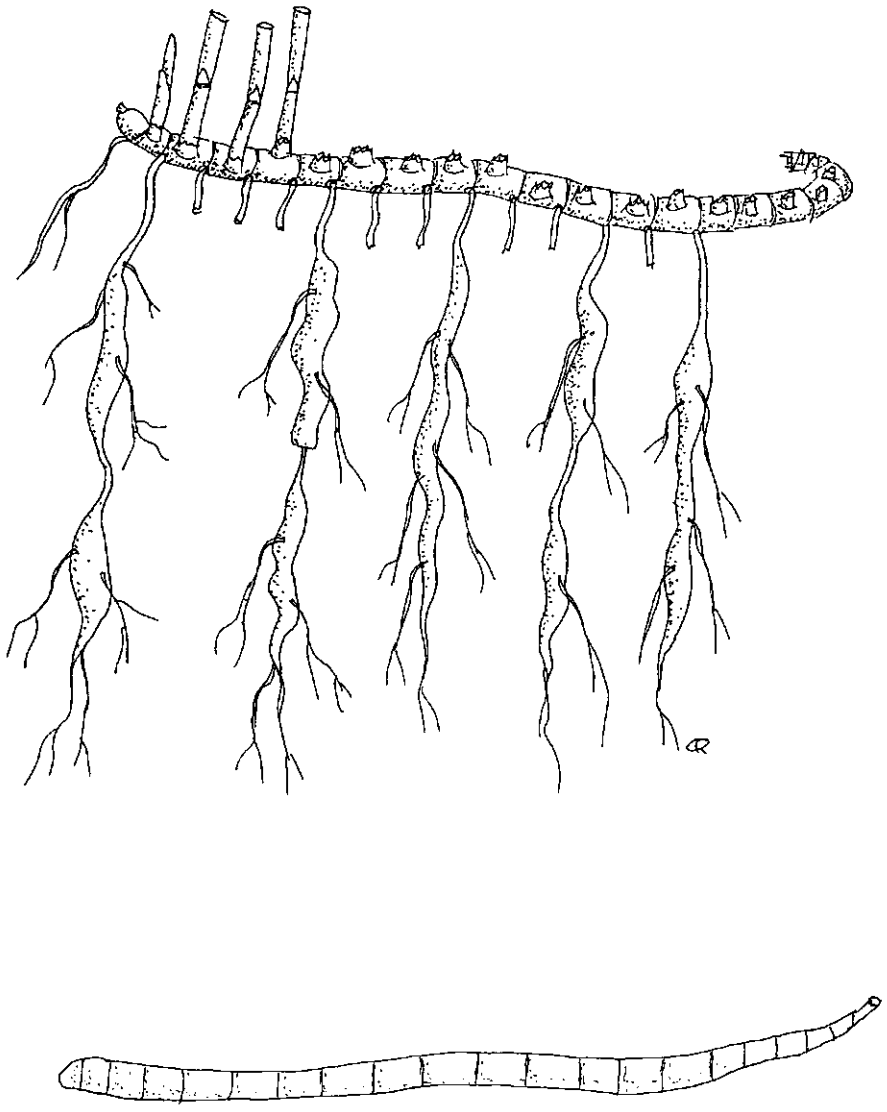

FIGURE 15. Side and bottom view of the rhizomes of the triploid hybrid cv 'Eleanor'.

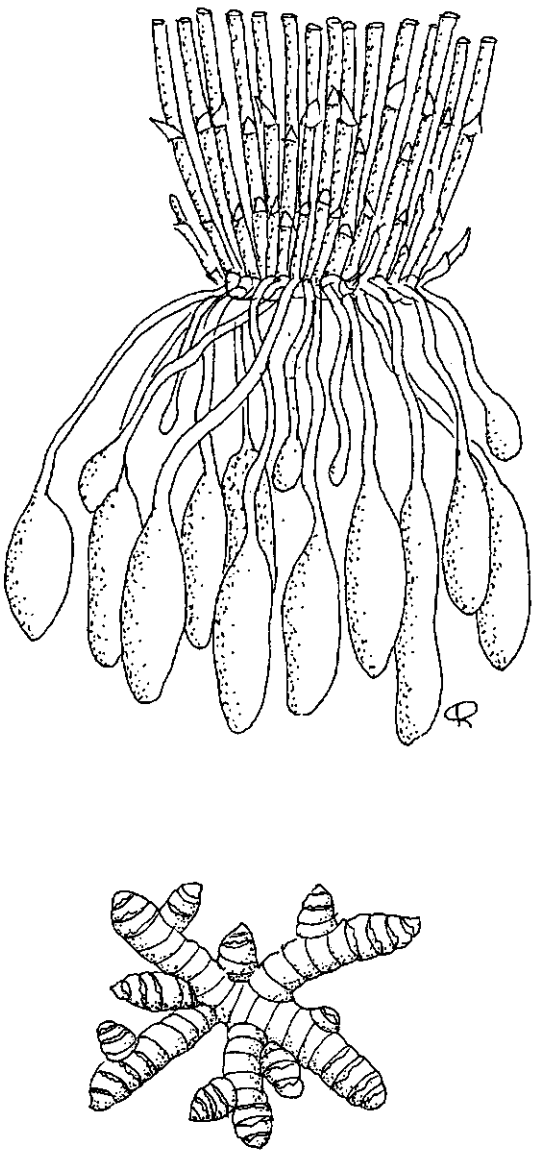

FIGURE 16. Side and bottom view of the rhizomes of a tetraploid hybrid between *A. pelegrina* and *A. psittacina*.

Acknowledgements

I gratefully acknowledge Robert-Jan Quené for many of the drawings and measurements of the underground organs of *Alstroemeria* taxa, and Dr. André van Lammeren for stimulating discussions at the early stages of this research.

Chapter 3

Giemsa C-banded karyotypes of eight species of *Alstroemeria* L. and some of their hybrids

J.H. Buitendijk and M.S. Ramanna. 1996. Annals of Botany 78: 449-457.

Abstract

Karyotype analysis of *Alstroemeria angustifolia* ssp. *angustifolia*, *A. aurea*, *A. inodora*, *A. ligtu* ssp. *ligtu*, *A. magnifica* ssp. *magnifica*, *A. pelegrina*, *A. philippii* and *A. psittacina* using Feulgen-staining and Giemsa C-banding techniques revealed for each species a characteristic chromosome morphology and C-banding pattern. These characteristics could be used to identify many individual chromosomes in diploid interspecific hybrids. Besides interspecific variation, some degree of intraspecific variation in C-banding pattern was observed within *A. angustifolia* ssp. *angustifolia*, *A. aurea*, *A. ligtu* ssp. *ligtu*, *A. magnifica* ssp. *magnifica* and *A. philippii*.

All species had large chromosomes (2n=2x=16) and asymmetric karyotypes. In many species the short arms of the acrocentric chromosomes were darkly stained upon Giemsa C-banding. These telomeric bands seemed satellites. B-chromosomes were observed in one species, *A. angustifolia* ssp. *angustifolia*. A variable number of large intercalary and telomeric C-bands was present in the Chilean species, whereas the Brazilian species showed only small C-bands. The differences in karyotypes suggest an early separation of the Chilean and Brazilian species, after which speciation followed different evolutionary pathways. In *Alstroemeria* the Giemsa C-banding technique can be valuable to plant taxonomists for unravelling species relationships.

Key words: Alstroemeria, Inca lily, evolution, Giemsa C-banding, karyotype.

Introduction

Alstroemeria (Inca lily) is cultivated for its ornamental value as a cut flower, bedding plant or potted plant. The species occur in Chile, Brazil, Venezuela, Ecuador, Peru, Bolivia, Paraguay and Argentina, where the plants grow in a wide variety of ecological niches (Aker and Healy, 1990). The centre of diversity is central Chile (Bayer, 1987), with a satellite distribution in central and eastern Brazil (Aker and Healy, 1990). All species are geophytic, herbaceous plants with large storage roots. They spread through the growth of rhizomes as well as through the dispersal of seeds. More than a hundred species names have been published so far (Uphof, 1952; Bayer, 1987; Ravenna, 1988). Bayer (1987) recently made a revision of the Chilean *Alstroemeria* species, but

several taxonomic questions remain unanswered. For example, the delimitation in the species complex *A. magnifica - A. pulchra* is probably not yet well defined (Bayer, 1987). The most urgent taxonomic questions concern the Brazilian species, on which there is no monograph available at present.

Besides the chromosome numbers of some species (Taylor, 1926; Whyte, 1929) and cultivars (Tsuchiya *et al.*, 1987; Hang and Tsuchiya, 1988) little is known on karyotypes (Lakshmi, 1976; Tsuchiya and Hang, 1989; Rustanius *et al.*, 1990; Stephens *et al.*, 1993) and on the degree of genome differentiation within the genus. The presence of satellites and their possible role in karyotype alteration was emphasized by Satô (1938). All species in which the chromosome number has been counted, are diploid (2n=2x=16). However, a chromosome number of 2n=4x=32 was reported for one accession of *A. ligtu* (Goodspeed, 1940). The occurrence of post-fertilization barriers to interspecific hybridization (Buitendijk *et al.*, 1995), could result from a differentiation of species genomes. To gain an insight into the degree of genomic differentiation, we investigated the karyotypes and the distribution of heterochromatin in some species and interspecific hybrids.

Materials and methods

Plant Material

Seeds and plants of species were obtained from botanical gardens and breeders. The collection is maintained at the Department of Plant Breeding of the Agricultural University of Wageningen and includes accessions of six Chilean species, two Brazilian species and eleven interspecific hybrids (Table 1). The Chilean species were identified on basis of Bayer (1987). The identity of the plants of *A. inodora* and *A. psittacina* was verified by consulting their first descriptions (Herbert, 1837; Schultes and Schultes, 1829). As to the separation of these species, much further taxonomical study is needed. For example, *A. psittacina* is considered to be conspecific with *A. pulchella* L.f. (Baker, 1888; Uphof, 1952). Material of the plants studied will be preserved at WAG (Wageningen Herbarium collection).

Pre-treatment, fixation, Feulgen-staining and Giemsa C-banding

Root or shoot tips were used for the preparation of somatic chromosome spreads. The material was collected during morning hours and pre-treated in 2mM 8-hydroxyquinoline for 7-8 h at 4 °C. Root tips were fixed in acetic acid : ethyl alcohol (1:3) and shoot tips in chloroform : acetic acid : ethyl alcohol (1:3:6). Material was fixed for at least 24 h.

Before Feulgen-staining, the fixed material was washed in water and hydrolysed in 1N HCl at 60 °C for 8 min. The hydrolysed tissue was stained with leuco basic fuchsin for 1 h and squashed in a drop of acetocarmine. Slide preparations were frozen in liquid nitrogen, air-dried and mounted with Euparal.

TABLE 1. Source and distribution of species accessions and interspecific hybrids.

Plant material	Distribution / altitude**	Accession / Source*
Species:		
A. angustifolia Herb. ssp. *angustifolia*	Chile, 33° S.L. / <1000 m[1)]	AN1W / W
A. aurea Graham	Chile, 36°-42° / 47°200-1800 m[1)]	A001 / W A002 / W A003 / W A005 / BGA A010 / BGW G015 / W
A. inodora Herb.	Central and Southern Brasil[2)]	P002 / S P003 / K P004 / Z
A. ligtu L. ssp. *ligtu*	Chile, 33°-38° S.L. / 0-800 m[1)]	F051 / W AL1S / S
A. magnifica Herb. ssp. *magnifica*	Chile, 29°-32° S.L. / 0-200 m[1)]	Q001 / Z Q008 / P F169 / W
A. pelegrina L.	Chile, 32°-33° S.L. / 0-50 m; Peru, coastal regions near Lima[1)]	C039 / W C042 / W C049 / W C063 / W
A. philippii Baker	Chile, 28° S.L. / <1000 m[1)]	B018 / W B013 / W
A. psittacina Lehm.	Northern Brasil[2)]	D031 / W D032 / W
Interspecific hybrids:		
A. aurea x *A. inodora*		A001PA004-1 / B
A. aurea x *A. magnifica* ssp. *magnifica*		A016Q001-1 / B
A. aurea x *A. psittacina*		A001D032-1 / B
A. inodora x *A. pelegrina*		P002C049-5 / B
A. ligtu ssp. *ligtu* x *A. aurea*		AL1SA002-1 / J AL5SA002-1 / J
A. magnifica ssp. *magnifica* x *A. inodora*		Q008P004-2 / B
A. pelegrina x *A. aurea*		C049A001-1 / B
A. pelegrina x *A. inodora*		C059P002-1 / B
A. pelegrina x *A. magnifica* ssp. *magnifica*		C042Q001-3 / B
A. psittacina x *A. philippii*		D032B018-1 / B

* B=Buitendijk *et al.*, 1995; BGA=Botanical Garden VUA Amsterdam; BGW=Botanical Garden Wageningen; J=De Jeu and Jacobsen, 1995; K=Könst Alstroemeria; P=Parigo (UK); S=Van Staaveren; W=Wülfinghoff Freesia; Z=Koninklijke Van Zanten.

** Literature source:1) Bayer, 1987; 2)Aker and Healy, 1990.

For Giemsa C-banding, the fixed material was washed in water and macerated for 30-65 min at 38 °C in an enzyme mixture of 15% pectinase (Sigma P-5146) and 1.5% cellulase (Onozuka, R-10) in 0.1 M citrate buffer, pH 4.8, and washed in citrate buffer. The Giemsa C-banding was carried out according to the procedure described by Ramachandran and Ramanna (1985).

Chromosome measurement

Chromosomes were measured on enlarged (4600x) photographs, using a digitizing tablet connected to MS DOS computer. Accuracy was ± 0.25 mm. Prior to measurement the chromosomes were identified on the basis of length, arm length ratio and C-banding pattern. Chromosomes were arranged in a sequence of decreasing relative length and classified according to their similarity with respect to arm length ratio in *A. aurea*. For estimation of relative chromosome length and the construction of idiograms of species, ten or more complete cells were measured from both Feulgen and C-banded preparations. To determine the relative length (RL) of the total chromosome complement of individual species, additional measurements were made in three or more selected metaphase plates in Feulgen-stained preparations of diploid interspecific hybrids. These measurements were related to the absolute length (AL) of the chromosome complement of *A. magnifica* ssp. *magnifica*, as measured in Feulgen-stained metaphase plates of accession Q008 of this species. Based on the idiograms of the parental species, nearly all chromosomes (with the exception of some acrocentrics) could be identified in the cells of eleven hybrids, and lengths of species karyotypes were established. In the hybrids with *A. magnifica* ssp. *magnifica*, the relative lengths of the species karyotypes ($RL_{species}$) were obtained by the ratio of the length of the two parental chromosome complements ($L_{species}$ / $L_{magnifica}$), multiplied by the absolute length of the haploid complement of *A. magnifica* ssp. *magnifica* ($AL_{magnifica}$):

$$RL_{species} = L_{species} / L_{magnifica} \times AL_{magnifica} \qquad (1)$$

In the hybrids in which *A. magnifica* ssp. *magnifica* was not one of the parents, the already calculated relative karyotype length ($RL_{species\ 2}$) of one of the parental species was used instead:

$$RL_{species\ 1} = L_{species\ 1} / L_{species\ 2} \times RL_{species\ 2} \qquad (2)$$

The length of the karyotype of most species was determined in different hybrids and averaged (Table 3).

Results

A comparison of the karyotypes of *A. angustifolia* ssp. *angustifolia*, *A. aurea*, *A. inodora*, *A. ligtu* ssp. *ligtu*, *A. magnifica* ssp. *magnifica*, *A. pelegrina*, *A. philippii* and *A. psittacina* revealed differences in relative chromosome lengths, arm length ratios and C-banding pattern (Figure 1, Table 2). Figures 2 and 3 show representative C-banded and Feulgen-stained chromosome portraits of these species and of diploid interspecific hybrids. In all species, the longest chromosome in the complement was metacentric

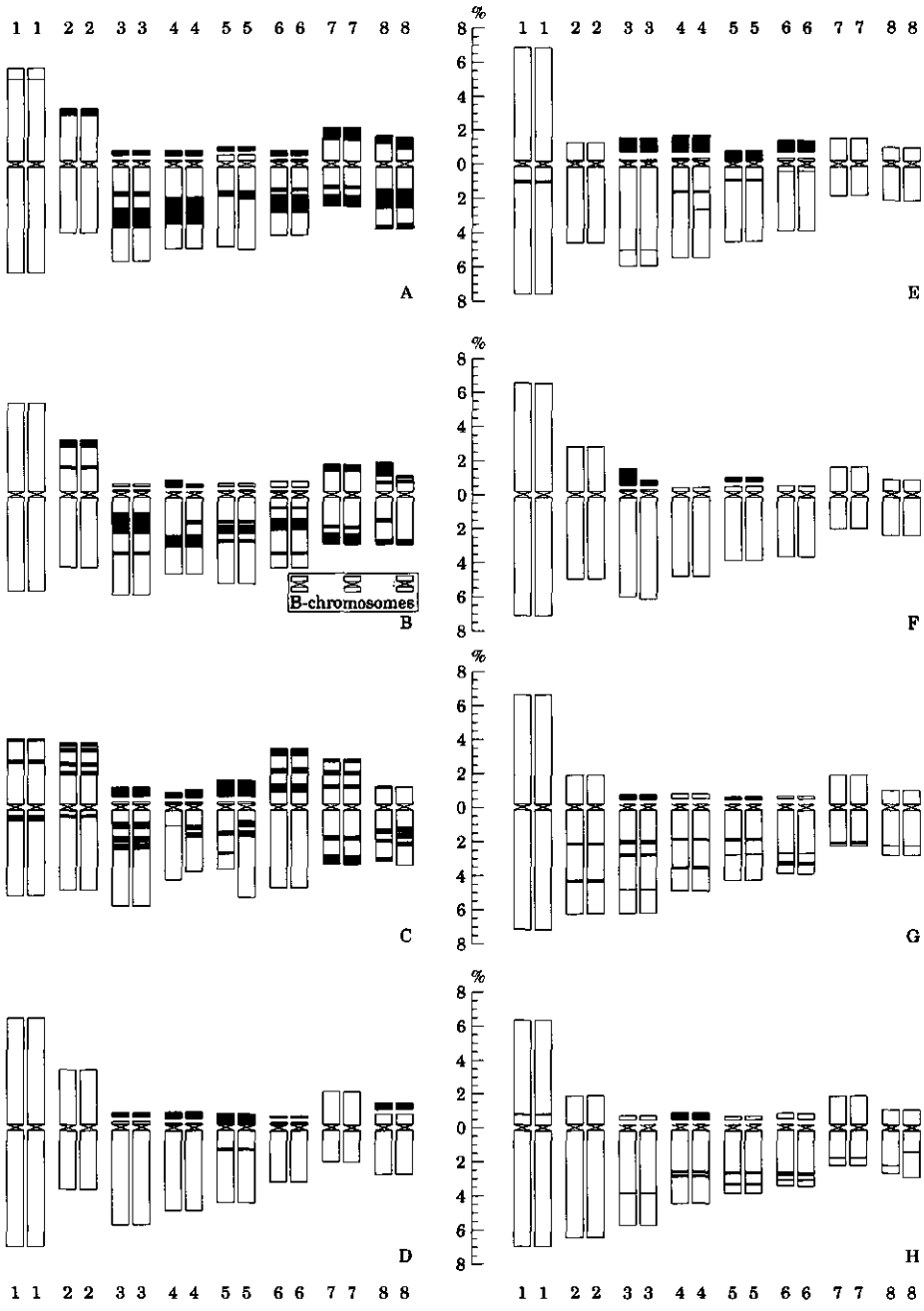

FIGURE 1. Diploid idiograms of C-banded chromosomes of *A. aurea* - A005 (A), *A. angustifolia* ssp. *angustifolia* - AN1W (B), *A. ligtu* ssp. *ligtu* - F051 (C), *A. pelegrina* - C063 (D), *A. magnifica* ssp. *magnifica* - Q008 (E), *A. philippii* - B018 (F), *A. inodora* - P002 (G) and *A. psittacina* - D031 (H).
Note heteromorphy in some chromosome pairs.

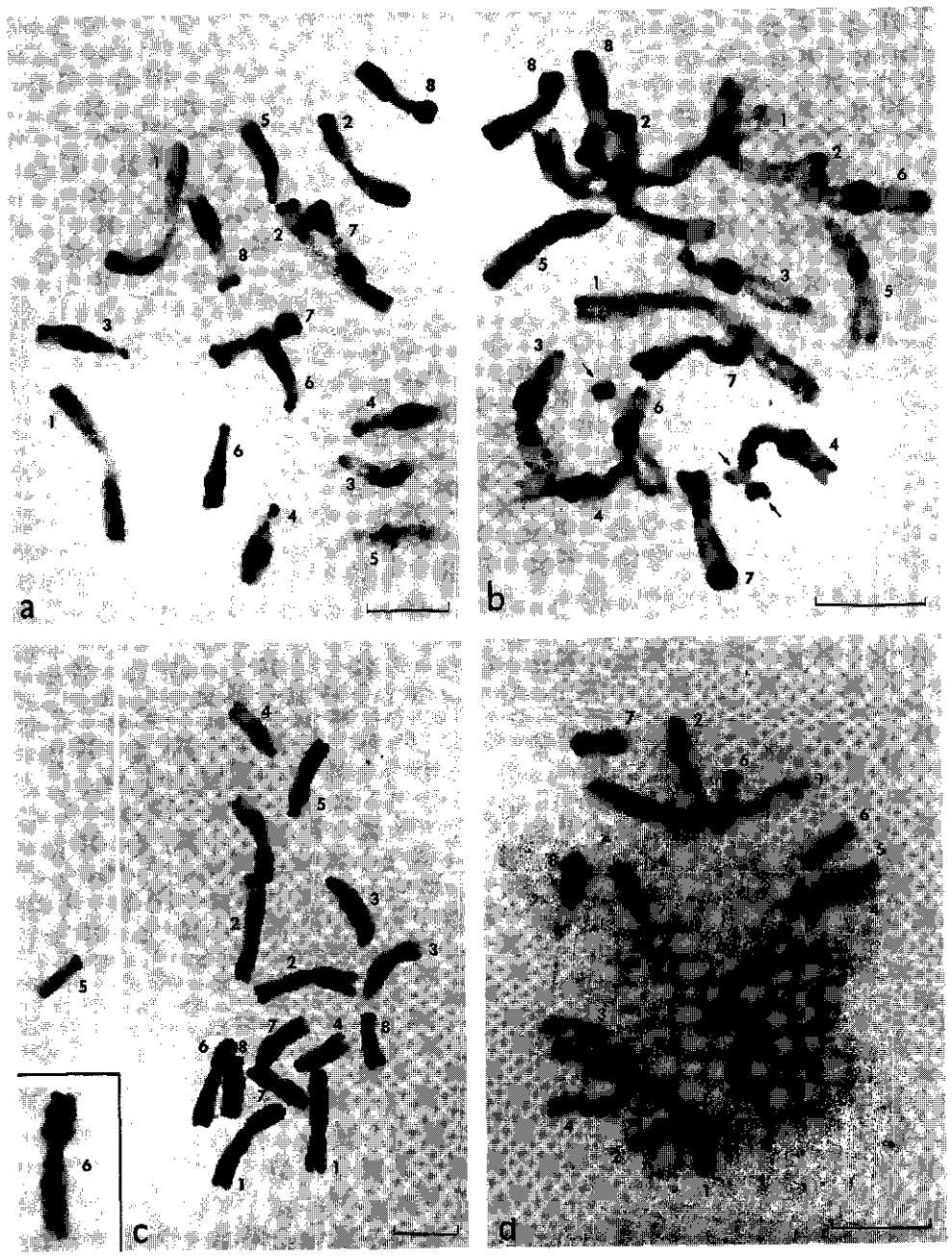

FIGURE 2. Giemsa C-banded chromosome portraits of *A. aurea* (a), *A. angustifolia* ssp. *angustifolia* (b), *A. ligtu* ssp. *ligtu* (c), with inset indicating differences in intensity of Giemsa staining in chromosome 6), *A. pelegrina* (d), *A. magnifica* ssp. *magnifica* Q008 (e) and F169-3 (f), *A. inodora* (g) and *A. psittacina* (h). Heteromorphic chromosome pairs in *A. aurea* (a), *A. angustifolia* ssp. *angustifolia* (b) and *A. ligtu* ssp. *ligtu* (c). Between accessions Q008 (e) and F169-3 (f) of *A. magnifica* ssp. *magnifica* there are differences for chromosomes 5 and 8. The B-chromosomes in *A. angustifolia* ssp. *angustifolia* (b) are indicated by arrows. Bars = 10 mm.

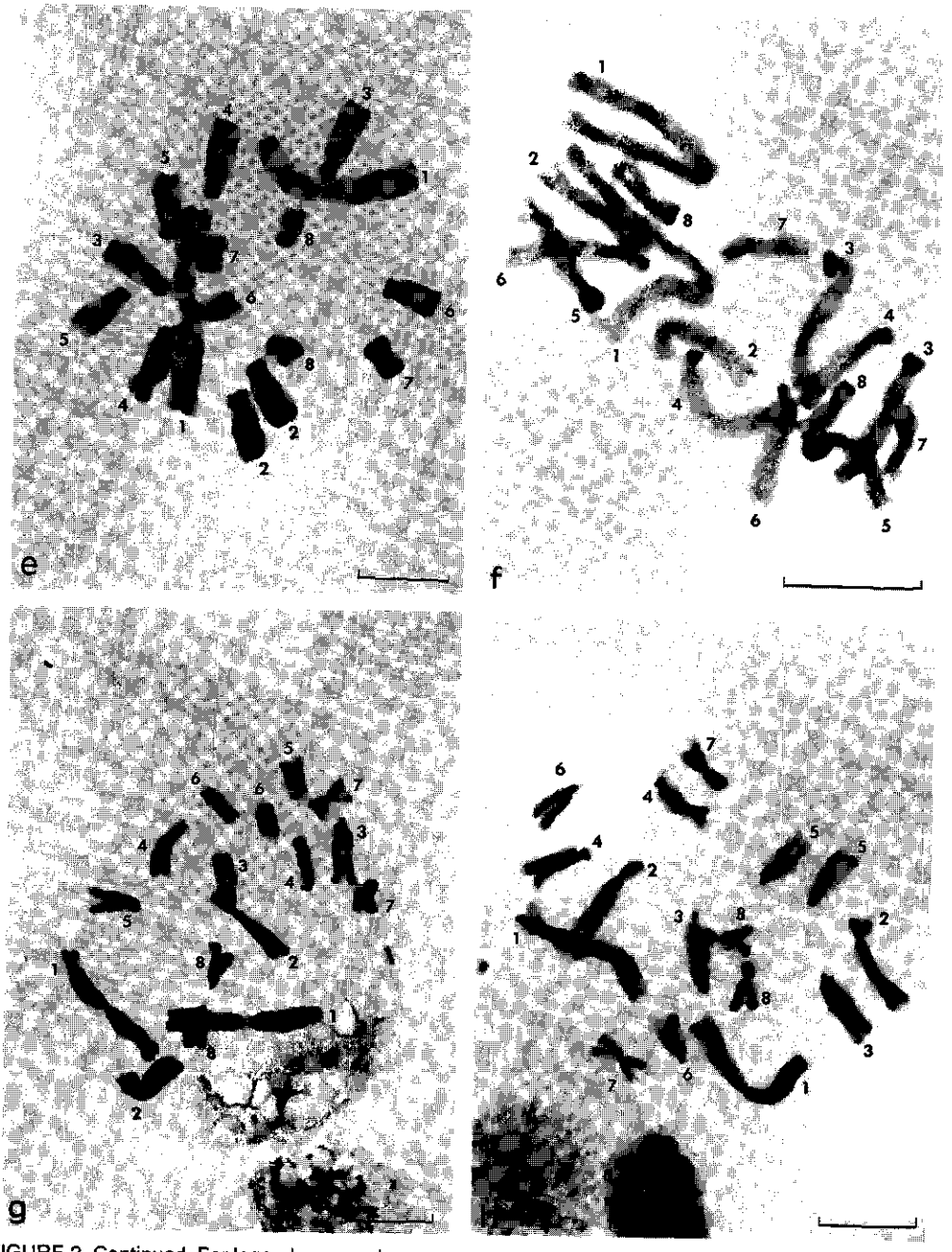

FIGURE 2. Continued. For legend see previous page.

TABLE 2. The relative length (%) per chromosome pair, the arm length ratio per chromosome and the relative length of constitutive heterochromatin (%) of eight *Alstroemeria* species (2n=2x=16).

Species	Relative length per chromosome pair (%)								Relative length of C-bands (%)
	1	2	3	4	5	6	7	8	
A. aurea (A005)	21.4	14.7	11.9	11.7	10.5	10.0	9.4	10.4	16.8
A. ligtu ssp. *ligtu* (F051)	17.4	16.2	13.0	9.1	9.0	15.3	11.7	8.3	12.9
A. angustifolia ssp. *angustifolia* (AN1W)	22.0	15.1	12.4	10.5	11.1	9.7	9.5	9.7	12.8
A. pelegrina (C063)	28.5	13.3	12.9	10.7	10.3	9.4	7.7	7.2	3.3
A. magnifica ssp. *magnifica* (Q008)	26.4	12.3	13.3	12.7	11.3	9.7	7.4	6.9	6.5
A. philippii (B018)	27.6	15.7	13.2	10.6	10.5	8.3	7.4	6.7	2.0
A. inodora (P002)	25.9	15.8	13.0	10.9	10.0	9.0	7.8	7.6	1.5
A. psittacina (D031)	26.4	16.2	12.3	11.0	9.9	8.4	8.2	7.6	<1.0

Species	Arm length ratio per chromosome							
	1	2	3	4	5	6	7	8
A. aurea (A005)	1.1	1.3	15.9	15.6	10.6	11.5	1.2	2.4
A. ligtu ssp. *ligtu* (F051)	1.3	1.3	8.2	6.9	3.2	1.3	1.2	2.7
A. angustifolia ssp. *angustifolia* (AN1W)	1.1	1.3	18.1	19.7	7.1	20.3	1.6	1.5
A. pelegrina (C063)	1.0	1.3	11.9	12.3	9.3	12.5	1.1	2.3
A. magnifica ssp. *magnifica* (Q008)	1.1	4.1	9.3	7.5	7.7	7.1	1.2	2.5
A. philippii (B018)	1.1	1.7	11.3	11.9	7.7	6.9	1.2	2.7
A. inodora (P002)	1.1	3.5	43.5	26.3	23.8	21.3	1.2	3.0
A. psittacina (D031)	1.1	3.5	14.3	12.8	11.4	9.5	1.2	2.6

and about two to four times as long as the shortest chromosome. The second chromosome was (sub)meta or subtelocentric. The third, fourth and fifth chromosomes were all acrocentric. Chromosome 6 was also acrocentric in most species, but metacentric in *A. ligtu* ssp. *ligtu*. Chromosomes 7 and 8 were meta- and submetacentrics, respectively. Secondary constrictions were present in the short arms of the acrocentric chromosomes, although they were not distinguishable in all cells. The distal parts of the short arms of these acrocentric chromosomes were darkly stained in a majority of cases, resembling satellites. In *A. pelegrina* there was a secondary constriction in the short arm of chromosome 8 (arrow in Figure 3B). *A. ligtu* ssp. *ligtu* had secondary constrictions in the short arms of chromosomes 2 and 6 in Feulgen-stained preparations. These secondary constrictions were, however, not visible in the C-banded preparations. Two pairs of acrocentric chromosomes with secondary constrictions were detected in Feulgen-stained metaphase plates of *A. philippii*, three pairs in *A. ligtu* ssp. *ligtu*, *A. pelegrina* and *A. magnifica* ssp. *magnifica* and four pairs in *A. angustifolia* ssp. *angustifolia*, *A. aurea*, *A. inodora* and *A. psittacina*. The accession of *A. angustifolia* ssp. *angustifolia* had three B-chromosomes (Figure 2B arrows). The lengths of the chromosome complements of seven species as measured in diploid interspecific hybrids are given in Table 3. The largest total chromosome length was

TABLE 3. Total chromosome length of seven haploid parental genomes as measured in eleven interspecific hybrids, relative to total chromosome length in *A. magnifica* ssp. *magnifica* (Q008) and combined total length of the haploid genomes of the seven species. Average values and standard deviations are presented.

		Total chromosome length (µm)	
Interspecific hybrid	Parental code	♀ parent	♂ parent
A. magnifica ssp. *magnifica* x *A. inodora*	Q008P004-2	58[1]	78 ± 1
A. pelegrina x *A. magnifica* ssp. *magnifica*	C042Q001-3	64 ± 2	58[1]
A. aurea x *A. magnifica* ssp. *magnifica*	A016Q001-1	75 ± 3	58[1]
A. pelegrina x *A. inodora*	C059P002-1	65 ± 3	78[2]
A. inodora x *A. pelegrina*	P002C049-5, -20	78[2]	63 ± 2
A. inodora x *A. aurea*	P004A001-1	78[2]	77 ± 4
A. pelegrina x *A. aurea*	C049A001-1	65 ± 2	76[2]
A. aurea x *A. psittacina*	A001D032-1	76[2]	78 ± 3
A. psittacina x *A. philippii*	D032B018-1	78[2]	63 ± 1
A. ligtu ssp. *ligtu* x *A. aurea*	AL1SA002-1	89 ± 1	76[2]
A. ligtu ssp. *ligtu* x *A. aurea*	AL5SA002-1	90 ± 1	76[2]

Species	Total chromosome length (µm)
A. magnifica ssp. *magnifica*	58 ± 1
A. philippii	63 ± 1
A. pelegrina	64 ± 2
A. aurea	76 ± 3
A. inodora	78 ± 1
A. psittacina	78 ± 3
A. ligtu ssp. *ligtu*	90 ± 2

[1] measured in *A. magnifica* ssp. *magnifica* (Q008)
[2] average value of measurements of parental genomes in other hybrids

found in *A. ligtu* ssp. *ligtu*. The chromosome complement of *A. magnifica* ssp. *magnifica* was the shortest one. Besides the differences in total chromosome length and C-banding pattern, the basic karyotype structure was apparently uniform.

Upon Giemsa C-banding, individual chromosomes could be identified more easily, since the banding patterns of many chromosomes in the complement were distinctive (Figures 1 and 2). The size and position of the C-bands within one accession were highly consistent among cells within a preparation as well as among preparations. Prominent C-bands were present in all Chilean species, whereas the Brazilian species possessed only inconspicuous C-bands. The Chilean species *A. ligtu* ssp. *ligtu*, *A. angustifolia* ssp. *angustifolia* and *A. aurea* had very prominent intercalary and telomeric C-bands in most of the chromosome arms. The latter two species had rather similar banding patterns, but differed in the arm length ratio of chromosomes 7 and 8 (Figures 1A-B and 2A-B and Table 2). Whereas the C-bands in *A. aurea* and *A. angustifolia* ssp. *angustifolia* were all darkly stained (Figures 2A and 2B), there was a slight gradation of intensity of the C-bands in *A. ligtu* ssp. *ligtu* (Figure 2C, inset). The Chilean species *A. pelegrina, A. philippii* and *A. magnifica* ssp. *magnifica* showed large terminal C-bands (Figures 1D-F, 2D-F). Within species there was some polymorphism

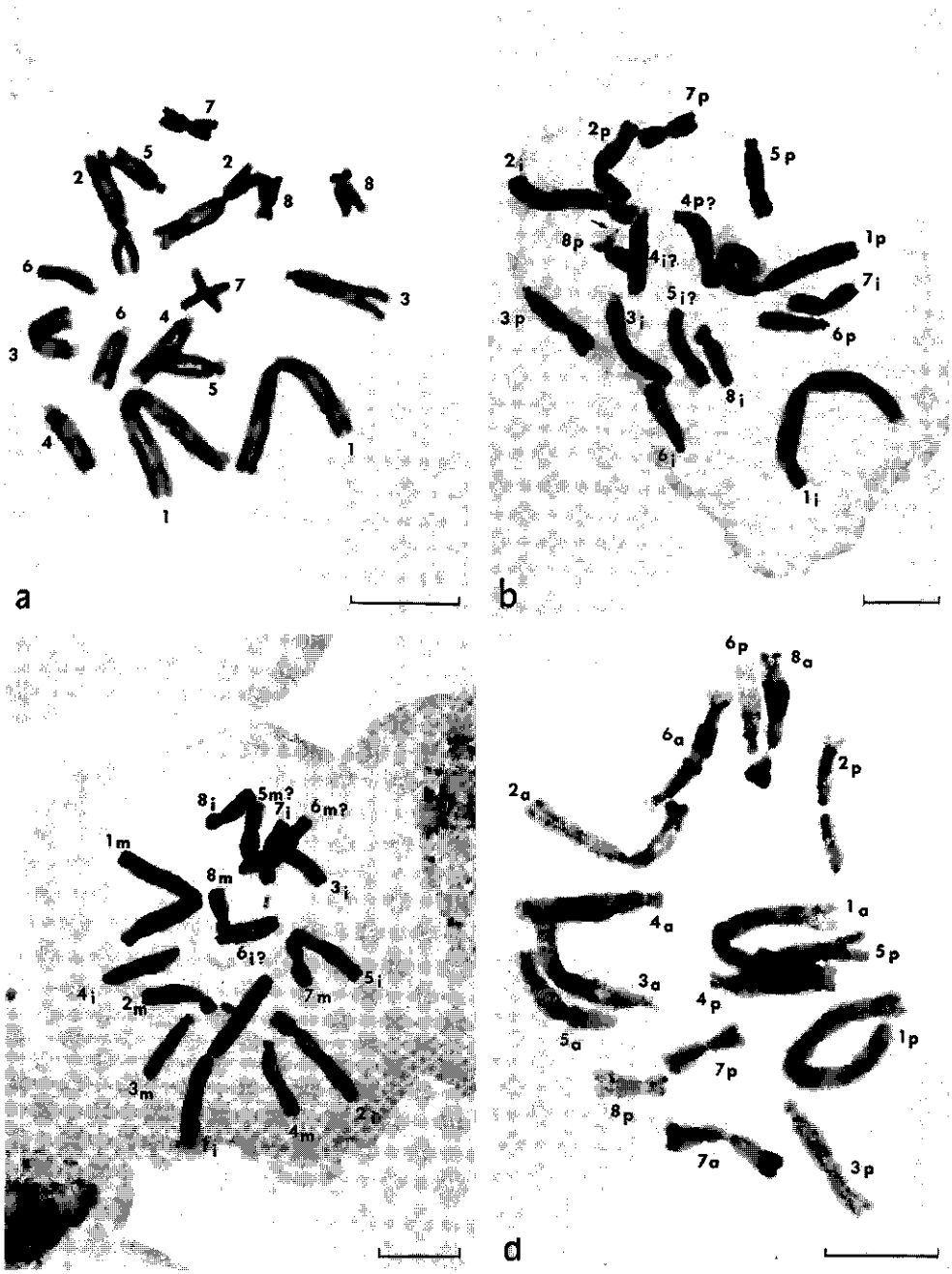

FIGURE 3. (a), Feulgen-stained chromosome portrait of *A. philippii* (B018). (b-c), Feulgen-stained chromosome portraits of interspecific hybrids *A. pelegrina* x *A. inodora* (C059P002-1) and *A. magnifica* ssp. *magnifica* x *A. inodora* (Q008P004-2). The secondary constriction of chromosome 8 of *A. pelegrina* in hybrid C059P002-2 (b) is indicated by an arrow. (d), Giemsa C-banded chromosome portrait of interspecific hybrid *A. pelegrina* x *A. aurea* (C039A001-8). Chromosomes of the parental genomes are numbered: a, *A. aurea*; i, *A. inodora;* m, *A. magnifica* ssp. *magnifica;* p, *A. pelegrina.* The identity of some chromosomes (indicated with a question mark) can not be determined with certainty. Bars = 10 mm.

in size and position of the C-bands, e.g., the accessions of *A. angustifolia* ssp. *angustifolia* (Figure 2B, chromosomes 4 and 8), *A. aurea* (Figure 2A, chromosomes 5 and 8), *A. ligtu* ssp. *ligtu* (Figure 2C, chromosomes 4, 5 and 8) and *A. magnifica* ssp. *magnifica* (Figures 2E and 2F, chromosomes 5 and 8).

With the exception of a few acrocentric chromosomes (of equal length), individual chromosomes could be recognized in the Feulgen-stained and C-banded karyotypes of many of the interspecific hybrids (Figure 3).

Discussion

Four conclusions emerge from this investigation on eight species of *Alstroemeria*. First, the species have asymmetric karyotypes with large chromosomes. Second, there is considerable interspecific variation in total chromosome length and C-banding pattern. Some species, however, have similar karyotypes (e.g. *A. aurea* and *A. angustifolia* ssp. *angustifolia*, and also *A. inodora* and *A. psittacina*, Table 2, Figures 1 and 2). Third, the intraspecific differences in C-banding pattern that were found among the accessions of *A. angustifolia* ssp. *angustifolia*, *A. aurea*, *A. ligtu* ssp. *ligtu* and *A. magnifica* ssp. *magnifica* warrant a further investigation on the extent of intraspecific variation of these and other species. Fourth, Giemsa C-banded and Feulgen-stained metaphase chromosomes can be used to confirm or analyse the hybrid nature of some interspecific hybrids.

In this study we measured total length of the haploid complements in hybrids and used these measurements to estimate relative genome length (Table 3). The validity of chromosome length as a measure of chromosome size has been questioned by Pegington and Rees (1970), who preferred the use of chromosome volume. Chromosome volume may indeed give a better estimate of chromosome size when large differences in DNA organization within the nucleus are to be expected, as was the case when they compared length, volume and DNA amount of diploids, tetraploids and hexaploids within the Triticinae. However, when the relative length of genomes of diploid species is estimated in diploid interspecific hybrids, there is no difference in ploidy level. Moreover, differences in condensation between cells cannot influence the estimates since measurements are established within one cell. On the other hand, although differential amphiplasty did not occur in the hybrids that were studied, it cannot be ruled out that the genomes in the hybrid influence each other's *total* length (amphiplasty, Navashin, 1928).

Because the present knowledge of *Alstroemeria* genomes is limited, we can only speculate on the evolutionary trends that occurred within the genus. The presence of prominent C-bands on the chromosomes of the Chilean species and the absence of such bands in the Brazilian species, suggests that the genomes of the two groups of species might have evolved differently. Those species within the Chilean group that have shorter genomes, possess few C-bands, whereas the larger genomes carry many C-bands (Tables 2 and 3 and unpublished results on *A. pulchra* and *A. werdermannii*,

which have few telomeric C-bands, and *A. hookeri* ssp. *hookeri* and *A. presliana* ssp. *presliana*, which carry many C-bands). The Brazilian species have large genomes, but hardly any C-bands. When we accept that interspecific differences in nuclear DNA content in various genera are largely due to variation in the highly repeated DNA fraction (Flavell, 1986; Narayan, 1988) and only a portion of it is located in C-bands, as was demonstrated in *Anemone* (Cullis and Schweizer, 1974), the majority of highly repeated DNA of the Brazilian species must be dispersed throughout the complement or aggregated in such a way that it cannot be revealed by C-banding.

The orogeny of the Andes mountain range and its consequences for the local climate have undoubtedly played a mayor role in the distribution and the isolation of species (Aker and Healy, 1990). We suspect that total chromosome length and length of C-bands might be associated with geographical distribution and climate. The species, that possess relatively small genomes and few C-bands, such as *A. magnifica* ssp. *magnifica*, *A. pelegrina* and *A. philippii*, are confined to comparatively restricted areas in the arid climate zone of Central Chile between 28° and 33° S, whereas the species with larger genomes and more C-bands, such as *A. aurea* and *A. ligtu* ssp. *ligtu*, have a wider distribution in the regions with higher rainfall at the more southern latitudes between 33° and 47° S (Table 1). However, before anything can be concluded on evolutionary trends within *Alstroemeria*, it will be necessary to study more species, including those with a trans-Andean distribution [*A. aurea*, *A. presliana* ssp. *presliana*, *A. pseudospathulata*, *A. patagonica* and perhaps *A. spathulata*, *A. exserens* and *A. andina*, Bayer (1987)].

Acknowledgements

We thank the breeding companies Koninklijke Van Zanten, Könst Alstroemeria, Van Staaveren and Wülfinghoff Freesia for providing plant material and financial support, and Dr. Arends, Dr. De Jong, Prof. Hermsen, Prof. Jacobsen and Prof. Sybenga for helpful comments.

Chapter 4

Nuclear DNA content in twelve species of *Alstroemeria* L. and some of their hybrids

J.H. Buitendijk, E.J. Boon and M.S. Ramanna. 1997. Annals of Botany 79: 343-353.

Abstract

Nuclear DNA content (2C-value), estimated through flow cytometry using propidium iodide (PI), was shown to vary from 36.5 pg. to 78.9 pg. among 29 accessions of 12 *Alstroemeria* species (2n=2x=16). The extremes were found in *A. magnifica* ssp. *magnifica* and in *A. ligtu* ssp. *simsii*, both belonging to the Chilean species group. The four Brazilian species exhibited less variation in nuclear DNA content (49.8 - 56.4 pg.), than the eight Chilean species (36.5 - 78.9 pg.). Nuclear DNA content was positively correlated ($r = 0.92$, $n = 7$, $P < 0.01$) with the total chromosome length. It was also positively correlated ($r = 0.85$, $n = 5$, $P < 0.01$) with the length of C-bands, when only the Chilean species were considered. When both karyotype parameters, length of non-C-banded chromosome regions (x) and length of C-bands (y) were determined, it was possible to predict the nuclear DNA content (z) with the formula $z = 0.65x + 1.31y - 0.45$ ($R^2 = 0.97$, $P = 0.004$).

The 4', 6-diamidino-2-phenylindole (DAPI) fluorescence of most accessions was proportional to the PI fluorescence ($r = 0.98$, $P < 0.001$), except for one accession of *A. ligtu*, that had a relatively high PI/DAPI ratio (1.88). The PI/DAPI ratios of the Brazilian species were lower (1.60 - 1.67) than those of the Chilean species (1.68 - 1.88), which might reflect a difference in base pair composition. Four groups of species could be distinguished on the basis of fluorescence values. Diploid interspecific hybrids were shown to have a DNA content intermediate to the values of the parents involved. Both the PI and the DAPI fluorescence values of these hybrids approximated the mid parent values. Tetraploids, derived from selfing of diploids, had PI and DAPI fluorescence values that were twice that of the diploid hybrids. It was possible to distinguish aneuploids from euploids based on fluorescence values.

Key words: Alstroemeria, Aneuploidy, C-banding, DAPI, Evolution, Flow cytometry, Genome size, Geophytes, Karyotypes, Inca lily, Nuclear DNA, Propidium iodide.

Introduction

Alstroemeria (Inca lily) is a cut flower of increasing importance. The cultivars have originated from interspecific crosses and consist of diploid, triploid or tetraploid forms. All *Alstroemeria* species are geophytes. They are rhizomatous and bear both tuberous and fibrous roots. The species are diploids (2n=2x=16) and possess asymmetrical

karyotypes, with large chromosomes and varying amounts of C-banded heterochromatin (Buitendijk and Ramanna, 1996). From the differences between karyotypes it is evident that the genomes of individual species are differentiated. Besides this, barriers to interspecific hybridization have been observed (Buitendijk *et al.*, 1995; De Jeu and Jacobsen, 1995).

Many monocotyledonous plants, that have a geophytic growth habit, have large genomes (> 20 pg./2C-value) e.g., *Allium, Amaryllis, Convallaria, Fritillaria, Hyacinthus, Hosta, Hippeastrum, Leucojum, Lilium, Muscari, Ornithogalum, Scilla, Trillium* and *Tulipa* (Bennett and Leitch, 1995; Bennett and Smith, 1976). The 2C-value of *Alstroemeria caryophyllaea* was estimated to be 51.5 pg. (Bharathan *et al.*, 1994). The presence of underground organs such as bulbs, corms and rhizomes seems to be indicative of high nuclear DNA amounts. However, some geophytic monocot genera include species with small genomes, e.g., *Sysirynchium* (Iridaceae, 2C=1-8 pg., Kenton *et al.*, 1986), while some non-geophytic monocot genera possess large genomes, e.g. *Echinodorus* and *Sagittaria* (Alismataceae, 2C=22-29 pg. and 27-28 pg. respectively, Bharathan *et al.*, 1994), *Tradescantia* (Comellinaceae, 2C=8-87 pg., Martinez and Ginzo, 1985) and *Voanioala* (Arecaea, 2C=60 pg., Johnson *et al.*, 1989), indicating that the relationship between genome size and growth habit may not be general.

Nuclear DNA content can be estimated through several methods, such as chemical analysis, Feulgen microdensitometry and flow cytometry. The latter offers a quick and sensitive method for quantifying DNA amounts (Michaelson *et al.*, 1991; Arumuganathan and Earle, 1991), but is still subject to debate (Bennett and Leitch, 1995; Doležel *et al.*, 1992). This might be attributed to technical differences and differences in the staining properties of the fluorochromes. Propidium iodide (PI) and ethidium bromide are considered to be useful fluorochromes for flow cytometric estimation of DNA content. These compounds intercalate with double stranded nucleic acids and are independent of base composition (Le Pecq and Paoletti, 1967). On the other hand, the staining with DNA intercalators (and non-intercalators) may be influenced by the spatial organization of the DNA molecule (Darzynkiewicz and Traganos, 1988). Other fluorochromes, such as DAPI (4',6-diamidino-2-phenylindole) and mithramycin bind preferentially to adenine-thymine (A-T) or guanine-cytosine (G-C) rich regions (Manzini *et al.*, 1983), leading to over- or underestimation of the nuclear DNA content. Although DAPI and mithramycin fluorescence alone cannot be used to estimate nuclear DNA content, it may give additional information on the arrangement of base pairs and chromatin structure, when compared with data obtained by using DNA intercalators (Doležel *et al.*, 1992).

The objectives of this study were three-fold: (1) to estimate the variation in nuclear DNA content of twelve *Alstroemeria* species and some of their hybrids, and to gain an insight into the overall base pair composition (2) to relate the variation in nuclear DNA content to the karyotypes of the species genomes and (3) to investigate the possibility of using flow cytometry to detect aneuploids in *Alstroemeria*.

TABLE 1. Distribution and source of species accessions (2n=2x=16).

Species	Distribution *	Accession / source**
A. angustifolia Herb. ssp. *angustifolia*	Chile, 33° S.L., 0-1000 m[1]	AN4S / S AN1S / S
A. aurea Graham	Chile, 36°-42°, 47° S.L. 200-1800 m[1]	A001 / W A002 / W A005 / BGA
A. brasiliensis Sprengel	central Brazil[2]	BA2K
A. caryophyllaea Jacq.	southern Brazil[2]	carS / S carZ / Z carP / Z
A. hookeri Lodd. ssp. *hookeri*	Chile, 35°-37° S.L., 0-300 m[1] Argentina, Mendoza[2]	AP3S / S I081 / W
A. inodora Herb.	central and southern Brazil[2]	P002 / S P004 / Z
A. ligtu L. ssp. *ligtu*	Chile, 33°-38° S.L., 0-800 m[1]	AL1S / S F051-2 / W
A. ligtu L. ssp. *simsii* (Sprengel) Bayer	Chile, 33°-35° S.L., 0-1800 m[1]; northern Brazil[2]	J091-4 / W K101-1 / W
A. magnifica Herb. ssp. *magnifica*	Chile, 29°-32° S.L., 0-200 m[1]	Q001 / Z Q008 / P F169-3 / W XIII-2 / LBO
A. pelegrina L.	Chile, 32°-33° S.L., 0-50 m; Peru, near Lima[1]	C049 / W C055 / W
A. philippii Baker	Chile, 28° S.L., 0-1000 m[1]	AS3Z / Z B018 / W
A. psittacina Lehm.	northern Brazil[2]	D031 / W D032 / W
A. pulchra Sims ssp. *pulchra*	Chile, 32°-34° S.L. 0-1000 m[1]	AB9Z / Z AB3W / W

* Literature source:[1] Bayer, 1987; [2] Aker and Healy, 1990.

** BGA=Botanical Garden VUA Amsterdam; K=Könst Alstroemeria; P=Parigo (UK); S=Van Staaveren; W=Wülfinghoff Freesia; Z=Koninklijke Van Zanten; LBO=Laboratorium voor Bloembollen Onderzoek.

Materials and methods

Plant Material

Seeds and plants of species were obtained from botanical gardens, breeders and research institutes. The collection is maintained at the Department of Plant Breeding of the Agricultural University of Wageningen. The accessions of species and interspecific hybrids that were used in this study are listed in the Tables 1 and 2. The identity of the plant material was verified by consulting a monograph on the Chilean *Alstroemeria*

TABLE 2. Parental code, ploidy level and source of interspecific hybrids.

Interspecific hybrid	Parental code	Ploidy level	Source*
A. aurea x *A. inodora*	A001P004-1	2x	B[1]
A. inodora x *A. ligtu* ssp. *simsii*	P002K101-1	2x	J[1]
A. inodora x *A. magnifica* ssp. *magnifica*	P004Q001-1	2x	B[1]
A. inodora x *A. pelegrina*	P002C049-3	2x	B[1]
	P002C049-9	2x	B[1]
	P002C049-10	2x	B[1]
A. ligtu ssp. *ligtu* x *A. aurea*	AL1SA002-1	2x	J[1]
A. magnifica ssp. *magnifica* x *A. inodora*	Q008P004-2	2x	B[1]
A. pelegrina x *A. aurea*	C049A001-2	2x	B[1]
A. psittacina x *A. aurea*	D032A001-1	2x	B[1]
A. psittacina x *A. magnifica* ssp. *magnifica*	D032Q003-1	2x	B[1]
A. inodora x *A. magnifica* ssp. *magnifica*	P004Q001-1-1	4x	B[2]
	P004Q001-1-2	4x	B[2]
	P004Q001-1-3	4x	B[2]
	P004Q001-1-4	4x+1	B[2]
	P004Q001-1-5	4x	B[2]
A. inodora x *A. pelegrina*	P002C049-3-1	4x	B[2]
	P002C049-3-2	4x+1	B[2]
	P002C049-3-3	4x	B[2]
	P002C049-9-25	4x	B[2]
	P002C049-9-26	4x	B[2]
	P002C049-9-27	4x	B[2]
	P002C049-10-1	4x	B[2]
	P002C049-10-10	4x+1	B[2]
	P002C049-10-12	4x	B[2]
A. inodora x *A. pelegrina*	P002C049-9-25-1	4x	B[3]
	P002C049-9-25-2	4x	B[3]
	P002C049-9-25-3	4x+1	B[3]
	P002C049-9-26-1	4x+1	B[3]
	P002C049-9-26-2	4x	B[3]
	P002C049-9-26-3	4x	B[3]

* [1] These diploid plants were obtained after crossing species, followed by embryo rescue, as described in Buitendijk *et al.*, 1995 (B) and De Jeu and Jacobsen, 1995 (J).
[2] These allotetraploid plants were obtained through selfing and normal seed set on diploid interspecific hybrids.
[3] These allotetraploid plants were obtained through selfing and normal seed set on tetraploid interspecific hybrids.

species (Bayer, 1987) and the first descriptions of the Brazilian species (*A. brasiliensis*: Sprengel, 1825; *A. caryophyllaea*: Jacquin, 1797; *A. inodora*: Herbert, 1837; *A. psittacina*: Lehmann in: Schultes and Schultes, 1829). Herbarium vouchers will be deposited at WAG (Wageningen Herbarium).

Nuclear DNA Measurements

Relative fluorescence and DNA content of nuclei stained with PI and DAPI were determined by flow cytometry using nuclei of young leaves of *in vivo* and *in vitro* grown *Alstroemeria* plants. For the DAPI measurements, nuclei of *Tulipa gesneriana* leaves, cv. 'West Point', with 2C=60.70 pg., were used as internal standard. This value was estimated via *Allium cepa* cv. 'Ailsa Craig', with 2C=33.50 pg., as the calibration standard. *Allium cepa* was avoided as an internal standard, because its DAPI fluorescence value is too close to that of *Alstroemeria*, so that the peaks in the histograms overlap. For the PI measurements, *Triticum aestivum* cv. 'Chinese Spring', with 2C=34.63 pg., was used as internal standard. Additionally, five plant species, that are recommended for calibration (Bennett and Leitch, 1995), were mixed and fluorescence ratios were determined. These plant species were *Allium cepa* cv. 'Ailsa Craig' (2C=33.50 pg.), *Hordeum vulgare* cv. 'Sultan' (2C=11.12 pg.), *Secale cereale* cv. 'Petkus Spring' (2C=16.57 pg.), *Triticum aestivum* cv. 'Chinese Spring' (2C=34.64 pg.) and *Vicia faba* PBI Inbred line 6 (2C=26.66 pg.).

To release nuclei from the cells, 0.5 cm^2 leaf tissue of *Alstroemeria* was chopped together with 0.25 cm^2 of leaf tissue of the internal standard in 0.5 ml buffer (High resolution DNA kit, Partec GmbH-Münster). The suspension, containing cell constituents and large tissue remnants, was passed through a nylon filter of 30 mm mesh size. In case of PI, RNAse (Sigma R9134, 5 units/ml) was added. Samples were stored for 20 minutes at room temperature. After this period, 2 ml of staining solution, containing either 40 $mg.l^{-1}$ PI or 4 $mg.l^{-1}$ DAPI, were added. Within two to three hours after staining, measurements were performed with a Partec CA-II flow cytometer. Excitation filters that were applied for PI fluorescence were KG1, BG38 and for DAPI fluorescence they were BG12 and KG1, UG1 and BG38. The dichromatic mirror TK500 was used for PI fluorescence and TK420 for DAPI fluorescence. The emission filters RG570 and GG345 were applied for PI and DAPI fluorescence respectively. All fluorescence values were converted to fluorescence ratios, relative to *Allium cepa* cv. 'Ailsa Craig', which was the primary reference standard.

Cytological Methods

Shoot tips were pre-treated with 2mM 8-hydroxyquinoline, fixed and hydrolysed. The hydrolysed tissue was stained with leuco basic fuchsin and squashed. The details of the procedure are given in Buitendijk and Ramanna (1996).

Statistical procedures

Statistical analyses of the variables 2C-value, PI/DAPI ratio, total chromosome length, length of C-bands and non-C-banded chromosome regions involved analyses of variance (Snedecor and Cochran, 1967), with application of Tukey's multiple range test using honestly significant differences. Also Pearson correlation analysis and multiple regression were run.

Results

The flow cytometric measurements yielded high resolution DNA histograms with coefficients of variation ranging from 5.0 to 7.0 % for the PI histograms and from 1.5 to 2.5% for the DAPI histograms (Figure 1).

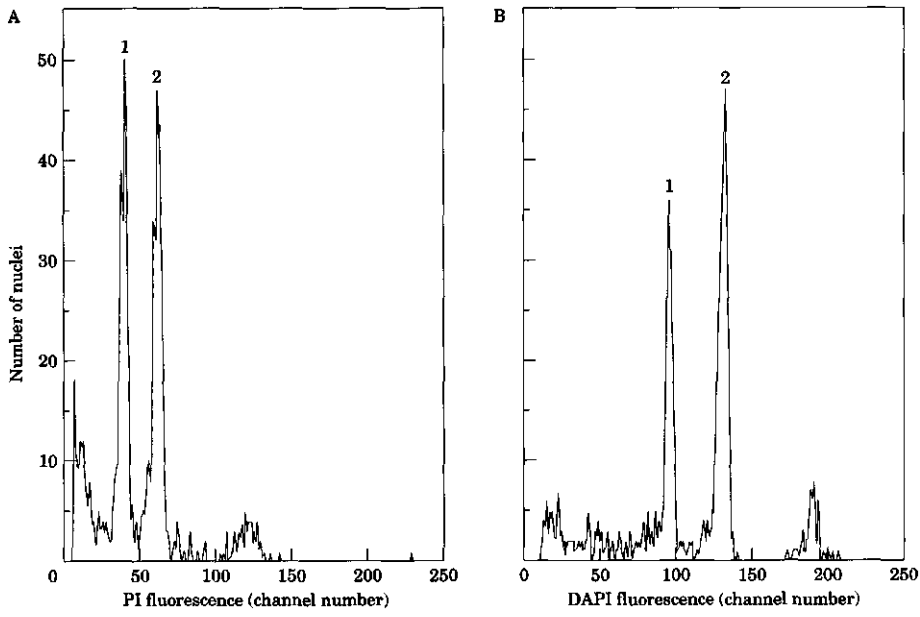

FIGURE 1. (A) DNA-histograms of propidium iodide stained nuclei from leaf tissue of *Triticum aestivum* cv. 'Chinese Spring' (peak 1) and *Alstroemeria aurea* A001 (peak 2), and (B) of DAPI stained nuclei of *Alstroemeria aurea* A001 (peak 1) and *Tulipa gesneriana* cv. 'West Point' (peak 2).

A series of ten runs on each of the accessions A001 and A002 of *A. aurea* and Q001 of *A. magnifica* ssp. *magnifica* confirmed that the estimates were highly reproducible (Table 3). When measured in different series, almost identical values were found.

TABLE 3. Propidium iodide and DAPI fluorescence ratios relative to *Allium cepa* cv. 'Ailsa Craig' and standard deviation in three accessions of *Alstroemeria*.

Accession	Number of measurements	PI ratio	DAPI ratio
A001	10	1.651 ± 0.014	0.977 ± 0.008
A002	10	1.630 ± 0.015	0.959 ± 0.001
Q001-4	10	1.247 ± 0.013	0.724 ± 0.003

The PI and DAPI fluorescence values and the 2C DNA values of *Alstroemeria* species relative to the fluorescence values of *Allium cepa*, cv. 'Ailsa Craig', are given in Table 4.

The 2C-values of the 29 accessions of 12 *Alstroemeria* species ranged from 36.5 pg. for *A. magnifica* ssp. *magnifica* to 78.9 pg. for *A. ligtu* ssp. *simsii*. Intraspecific variation was found among accessions of *A. ligtu*, *A. aurea* and *A. magnifica* ssp. *magnifica* (Table 4). In certain cases the intraspecific variation was wider than the interspecific variation (cf. the accessions of *A. magnifica* ssp. *magnifica*, *A. philippii* and *A. pulchra* ssp. *pulchra*). The PI and the DAPI fluorescence ratios were strongly correlated ($r = 0.98$, $n = 29$, $P < 0.001$). The PI/DAPI ratio of the *Alstroemeria* species was higher than that of *Allium cepa* cv. 'Ailsa Craig', *Vicia faba* PBI inbred line 6, and *Tulipa gesneriana* cv. 'West Point', but lower than that of *Hordeum vulgare* cv. 'Sultan', *Secale cereale* cv. 'Petkus Spring' and *Triticum aestivum* cv. 'Chinese Spring'. The variation within *Alstroemeria* (1.60 - 1.88) was smaller than the variation among the other taxa that were measured (1.00 - 2.00). The Brazilian species had lower PI/DAPI ratios (1.60 - 1,67) than the Chilean species (1.68 - 1.88). The accession K101-1 of *A. ligtu* ssp. *simsii* had a notably high ratio of 1.88. Analysis of variance for the 2C-value and for the PI/DAPI ratio (Table 5) showed that: (a) the Chilean and the Brazilian group of species do not differ significantly in their 2C-value, but they do differ in their PI/DAPI ratio (b) both the 2C-value and the PI/DAPI-ratio vary significantly among species and (c) among accessions within species. After a comparison of 2C-values with the karyotypes of eight *Alstroemeria* species (Buitendijk and Ramanna, 1996), it was found that there was a significant positive correlation between nuclear DNA content and total chromosome length ($r = 0.92$, $n = 8$, $P < 0.01$). Generally, there was no correlation between the length of C-bands and the nuclear DNA content ($r = 0.72$, $n = 8$, ns), but when only the six Chilean species were considered, the correlation was significant ($r = 0.85$, $n = 6$, $p < 0.01$). Multiple regression of nuclear DNA content (z) on the length of non-C-banded (x) and C-banded (y) regions of the chromosomes led to the linear relationship $z = 0.65x + 1.31y - 0.45$ ($R^2 = 0.97$, $P = 0.004$). The PI/DAPI ratio was not correlated with either the total length of the chromosomes, or with the amount of C-banded heterochromatin or nuclear DNA content. When both the qualitative and quantitative differences in nuclear DNA between *Alstroemeria* species that were observed in this investigation are presented graphically (Figure 2), four groups of species could be distinguished. These groups are (1) *A. magnifica* ssp. *magnifica*, *A. pelegrina*, *A. philippii* and *A. pulchra* ssp. *pulchra*; (2) *A. angustifolia* ssp. *angustifolia*, *A. aurea* and *A. hookeri* ssp. *hookeri*; (3) *A. ligtu* ssp. *ligtu* and *A. ligtu* ssp. *simsii*; and (4) *A. brasiliensis*, *A. caryophyllaea*, *A. inodora* and *A. psittacina*.

Diploid interspecific hybrids had PI and DAPI fluorescence ratios that approached the individual mid parent values (Table 6). Tetraploids that were obtained through selfing of diploid and tetraploid interspecific hybrids possessed values close to twice the combined value of the diploids (Table 7). Some of these allotetraploid plants, however, had a higher DAPI fluorescence ratio, than was expected on the basis of mid parent value. Most of these plants were aneuploid ($2n = 4x+1$; Figure 3). In those cases in which the DAPI fluorescence ratio exceeded the mid parent value by 6% or more, the aneuploidy was due to an extra chromosome 1.

TABLE 4. Propidium iodide and DAPI fluorescence ratios and 2C-values of calibration standards and of *Alstroemeria* species accessions

Species	Accession	Fluorescence ratio (fluorescence sample/fluorescence *Allium cepa*) PI	DAPI	PI/DAPI	Measured 2C-value (pg)	Reported 2C-value (pg)	Ranges of Tukey[1] for 2C-value Species	Accession	PI/DAPI ratio Species	Accession
Allium cepa[2]	cv. 'Ailsa Craig'	1.000	1.000	1.00	33.5	33.50[3]				
Hordeum vulgare	cv. 'Sultan'	0.326	0.163	2.00	10.9	11.12[3]				
Secale cereale	cv. 'Petkus Spring'	0.480	0.248	1.94	16.1	16.57[3]				
Triticum aestivum	cv. 'Chinese Spring'	1.016	0.519	1.96	34.0	34.64[3]				
Tulipa gesneriana	cv. 'West Point'	1.812	1.346	1.35	60.7	-				
Vicia faba	PBI Inbred line 6	0.725	0.613	1.18	24.4	26.66[3]				
Vicia faba[4]	Unknown4	0.774[4]	0.613[4]	1.26[4]	26.9[4]	26.66[3]				
Alstroemeria angustifolia	AN4S	1.546	0.901	1.72	51.8	-	c	ghi	b	ghi
ssp. *angustifolia*	AN1S	1.516	0.893	1.70	50.8			fgh		efghi
A. aurea	A1	1.651	0.979	1.69	55.3	-	d	k	b	cdefgh
	A2	1.630	0.958	1.70	54.6			jk ij		cdefghi
	A5	1.594	0.937	1.71	53.4					efghi
A. brasiliensis	BA2K	1.499	0.922	1.63	50.2	-	c	f	a	ab
A. caryophyllaea	carS	1.639	0.997	1.64	54.9	51.2[5]	e	jk	a	abcde
	carZ	1.657	1.018	1.63	55.5			k		ab
	carP	1.684	1.006	1.67	56.4			k		bcdefgh
A. hookeri ssp. *hookeri*	AP3S	1.552	0.899	1.73	52.0	-	c	hi	bc	hi
	I081	1.534	0.884	1.74	51.4			fgh		hi
A. inodora	P002	1.507	0.919	1.64	50.5	-	c	fg	a	abc
	P004	1.504	0.919	1.64	50.4			fg		abcde

TABLE 4. Continued

Species	Accession	Fluorescence ratio (fluorescence sample/fluorescence *Allium cepa*) PI	DAPI	PI/DAPI	Measured 2C-value (pg)	Reported 2C-value (pg)	Ranges of Tukey[1] for 2C-value Species	2C-value Accession	PI/DAPI ratio Species	PI/DAPI ratio Accession
A. ligtu ssp. *ligtu*	AL1S	2.072	1.194	1.74	69.4	-	f	l	c	hi
	F051-2	2.033	1.167	1.74	68.1			l l		i
A. ligtu ssp. *simsii*	J091-4	2.045	1.176	1.74	68.5			m		hi
	K101-1	2.355	1.254	1.88	78.9					j
A. magnifica ssp. *magnifica*	Q001	1.251	0.728	1.72	41.9	-	a	d	b	ghi
	Q008	1.245	0.722	1.72	41.7			cd		ghi
	F169-3	1.224	0.713	1.72	41.0			bcd		ghi
	XIII-2	1.090	0.651	1.68	36.5			a		bcdefghi
A. pelegrina	C049	1.313	0.758	1.73	44.0	-	b	e	bc	hi
	C055	1.319	0.764	1.73	44.2			e		ghi
A. philippii	AS3Z	1.239	0.728	1.70	41.5	-	a	bcd	bc	efghi
	B018	1.230	0.716	1.71	41.2			bcd		efghi
A. psittacina	D031	1.487	0.931	1.60	49.8	-	c	f	a	a
	D032	1.490	0.901	1.65	49.9			f		abcdef
A. pulchra ssp. *pulchra*	AB9Z	1.197	0.687	1.74	40.1	-	a	bc	bc	hi
	AB3W	1.191	0.684	1.74	39.9			b		hi

[1] The letters denote whether or not pairs of *Alstroemeria* species or accessions are significantly different at the P=0.05 level for their 2C-value and PI/DAPI ratio (Tukey's multiple range test). Species or accessions with the same letter do not differ significantly.

[2] *Allium cepa*, cv. 'Ailsa Craig', was the primary reference standard with PI and DAPI fluorescence ratios of 1.00 and 2C-value of 33.50 pg.

[3] 2C-value as reported by Bennett and Leitch, 1995.

[4] Fluorescence ratios of *Vicia faba* relative to *Allium cepa* and 2C-value as reported by Doležel *et al.*, 1992.

[5] 2C-value as reported by Bharathan *et al.*, 1994.

TABLE 5. Analysis of variance of 2C-value and PI/DAPI ratio for 78 measurements on 29 accessions of 12 *Alstroemeria* species, belonging to two groups (the Chilean and the Brazilian species).

Analyses of variance of 2C-value Source of variation	d.f.	MS	Expected MS	F	$P(F^x_y>F)=p$
Among groups	1	7.1		0.08 NS	$P(F^1_{76}>0.08)=0.78$
Residual (within groups)	76	93.1			
Among species	11	622.5		175.2***	$P(F^{11}_{66}>175.2)=0.000$
Residual (within species)	66	3.6			
Among accessions	28	252.5		1129.2***	
Residual (within accessions)	49	0.2			
Analysis of variance in nested classifications with unequal numbers					
Among groups	1	7.1	$\sigma_r^2+1.8\sigma_a^2+5.7\sigma_s^2+27.7\sigma_g^2$		
Among species within groups	10	684.0	$\sigma_r^2+2.9\sigma_a^2+6.5\sigma_s^2$		
Among accessions within species	17	13.2	$\sigma_r^2+2.5\sigma_a^2$	(F=59.8***)	
Residual (within accessions)	49	0.2	σ_r^2		$P(F^{17}_{49}>59.8)=0.000$
$s_r^2=0.22$	$s_a^2=5.09$	$s_s^2=103.37$	$s_g^2=-21.35$		
Analysis of variance of PI/DAPI ratio					
Among groups	1	0.1080		100.0***	$P(F^1_{76}>100.0)=0.000$
Residual	76	0.0011			
Among species	11	0.0123		15.8***	$P(F^{11}_{66}>15.8)=0.000$
Residual	66	0.0008			
Among accessions	28	0.0063		22.5***	
Residual (within accessions)	49	0.0003			$P(F^{28}_{49}>22.5)=0.000$
Analysis of variance in nested classifications with unequal numbers					
Among groups	1	0.1080	$\sigma_r^2+1.8\sigma_a^2+5.7\sigma_s^2+27.7\sigma_g^2$		
Among species within groups	10	0.0030	$\sigma_r^2+2.9\sigma_a^2+6.5\sigma_s^2$		
Among accessions within species	17	0.0008	$\sigma_r^2+2.5\sigma_a^2$	(F=2.9**)	
Residual (within accessions)	49	0.0003	σ_r^2		$P(F^{17}_{49}>2.9)=0.002$
$s_r^2=0.0003$	$s_a^2=0.0002$	$s_s^2=0.0003$	$s_g^2=0.0038$		

Analysis of variance in nested classifications with unequal numbers and estimates (s_x^2) of the components of variance (σ_x^2), with x= r (residual), a (accessions), s (species), g (groups). *** P<0.001, ** P<0.01, NS not significant.

TABLE 6. Propidium iodide and DAPI fluorescence ratio and 2C-value of diploid interspecific hybrids of *Alstroemeria*.

Interspecific hybrid	Parental code	Ploidy level	PI ratio	2C value (pg.)	mpv* 2C (pg.)	Prop. div.** (%)	DAPI ratio	mpv* DAPI	Prop. div.** (%)
A. aurea x *A. inodora*	A001P004-1	2x	1.573	52.7	52.9	+0.38	0.940	0.949	-0.94
A. inodora x *A. magnifica* ssp. *magnifica*	P004Q001-1	2x	1.379	46.2	46.2	+0.00	0.830	0.821	+0.94
A. inodora x *A. ligtu* ssp. *simsii.*	P002K101-1	2x	1.937	64.9	64.9	+0.03	1.099	1.087	+1.18
A. inodora x *A. pelegrina*	P002C049-3	2x	1.439	48.2	47.2	+2.11	0.850	0.839	+1.31
	P002C049-9	2x	1.442	48.3	47.2	+2.34	0.860	0.839	+2.50
	P002C049-10	2x	1.415	47.4	47.2	+0.42	0.840	0.839	+0.12
A. ligtu ssp. *ligtu* x *A. aurea*	AL1SA2-1	2x	1.788	59.9	62.0	-3.39	1.051	1.078	-2.36
A. magnifica ssp. *magnifica* x *A. inodora*	Q008P004-2	2x	1.370	45.9	46.1	-0.04	0.814	0.820	-0.99
A. pelegrina x *A. aurea*	C049A001-2	2x	1.457	48.8	49.7	-1.81	0.863	0.869	-0.69
A. pelegrina x *A. magnifica* ssp. *magnifica*	C042Q001-3	2x	1.275	42.7	42.9	-0.58	0.743	0.742	+0.20
A. psittacina x *A. aurea*	D032A001-1	2x	1.603	53.7	52.6	+2.09	0.937	0.940	-0.34
A. psittacina x *A. magnifica* ssp. *magnifica*	D032Q003-1	2x	1.301	43.6	43.2	+0.93	0.776	0.774	+0.27

* mpv= mid parent value = (value of ♀ + value of ♂) / 2

** prop. div. = proportional divergence = (value - mpv) / 100

TABLE 7. DAPI fluorescence ratio of euploids (4x) and aneuploids (4x+1) of Alstroemeria at the tetraploid level.

Interspecific hybrid	Parental code	Ploidy level	DAPI ratio	mpv*	Prop. div.** (%)
A. inodora x *A. magnifica* ssp. *magnifica*[1]	P004Q001-1-1	4x	1.644	1.645	-0.05
	P004Q001-1-2	4x	1.642	1.645	-0.18
	P004Q001-1-3	4x	1.585	1.645	-2.31
	P004Q001-1-4	4x+1	1.696	1.645	+3.07
	P004Q001-1-5	4x	1.649	1.645	+0.28
A. inodora x *A. pelegrina*[1]	P002C049-3-1	4x	1.672	1.678	-0.32
	P002C049-3-2	4x+1	1.859	1.678	+10.84
	P002C049-3-3	4x	1.692	1.678	+0.85
	P002C049-9-25	4x	1.712	1.678	+2.03
	P002C049-9-26	4x	1.745	1.678	+4.00
	P002C049-9-27	4x	1.719	1.678	+2.43
	P002C049-10-1	4x	1.684	1.678	+0.36
	P002C049-10-10	4x+1	1.788	1.678	+6.58
	P002C049-10-12	4x	1.660	1.678	-1.07
A. inodora x *A. pelegrina*[2]	P002C049-9-25-1	4x	1.690	1.678	+0.72
	P002C049-9-25-2	4x	1.678	1.678	+0.03
	P002C049-9-25-3	4x+1	1.834	1.678	+9.27
	P002C049-9-26-1	4x+1	1.833	1.678	+9.24
	P002C049-9-26-2	4x	1.639	1.678	-2.32
	P002C049-9-26-3	4x	1.701	1.678	+1.40

* mpv= mid parent value = (value of ♀ + value of ♂ / 2)

** prop. div. = proportional divergence = (value - mpv) / 100

[1] meiotically doubled, obtained through selfing of diploid hybrids.

[2] meiotically doubled, obtained through selfing of tetraploid hybrids.

Discussion

This investigation on 12 species of *Alstroemeria* has provided a further insight into the genomic differentiation within the genus. First, the 12 species possess large amounts of nuclear DNA that vary significantly among species. Second, the variation in nuclear DNA content among eight species representing both the Chilean and the Brazilian group of species can be largely ascribed to the variation in total chromosome length and the variation in total length of C-bands. Species that possess longer chromosome complements and more C-banded heterochromatin, have higher 2C-values. Third, in some species a considerable amount of intraspecific variation in 2C-value was observed (up to 16%). Part of this variation may be explained by the occurrence of C-band polymorphisms (Buitendijk and Ramanna, 1996). Fourth, when compared to the Chilean species, the Brazilian species have a relatively low PI/DAPI ratio, suggesting a higher AT content in their genomes. Fifth, the PI/DAPI ratio does not correlate with the amount of C-banded heterochromatin. In *Zea mays* Rayburn *et al.*, (1992) observed a significant negative correlation between these parameters.

Figure 4 depicts the relationship between the DNA content of the calibration standards *Allium cepa*, *Hordeum vulgare*, *Secale cereale*, *Triticum aestivum* and *Vicia faba* as reported in Bennett and Leitch (1995) and the relative DNA content as obtained from the flow cytometric measurements of PI fluorescence. The strong correlation between the reported and the observed 2C-values ($r = 0.99$, $n = 5$, $P < 0.01$) illustrates that the relative PI fluorescence could safely be used to estimate nuclear DNA content. When comparing plants within the genus *Alstroemeria*, the flow cytometric measurement of the DAPI fluorescence can be of even greater practical value than the PI fluorescence, since the DAPI measurements showed a higher sensitivity (cf. the coefficients of variation in Figure 1A and 1B). An important limitation to increase the resolution of flow cytometric analysis in animal and human systems was the occurrence of tissue related differences in fluorescence (Vindelov *et al.*, 1983). These authors examined and discussed the limits of detection of nuclear deviations in human and mouse systems. Differences of 1.5% between blood cells of males and females could be detected, as well as differences of 2.7% between euploid and aneuploid cells. In dioecious plants of *Silene*, Costlich *et al.*, (1991) distinguished between male and female individuals, that differed by 5% in nuclear DNA. Bashir *et al.*, (1993) and Pfosser *et al.*, (1995) showed that flow cytometry can be used to detect aneuploidy in wheat-rye addition lines (5-8% and 2% difference, respectively). The currently attainable resolution in *Alstroemeria* is determined by the coefficients of variation of the histograms. Because of the low chromosome number in *Alstroemeria*, it is possible to detect aneuploids. Depending on the species and the chromosome that is involved, an increase or decrease in nuclear DNA content of 3.4 to 14.3% can be expected on the basis of relative chromosome lengths (Buitendijk and Ramanna, 1996). Among a population of tetraploids we found aneuploid plants, that showed an increase in DAPI fluorescence of 3.07 to 10.84%. Chromosome heteromorphism can, however, lead to deviations from the expected fluorescence value as well. The deviation from the

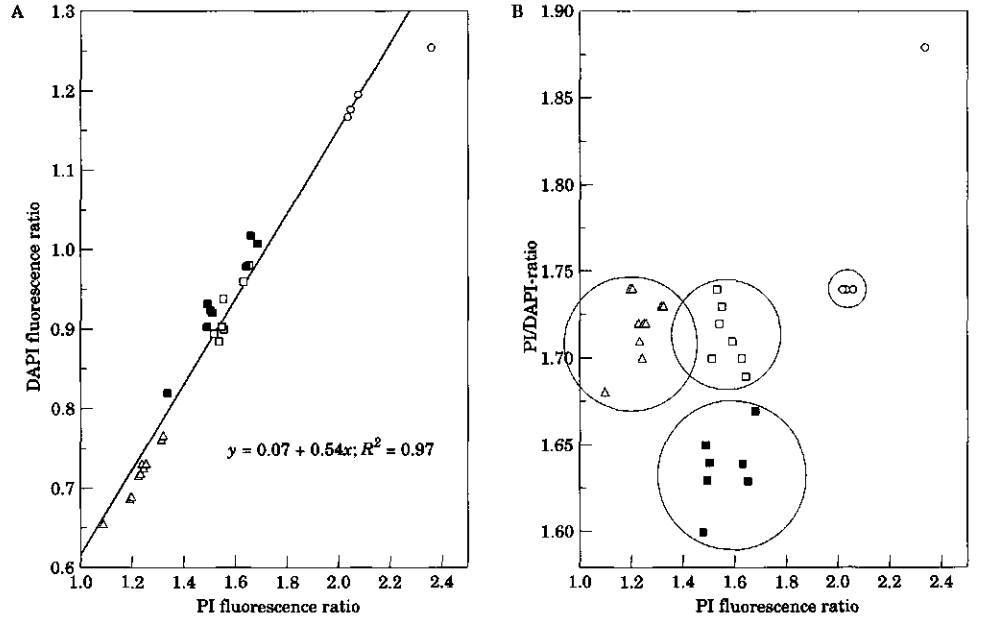

FIGURE 2. A, Scatter diagram of PI fluorescence ratios and DAPI fluorescence ratios of 29 accessions of 12 Alstroemeria species and B, scatter diagram of PI fluorescence ratio and PI/DAPI ratio. Accessions that are indicated with the same symbols have karyotype similarities. The four groups of species that can be distinguished show much similarity between karyotypes (cf. Buitendijk and Ramanna, 1996). Group 1: (△), *A. magnifica* ssp. *magnifica*, *A. pelegrina*, *A. philippii* and *A. pulchra* ssp. *pulchra*. Group 2: (□), *A. angustifolia* ssp. *angustifolia*, *A. aurea* and *A. hookeri* ssp. *hookeri*. Group 3: (○), *A. ligtu* ssp. *ligtu* and *A. ligtu* ssp. *simsii*. Group 4: (■), *A. brasiliensis*, *A. caryophyllaea*, *A. inodora* and *A. psittacina*.

expected DAPI fluorescence values among the euploid interspecific hybrids ranged from -2.36 to +2.50% for the diploids and from -2.32 to +4.00% for the allotetraploids. There are several reports on DNA variation in polyploid populations (Grant, 1969; Kenton *et al.*, 1986). Most of them describe a decrease in DNA amount in subsequent generations accompanied by a stabilization of the polyploid. Comparisons of the DNA values of polyploids with their true diploid ancestors are, however, rare (e.g. Raina *et al.*, 1994).

A two-fold variation in nuclear DNA content as was shown to occur in *Alstroemeria* is not unusual among geophytes (Bennett and Leitch, 1995). Grime and Mowforth (1982) surveyed the nuclear DNA content of 162 species of the British Flora, including many geophytes. They found that plants, in which growth is confined to winter and early spring, usually possess large genomes, whereas plants that grow under summer conditions have relatively small genomes. According to these authors, the simultaneous occurrence of a geophytic growth habit and large DNA amounts is not merely incidental, but results from adaptation to ecological niches where a temporal

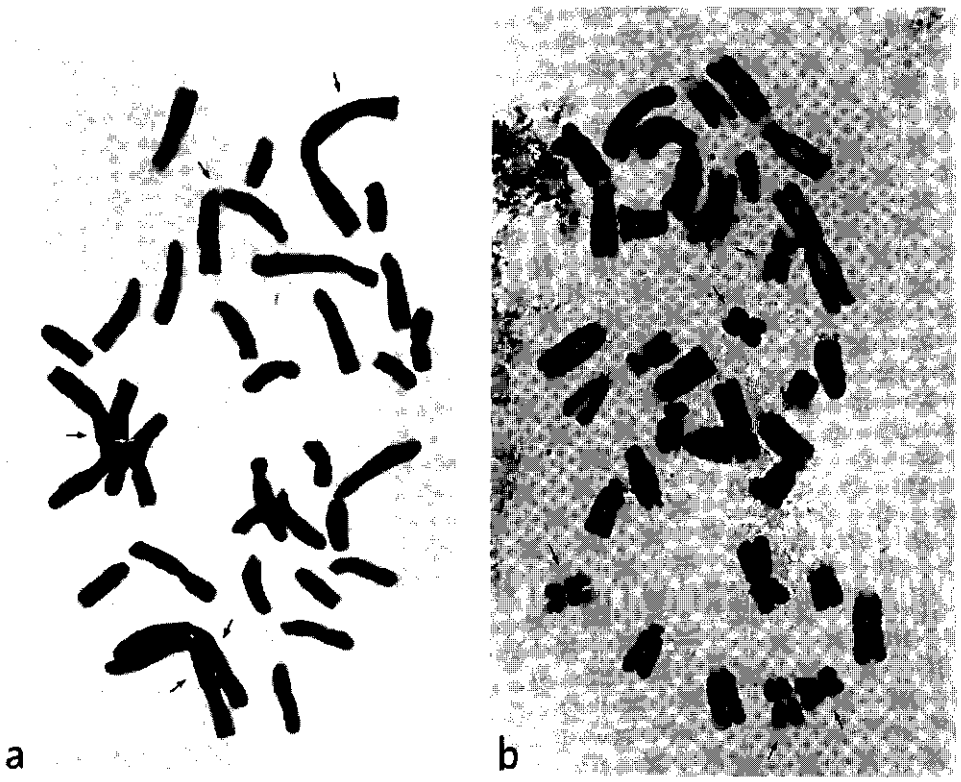

FIGURE 3. (a), Feulgen-stained metaphase plates of somatic chromosomes of hybrid P002C049-3-2 and (b), of hybrid P004Q001-1-4, both with 2n=4x+1=33 chromosomes. Hybrid P002C049-3-2 has chromosome 1 extra (arrowed). Hybrid P004Q001-1-4 has chromosome 7 extra (arrowed).

separation between cell division and cell elongation is advantageous. Such evolutionary adaptation may have taken place in the *Alstroemeria* species, in which dormancy is essential to survive periods of extreme drought and high temperature. The hypothesis that changes in nuclear DNA content have adaptational significance was first postulated by Bennett (1972). It was sustained by many reports on positive correlations between nuclear DNA content and growth related parameters, such as cell volume, duration of the mitotic and meiotic cycle, minimum generation time, speed of germination and general performance (reviewed by Price, 1988).

There were considerable differences in the PI/DAPI ratio between the genera, *Allium, Alstroemeria, Hordeum, Secale, Tulipa, Triticum* and *Vicia*. These differences can probably be ascribed to differences in overall base pair composition. The low PI/DAPI ratio for *Allium cepa*, is in agreement with the extremely low GC content that was reported by Stack and Comings (1979). The fluorescence values of *Vicia faba* approach the values that were found by Doležel *et al.*, (1992; Table 4). Within *Alstroemeria*, the PI/DAPI ratio was more constant, with the exception of one accession of *A. ligtu*. There was a tendency of lower values for the Brazilian species. Since the Brazilian species *A. psittacina* and the Chilean species *A. philippii*, for example, have

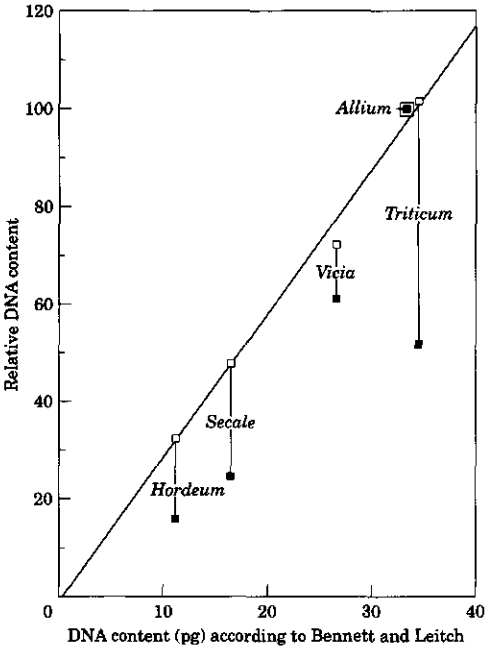

FIGURE 4. The relationship between DNA content of five plant species according to Bennett and Leitch (1995) (x-axis) and the relative DNA content estimated via the relative PI fluorescence, r=0.99, n=5, P<0.001. The relative DAPI values are also presented. The relative DNA content and the relative DAPI value of *Allium cepa* cv. 'Ailsa Craig' is 100. (□), PI; (■), DAPI.

equally low amounts of C-banded heterochromatin (Buitendijk and Ramanna, 1996), but differ in their PI/DAPI ratio (Table 4), it must be concluded that the differences between these species in binding capacity of PI and DAPI, find their origin in the non-C-banded chromatin. This probably reflects quantitative and qualitative differences in families of dispersed repeats.

The relationship between karyotype characteristics and genome size is not unexpected, since an increase in total length of chromosomes and in total length of C-bands imply an increase in DNA amount. A significant positive correlation between the total chromosome length and nuclear DNA content, as demonstrated in *Alstroemeria*, has also been found in *Allium* (Jones and Rees, 1968), *Avena* (Bullen and Rees, 1972) and other genera (Marks and Schweizer, 1974; Pringle and Murray, 1993). However, such a correlation was not found in *Secale* (Bennett, Gustafson and Smith, 1977). In *Secale* (Bennett *et al.*, 1977), *Allium* (Narayan, 1988), *Lathyrus* (Narayan and Rees, 1977) and *Zea mays* (Rayburn *et al.*, 1985; Tito, Poggio and Naranjo, 1991), however, changes in DNA content have been attributed to the amount of C-banded heterochromatin. Whether or not the total chromosome length and the length of C-bands are correlated with DNA content is determined by the mechanisms through which genomic changes occurred during the evolution of a taxon, such as amplification,

transposition, deletion, base pair divergence, rearrangement and translocation (Flavell, 1986; Dean and Schmidt, 1995). The succession of these events results in a colinearity of families of repeated sequences and a gradual divergence of genomic structure. It implies that the older a repetitive DNA family, the more likely it is to exist in different forms and at many locations within a species genome (Flavell, 1986). Within each of the four groups of *Alstroemeria* species, that could be distinguished on the basis of relative fluorescence of PI and DAPI (Figure 2), there are remarkable karyotype similarities (cf. Buitendijk and Ramanna, 1996). The first three groups consist of Chilean species. Whereas the species of group 1 have relatively small genomes with few terminal C-bands, group 2 comprises species with larger genomes and many terminal and interstitial C-bands and group 3 consists of species with very large genomes, many C-bands, relatively symmetrical karyotypes and an exceptional, large, metacentric chromosome 6. Group 4 is composed of Brazilian species, with large genomes and very few C-bands. Besides karyotype similarities, there are also similarities regarding the geographical distribution of species, as was discussed in Buitendijk and Ramanna (1996). Herbert (1837), who recognized 29 species, divided the genus *Alstroemeria* into nine groups based on morphological traits. Bayer (1987, p. 8) made no subdivision of the Chilean species and only mentioned three groups of species which are more closely related than others. These groups correspond to a great extent with the first three groups that are distinguished in the present work. It is very well possible, that a finer subdivision of the genus can be made after many more species are flow cytometrically studied and it will be interesting to compare this with the groups of Herbert (1837).

Individual repeated sequences have been cloned, characterized and mapped onto the chromosomes by *in situ* hybridization. Major tandem arrays are often localized in similar positions on the chromosomes, for instance at telomeres (in *Secale*: Jones and Flavell, 1982) or interstitially (in *Lycopersicon*: Ganal, Lapitan and Tanksley, 1988; in *Zea*: Peacock *et al.*, 1981). Other clusters of repeated sequences were shown to occur dispersed over the genome (Smyth, 1991; Hagemann, Scheer and Schweizer, 1993). It is expected that recent developments in the application of molecular techniques will enable us to verify and specify the observed differences and similarities between the species genomes of *Alstroemeria*.

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Chapter 5

Genome size variation and C-band polymorphism in *Alstroemeria aurea* Graham, *A. ligtu* L. and *A. magnifica* Herb. (Alstroemeriaceae)

J.H. Buitendijk, A. Peters, R.J. Quené and M.S. Ramanna. 1998. Plant Systematics and Evolution, in press.

Abstract:

Among a total of 43 accessions of *Alstroemeria aurea*, *A. ligtu* and *A. magnifica* nuclear DNA amounts (2C-values) showed significant intraspecific variation, 1.09, 1.21 and 1.15 fold, respectively, when determined through flow cytometric measurements of fluorescence of propidium iodide (PI) stained nuclei. After staining with another fluorochrome, 4',6-diamidino-2-phenylindole (DAPI), an intraspecific variation of 1.10, 1.11 and 1.12 fold, respectively, was found. C-band polymorphisms were present among and within the accessions of all three species. In some cases only very small differences in C-banding pattern were observed. In other cases, however, differences were more prominent. Besides C-band polymorphism, there were also instances of chromosome length polymorphism, which concerned the total chromosome complement or single chromosomes. The variation in nuclear DNA amount in *A. aurea* and *A. ligtu* was more or less continuous, except for one accession of *A. ligtu* ssp. *simsii*. Artificial selection and possibly introgression of chromosomes from other species may have moulded the karyotypes of some of the accessions of *A. aurea*, a species that has been under cultivation for more than 160 years. The variation as observed in *A. magnifica* ssp. *magnifica* was discontinuous and could be due to a broad species concept.

Key words: *Alstroemeriaceae*, *Alstroemeria aurea*, *A. ligtu*, *A. magnifica*, - C-band polymorphism, evolution, flow cytometry, genome size

Introduction

Karyotypes of *Alstroemeria* species are characterized by high nuclear DNA amounts and bimodal karyotypes. There are eight pairs of chromosomes (2n=2x=16), of which three or four are acrocentric and four or five are (sub)metacentric or subtelocentric. Upon Giemsa C-banding, the chromosomes reveal a banding pattern that is characteristic for the species concerned. Between species, there is considerable variation both in nuclear DNA content (at least two-fold) and in the amount of C-banded heterochromatin (Buitendijk and Ramanna, 1996; Buitendijk *et al.*, 1997).

Genomic differentiation was also confirmed through slot blot analysis and genomic *in situ* hybridization (Kuipers *et al.*, 1997).

The three Chilean species, *A. aurea*, *A. ligtu* and *A. magnifica* are representatives of the total range of DNA variation, with *A. magnifica* representing the small genomes and *A. ligtu* the large genomes. Some degree of intraspecific variation concerning both nuclear DNA amounts and C-band polymorphisms has been reported for these species (Buitendijk and Ramanna, 1996; Buitendijk *et al.*, 1997).

Both *A. aurea* (Hang and Tsuchiya, 1988; Ramanna, 1992) and *A. magnifica* are progenitors of many of the modern cultivars. *A. aurea* has been cultivated in Europe and America (Herbert, 1837; Robinson, 1963) for more than 160 years, and in Australia (Errey, 1962) and Japan (Miyake, 1989) for more than 70 years. The species *A. ligtu*, as described by Bayer (1987), comprises three subspecies: (1) *A. ligtu* ssp. *ligtu*, (2) *A. ligtu* ssp. *simsii* and (3) *A. ligtu* ssp. *incarnata*. In the past, there has been much confusion about the identification and nomenclature of this species (among others Stinson 1945 and 1952; for synonyms see Bayer, 1987). This confusion was probably enhanced by the fact that the plants known as *A. chilensis*, *A. haemantha*, *A. ligtu* and *A. hookeri* intercross readily (Hannibal, 1941; Comber, 1946; Sahin, 1973) and many intermediate forms of the subspecies of *A. ligtu* occur in nature (Bayer, 1987: 128). The current view is to include *A. chilensis* and *A. haemantha* in *A. ligtu* (Bayer, 1987). *A. ligtu* is not (yet) involved in the modern West European cultivars. In Japan *A. ligtu* was successfully used in breeding some of the spotless strains of *Alstroemeria* (Miyake, 1989).

The aim of the present investigation was to explore the extent of the intraspecific variation concerning nuclear DNA content and C-banding pattern among a number of accessions of *A. aurea*, *A. ligtu* and *A. magnifica*. The C-banding patterns of *A. ligtu* ssp. *simsii* and *A. ligtu* ssp. *incarnata* have not been published before. In order to establish homoeology between the chromosomes of the different genomes, meiotic pairing was monitored. The results are discussed in the context of the methodology of estimating DNA variation and the mechanisms that generate and maintain chromosome structural polymorphisms.

Materials and methods

Plant material

A total of 43 accessions of the species *A. aurea*, *A. ligtu* and *A. magnifica* was obtained from botanical gardens, breeders and institutes and maintained at the Department of Plant Breeding of the Agricultural University of Wageningen (Table 1). The identity of the accessions could be verified (Bayer, 1987). The material included wild accessions, and in the case of *A. aurea* also a cultivar, and other selected genotypes. Although the material could be propagated clonally, it had mostly been reproduced sexually at nurseries for an unknown number of generations. The accessions of *A. ligtu* were compared to the descriptions of the three subspecies of *A. ligtu* given by Bayer (1987).

TABLE 1. Details on accession codes, morphological features, state of cultivation and source of accessions of *Alstroemeria aurea*, *A. ligtu* and *A. magnifica* and four of their interspecific hybrids.

Species*		State of cultivation	Flower colour/ Hairs on leaf edges
A. aurea Graham	A001/W	selected genotype	yellow
	A002/W	selected genotype	orange-red
	A003/W	selected genotype	yellow
	A005/BGA	selected genotype	orange
	A006/BGW	selected genotype	yellow
	A010/BGW	selected genotype	yellow
	A011/Z	selected genotype	yellow
	A014/Z	cultivar 'Orange King'	orange
	A016/S	selected genotype	orange
	A017/W	selected genotype	orange
	A023/Z	selected genotype	yellow
	A024/C	selected genotype	yellow
	Aw11/W	selected genotype	orange
	Aw12/W	selected genotype	orange
	Aw14/W	selected genotype	orange
	Aw15/W	selected genotype	yellow
	Aw16/W	selected genotype	yellow
	Aw28/W	selected genotype	orange
	AwM23/W	selected genotype	yellow
	AwM60/W	selected genotype	yellow
A. ligtu L. ssp. *ligtu*	AL1S/S	wild	light pink/0.1 mm
	AL5S/S	wild	pink/<0.1 mm
	FB190S/S	wild	white-pink/<0.1 mm
	F051-2/W	wild	pink/0.1-0.2 mm
A. ligtu L. ssp. *simsii* (Sprengel) Bayer	J091-4/W	wild	orange-red/0.2-0.5 mm
	AM3S/S	wild	orange-red/0.2-0.5 mm
	K101-1/W	wild	orange-red/>0.6 mm
	L111-2/W	wild	orange-red/0.2-0.5 mm
	K470/W	wild	orange-red/0.2-0.5 mm
A. ligtu L. ssp. *incarnata* Bayer	AJ4S/S	wild	light pink/0.5-0.6 mm
	AJ7S/S	wild	light pink/0.4-0.6 mm
A. magnifica Herb. ssp. *magnifica*	XIII-1/LBO	wild	light purple
	XIII-2/LBO	wild	light purple
	F169-3/W	wild	?
	Q002/PBA	wild	purple-violet
	Q003/PBA	wild	purple-violet
	Q001/Z	wild	purple-violet
	Q008/P	wild	purple-violet
	Qw1/W	wild	purple-violet
	Qw2/W	wild	purple-violet
	Qw3/W	wild	purple-violet
	Qw4/W	wild	purple-violet
	Qw5/W	wild	purple-violet

Interspecific hybrid	Parental code/Source**
A. ligtu ssp. *ligtu* x *A. aurea*	AL1SA002-2/J
A. ligtu ssp. *ligtu* x *A. aurea*	AL5SA002-2/J
A. inodora x *A. ligtu* ssp. *simsii*	P002K101-1/J
A. aurea x *A. magnifica* ssp. *magnifica*	A016Q001-3/B

* Classification according to Bayer (1987).

** Source of plant material. BGA=Botanical Garden VUA Amsterdam; BGW=Botanical Garden Wageningen; B=Buitendijk *et al.*, 1995; C=Chiltern Seeds (UK); J=De Jeu and Jacobsen 1995; LBO=Laboratorium voor Bloembollen Onderzoek; P=Parigo (UK); PBA=Proefstation voor de Bloemisterij Aalsmeer; S=Van Staaveren; W=Wülfinghoff Freesia; Z=Koninklijke Van Zanten.

They were identified as *A. ligtu* ssp. *ligtu*, *A. ligtu* ssp. *simsii* and *A. ligtu* ssp. *incarnata* (Table 1). No instances of intermediate forms of these subspecies were found. According to Bayer (1987: 198), the large leafed and large flowered *A. gayana* and *A. sierrae* (formerly separated as species) are conspecific with *A. magnifica* ssp. *magnifica*. No accessions of the other subspecies of *A. magnifica*, *A. magnifica* ssp. *maxima*, were available. Two species accessions, A017 and Qw1, had a triploid chromosome number (2n=3x=24). All other species had a diploid chromosome complement. Herbarium vouchers are being deposited in Wageningen Herbarium (WAG). Four diploid interspecific hybrids were used for measuring the length of the haploid chromosome complements of some of the accessions of *A. ligtu* ssp. *ligtu*, *A. ligtu* ssp. *simsii* and *A. magnifica* ssp. *magnifica* (for methodology see Buitendijk and Ramanna, 1996). Two of these interspecific hybrids were used to study homoeology of the chromosomes in male meiosis.

Estimation of nuclear DNA amount

Relative fluorescence of nuclei, that were stained with propidium iodide (PI) or 4',6-diamidino-2-phenylindole (DAPI), was determined with a flow cytometer, according to a method described before (Buitendijk *et al.*, 1997). In each of the accessions, two to six samples were analysed. In both PI-and DAPI-stained samples about 2,000 nuclei were analysed. The fluorescence of each *Alstroemeria* accession, relative to the fluorescence of *Allium cepa*, cv. 'Ailsa Craig', was expressed as fluorescence ratio (Table 2). Nuclear DNA amounts of the *Alstroemeria* samples, in terms of picograms of DNA per nucleus, (2C-value) were estimated via their PI-fluorescence ratio and the 2C-value of *Allium cepa*, cv. 'Ailsa Craig' (33,5 pg., Bennett and Smith, 1976).

Karyotype analysis

Root or shoot tips were collected during morning hours. The material was pre-treated with 2mM 8-hydroxyquinoline, fixed in acetic alcohol (3:1), metaphase plates were Giemsa C-banded or Feulgen-stained, and the lengths of chromosomes and C-bands were measured (for details see Buitendijk and Ramanna, 1996). The chromosomes in the karyograms of the three species are numbered according to their homoeology with the *A. aurea* chromosomes (Buitendijk and Ramanna, 1996) as far as known from meiotic preparations, otherwise according to their similarity with the complement of *A. aurea*. When homologous chromosomes showed structural heterozygosities, that were sufficiently pronounced to distinguish between them, they were measured separately and designated as A,B,C, etcetera. When no or only very minor differences were observed, the values of the homologues were pooled.

Identification of homoeologous chromosomes in meiosis of diploid interspecific hybrids

Young flower buds of about 0.5 cm length were collected during morning hours in Carnoy solution. The material was fixed for at least 24 hours. The anthers were cut, pressed onto a glass slide so that their contents was shed, and gently squashed after heating in a gas flame. Preparations were checked for meiotic metaphase I stages and

Giemsa C-banded. Bivalents and univalents were analysed so that homoeologous chromosomes could be identified.

Statistical Procedures

Statistical analysis involved an analysis of variance of 2C-value, with Tukey's multiple range test using honestly significant differences to indicate significance of differences in DNA amount between species accessions, and Pearson correlation analysis of 2C-value, relative heterochromatin amount and PI/DAPI ratio.

Results

Nuclear DNA amounts

The relative PI-fluorescence and DAPI-fluorescence values of the *Alstroemeria* accessions are given in Table 2. Nuclear DNA amounts (2C-values) are deduced from the relative PI-fluorescence values. The 2C-values of the diploid accessions of *A. aurea* varied from 50.7 to 55.3 pg. DNA (1.09 fold), those of *A. ligtu* from 65.2 to 78.9 pg. DNA (1.21 fold) and those of *A. magnifica* ssp. *magnifica* from 36.5 to 41.9 pg. DNA (1.15 fold). Significance of differences in DNA content between individual accessions is indicated in Table 2. The DAPI-fluorescence values showed an intraspecific variation of 1.10 fold for *A. aurea*, 1.11 fold for *A. ligtu* and 1.12 fold for *A. magnifica* ssp *magnifica*. The PI/DAPI ratio of the diploid *A. aurea* accessions ranged from 1.53 to 1.83, with most values between 1.66 and 1.70. Among the *A. ligtu* accessions this PI/DAPI ratio varied from 1.73 to 1.77 with one exceptionally high value of 1.88 for the accession K101-1 of *A. ligtu* ssp. *simsii*. The PI/DAPI ratio in *A. magnifica* ssp. *magnifica* indicated two groups of accessions: one group with ratios between 1.67 and 1.68, and another group with ratios between 1.72 and 1.73. The first group coincided with the accessions that had 2C-values of about 36.6 pg. and the second group with the accessions that had about 41.6 pg. (Table 2). The triploid species accessions of *A. aurea* (A017) and *A. magnifica* ssp. *magnifica* (Qw1) had fluorescence values and DNA amounts of about 1.5 times the values of the diploid accessions. Their PI/DAPI ratios were in the same range as those of the diploid accessions.

Giemsa C-banded and Feulgen-stained karyotypes

The C-banded karyotypes of 16 *A. aurea*, six *A. ligtu* and four *A. magnifica* accessions were analysed (Figure 1; Table 2) and they revealed differences between homologous chromosomes. These differences concerned both the C-band patterns and the relative chromosome length. Many accessions showed heterozygous banding patterns, but homozygosity was more frequent (Figures 2, 3 and 4; Table 2). Whereas some homologous chromosomes showed only a limited number of polymorphic forms (e.g. chromosomes 1,2,3,4 and 7 of *A. aurea*), other chromosomes were highly variable (e.g. chromosomes 5 and 8 of *A. aurea*).

TABLE 2. The PI- and DAPI-fluorescence ratios of 43 *Alstroemeria* accessions relative to the fluorescence values of *Allium cepa*, the PI/DAPI ratio, the 2C-values as derived from relative PI-fluorescence ratios in picograms, the amount of C-banded heterochromatin expressed as the percentage of the total chromosome length that consists of C-bands, and the composition of polymorphic forms in 26 accessions of *A. aurea*, *A. ligtu* and *A. magnifica* (cf. Figure 1).

Species	Accession[1]	PI ratio	DAPI ratio	PI/DAPI[2]	2C-value[2] (pg.) ±SD	Ranges of Tukey[3]	Hetero-chromatin[2] (%)	Polymorphic forms in chromosome pair 1	2	3	4	5	6	7	8
Allium cepa	'Ailsa Craig'	1.000	1.000	1.00	33.5[4]		-								
Alstroemeria aurea	A001[5]	1.651	0.979	1.69	55.3 ± 0.5	h	18.4	AA	AA	AA	BB	FF	DD	CC	EE
	A002[5]	1.630	0.958	1.70	54.6 ± 0.1	efgh	17.7	AA	AB	AA	AA	CC	DD	BB	DE
	A003	1.641	0.972	1.69	55.0 ± 0.1	fgh	17.7	AA	BB	AA	AB	BF	DD	BC	EE
	A005[5]	1.594	0.937	1.70	53.4 ± 0.3	def	16.8	BB	AA	AA	AA	BD	DD	CC	EF
	A006	1.630	0.970	1.68	54.6 ± 0.9	efgh	18.0	AA	AA	AA	AB	BB	EE	CC	FF
	A010	1.588	0.942	1.69	53.2 ± 0.0	de	16.1	BB	BB	AA	BB	CE	DD	BC	CE
	A011	1.606	0.957	1.68	53.8 ± 0.6	defg	-	-	-	-	-	-	-	-	-
	A014	1.594	0.937	1.70	53.4 ± 0.2	de	15.9	AA	AA	AA	AA	BE	DD	CC	EE
	A016	1.618	0.958	1.69	54.2 ± 0.0	efgh	17.3	AA	AA	AA	AA	CC	DD	CC	EE
	A017[1]	2.415	1.422	1.70	80.9 ± 0.2		16.7	AAA	ABB	AAA	AAA	EE?	DDD	BBC	DEE
	A023	1.636	0.939	1.74	54.8 ± 0.4	fgh	-	-	-	-	-	-	-	-	-
	A024	1.591	0.941	1.69	53.2 ± 0.3	de	-	-	-	-	-	-	-	-	-
	AwO11	1.654	0.973	1.70	54.0 ± 0.4	defgh	17.2	AA	AA	AA	AA	DE	DE	AC	EG
	AwO12	1.567	0.937	1.67	52.5 ± 0.0	d	14.3	AA	AA	AA	AA	AB	DD	AB	AE
	AwO14	1.597	0.937	1.70	53.5 ± 0.2	def	15.8	CC	AA	AA	BC	AD	DD	AC	BE
	AwG15	1.582	0.931	1.67	53.0 ± 0.2	de	16.1	AA	AA	AA	AB	DG	BC	CC	CE
	AwG16	1.558	0.922	1.69	52.2 ± 0.1	d	15.4	AA	AA	AA	AA	BB	AD	BC	CE
	AwM23	1.513	0.988	1.53	50.7 ± 0.5	c	11.9	AA	A?	AA	B?	D?	DE	AC	AE
	AwO28	1.588	0.949	1.67	53.2 ± 0.4	de	15.8	AA	AA	AA	AA	BC	DD	BB	DD
	AwM60	1.645	0.899	1.83	55.1 ± 0.5	gh	-	-	-	-	-	-	-	-	-

TABLE 2. Continued

Species	Accession[1]	PI ratio	DAPI ratio	PI/DAPI[2]	2C-value[2] (pg.) ±SD	Ranges of Tukey[3]	Hetero-chromatin[2] (%)	Polymorphic forms in chromosome pair 1	2	3	4	5	6	7	8
A. ligtu ssp. *ligtu*	AL1S[5]	2.072	1.194	1.74	69.4 ± 0.4	jkl	13.9	AA	AA	AA	AB	AA	AA	AA	AB
	AL5S	2.040	1.167	1.75	68.3 ± 0.4	jk	13.1	AA	AA	AA	AB	AA	AA	AB	AA
	FB190S	2.036	1.176	1.73	68.2 ± 0.3	jk	-	-	-	-	-	-	-	-	-
	F051-2[5]	2.033	1.167	1.74	68.1 ± 0.6	j	12.9	AA	AA	AA	AB	BC	AA	BB	AB
A. ligtu ssp. *simsii*	J091-4[5]	2.045	1.176	1.74	68.5 ± 0.2	jk	-	-	-	-	-	-	-	-	-
	AM3S	2.065	1.196	1.73	69.2 ± 0.1	jkl	19.1	BB	BB	CC	CC	DE	CC	EE	DE
	K101-1[5]	2.355	1.254	1.88	78.9 ± 0.4	m	17.6	CC	CC	BB	DE	FF	BC	CD	CC
	L112	1.946	1.125	1.73	65.2 ± 0.2	i	-	-	-	-	-	-	-	-	-
	K470	1.961	1.134	1.73	66.7 ± 0.6	i	-	-	-	-	-	-	-	-	-
A. ligtu ssp. *incarnata*	AJ4S	2.081	1.176	1.77	69.7 ± 0.9	kl	22.1	DD	DE	EF	FF	GH	DE	FF	FG
	AJ7S	2.090	1.185	1.76	70.0 ± 0.5	l	-	-	-	-	-	-	-	-	-
A. magnifica ssp. *magnifica*	XIII-1	1.098	0.657	1.67	36.8 ± 0.1	a	-	-	-	-	-	-	-	-	-
	XIII-2[5]	1.090	0.651	1.67	36.5 ± 0.2	a	-	-	-	-	-	-	-	-	-
	F169-3[5]	1.224	0.713	1.72	41.0 ± 0.4	b	6.5	AA	BB	CC	CC	CC	BB	AA	CC
	Q002	1.093	0.651	1.68	36.6 ± 0.0	a	-	-	-	-	-	-	-	-	-
	Q003	1.089	0.650	1.68	36.5 ± 0.1	a	-	-	-	-	-	-	-	-	-
	Q001[5]	1.251	0.728	1.72	41.9 ± 0.6	b	6.3	AA	BB	BB	CC	BB	BB	AA	BB
	Q008[5]	1.245	0.722	1.72	41.7 ± 0.4	b	6.3	BB	AA	CC	CD	BB	AA	BB	AA
	Qw1[1]	1.839[1]	1.099	1.67	61.6 ± 0.2		-	-	-	-	-	-	-	-	-
	Qw2	1.245	0.722	1.73	41.7 ± 0.4	b	6.9	AA	AA	AA	AA	AA	AC	BB	AA
	Qw3	1.248	0.722	1.73	41.8 ± 0.2	b	-	-	-	-	-	-	-	-	-
	Qw4	1.248	0.725	1.72	41.8 ± 0.4	b	-	-	-	-	-	-	-	-	-
	Qw5	1.245	0.722	1.73	41.7 ± 0.5	b	-	-	-	-	-	-	-	-	-

[1] All *Alstroemeria* species accessions had a diploid chromosome number (2n=2x=16), except for the accessions A017 of *A. aurea* and Qw1 of *A. magnifica* ssp. *magnifica*, which were triploid (2n=3x=24).

[2] Significant positive correlations were found between % heterochromatin and 2C-value for the accessions of *A. aurea* (r=0.96, n=15, P<0.001) and between PI/DAPI ratio and 2C-value for the accessions of *A. aurea* (r=0.77, n=15, P=0.001) and *A. ligtu* (r=0.97, n=6, P=0.001).

[3] The letters denote whether or not pairs of *Alstroemeria* species accessions are significantly different at the p=0.05 level for their 2C-value (Tukey's multiple range test). Accessions with the same letter do not differ significantly.

[4] 2C-value of the calibration standard *Allium cepa* cv. 'Ailsa Craig' as reported in Bennett and Leitch (1995)

[5] Data from Buitendijk *et al.*, 1997.

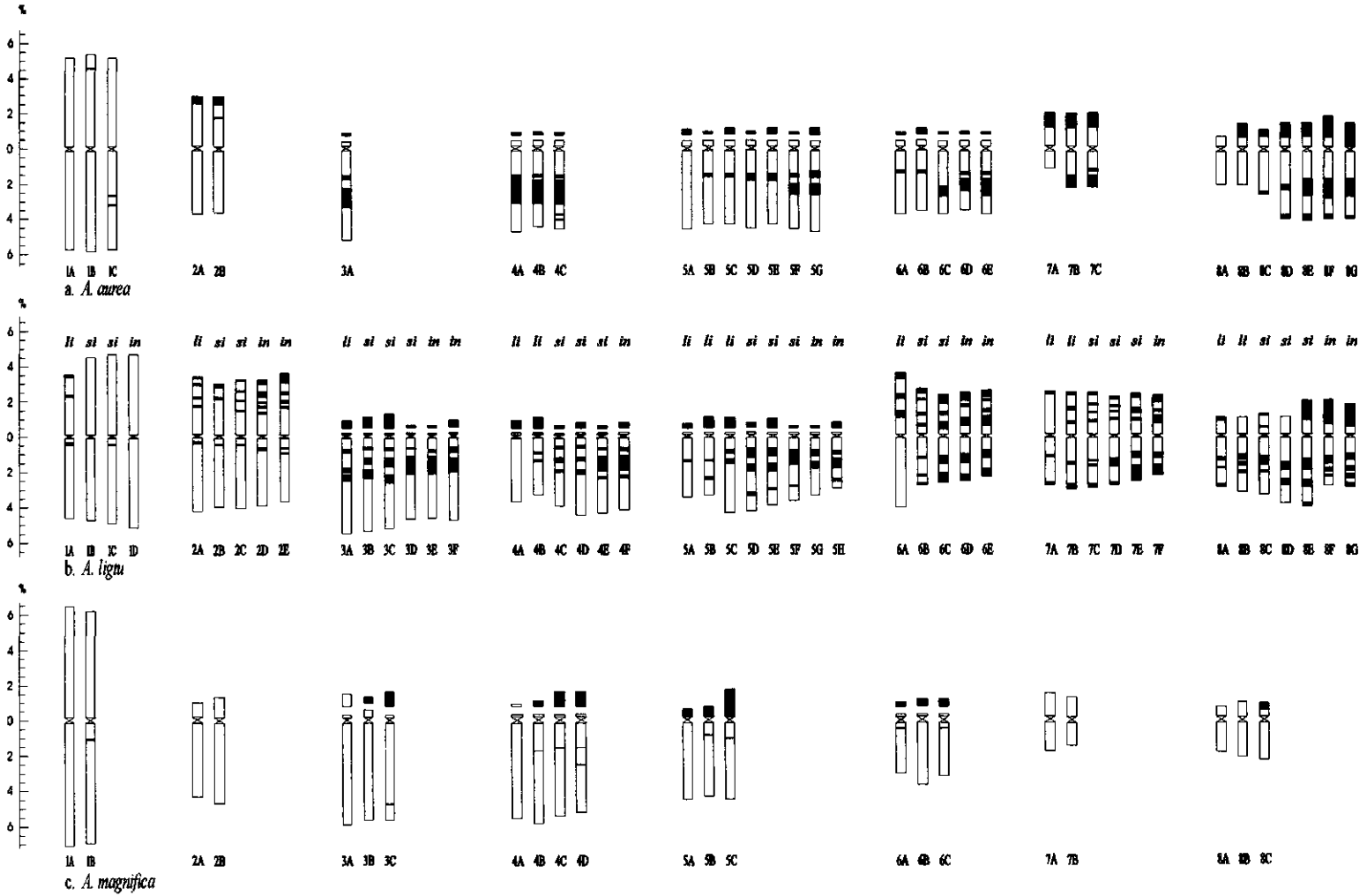

FIGURE 1. Schematic presentation of C-band polymorphism as observed in the A. aurea (a), A. ligtu (b) and A. magnifica accessions (c). The idiograms present the length of chromosomes and their C-banded segments relative to the total length of the respective diploid karyotype (100%). li = A. ligtu ssp. ligtu, si = A. ligtu ssp. simsii, in = A. ligtu ssp. incarnata.

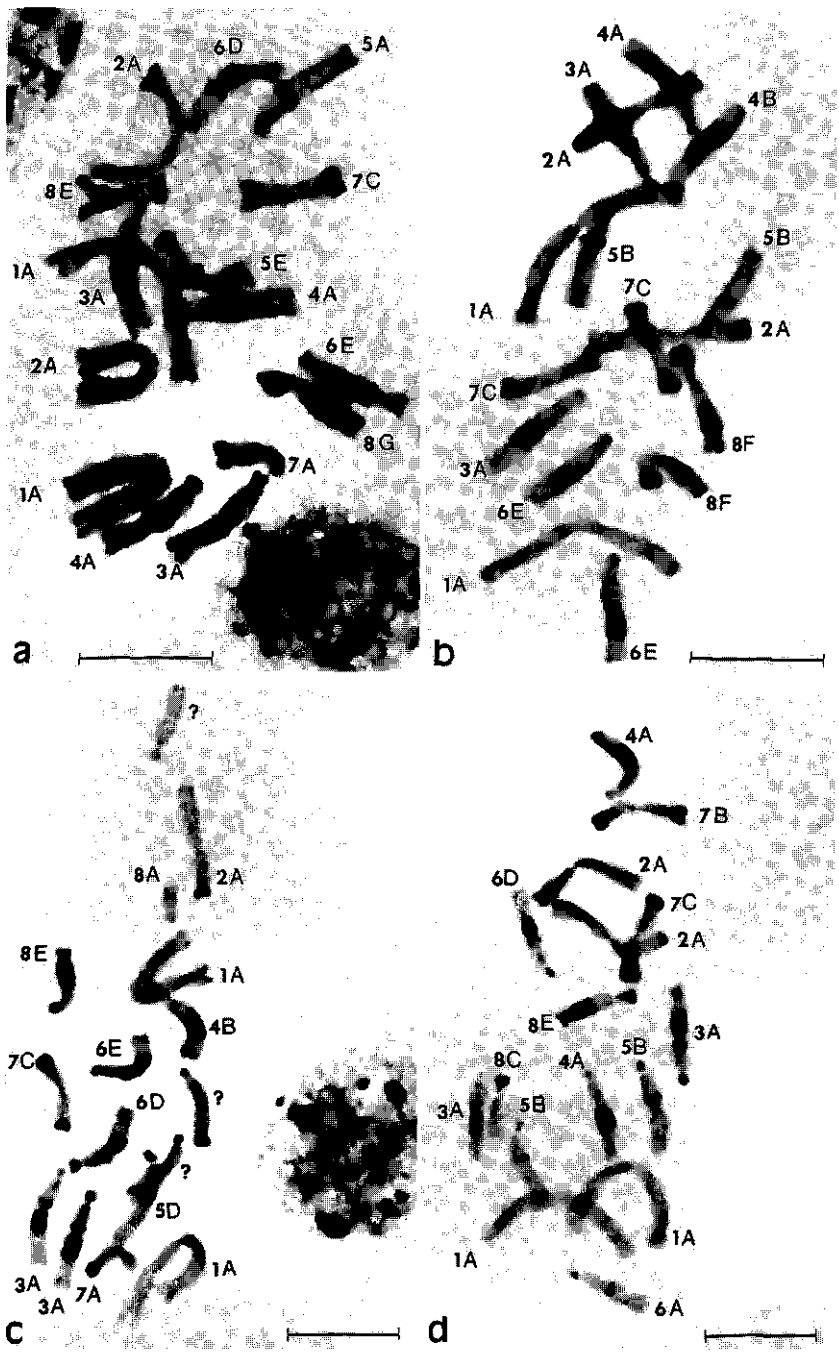

FIGURE 2. C-band polymorphism in *A. aurea* accessions AwO11 (a), A006 (b), AwM23 (c) and AwG16 (d). The numbers and letters correspond with the polymorphic forms as depicted in Figure 1a. ? = unidentified chromosome. Bar = 10 mm.

This led to varying numbers of types per chromosome in Figure 1. The *A. aurea* accessions revealed many differences in size and location of C-bands as well as in chromosome length (Figures 1a and 2). The total amount of C-banded heterochromatin ranged from 11.9 to 18.4 % of the total chromosome length (Table 2). Accession AwM23 had the lowest amount of heterochromatin, the lowest DNA content and the lowest PI/DAPI ratio. Accession A001 showed the highest amount of heterochromatin and the highest DNA content. There was a significant positive correlation between C-banded heterochromatin and DNA content ($r = 0.96$, $n = 15$, $P < 0.001$).

The total range of polymorphic forms that was found in the accessions of *A. ligtu* that were used for C-banding, is presented in Figure 1b. The C-banded karyotype of *A. ligtu* ssp. *ligtu* was clearly different from the other two subspecies, having less C-banded heterochromatin and a relatively long chromosome 6 (Figures 1b and 3). There were many similarities between the C-banded karyotypes of the *A. ligtu* ssp. *simsii* and the *A. ligtu* ssp. *incarnata* accessions. The lowest amount of C-banded heterochromatin (12.9%) was found in an accession of *A. ligtu* ssp. *ligtu* and the highest amount (22%) was found in *A. ligtu* ssp. *incarnata* (Table 2). The secondary constrictions, especially those of the chromosomes 2, 6 and 7 of *A. ligtu* of the C-banded preparations, could not be detected in all cases, whereas they were usually well discernible in Feulgen-stained preparations (arrowed in Figure 5). The exact position of these secondary constrictions in the C-banded karyotypes could not be determined with certainty and was not indicated in Figure 1b.

TABLE 3. The lengths (mm) of the haploid chromosome complements as measured in Feulgen-stained preparations of the diploid interspecific hybrids between *A. ligtu* ssp. *ligtu* and *A. aurea*, between *A. inodora* and *A. ligtu* ssp. *simsii* and between *A. aurea* and *A. magnifica* ssp. *magnifica* (for methodology see Buitendijk and Ramanna, 1996).

		Total chromosome length (µm)	
Interspecific hybrid	Parental code	♀-parent	♂-parent
A. ligtu ssp. *ligtu* x *A. aurea*	AL1SA002-2	90 ± 1	76*
A. ligtu ssp. *ligtu* x *A. aurea*	AL5SA002-2	91 ± 1	76*
A. inodora x *A. ligtu* ssp. *simsii*	P002K101-1	78*	119 ± 2
A. aurea x *A. magnifica* ssp. *magnifica*	A016Q001-3	76*	59 ± 2

Species	Total chromosome length (µm)	Measurements obtained and described in:
A. magnifica ssp. *magnifica*	58 ± 1	Buitendijk and Ramanna, 1996
	59 ± 2	this paper
A. ligtu ssp. *ligtu*	90 ± 2	Buitendijk and Ramanna, 1996
	91 ± 1	this paper
A. ligtu ssp. *simsii*	119 ± 2	this paper
A. aurea	76 ± 3	Buitendijk and Ramanna, 1996

* Average value of measurements of parental genomes in other hybrids (Buitendijk and Ramanna, 1996).

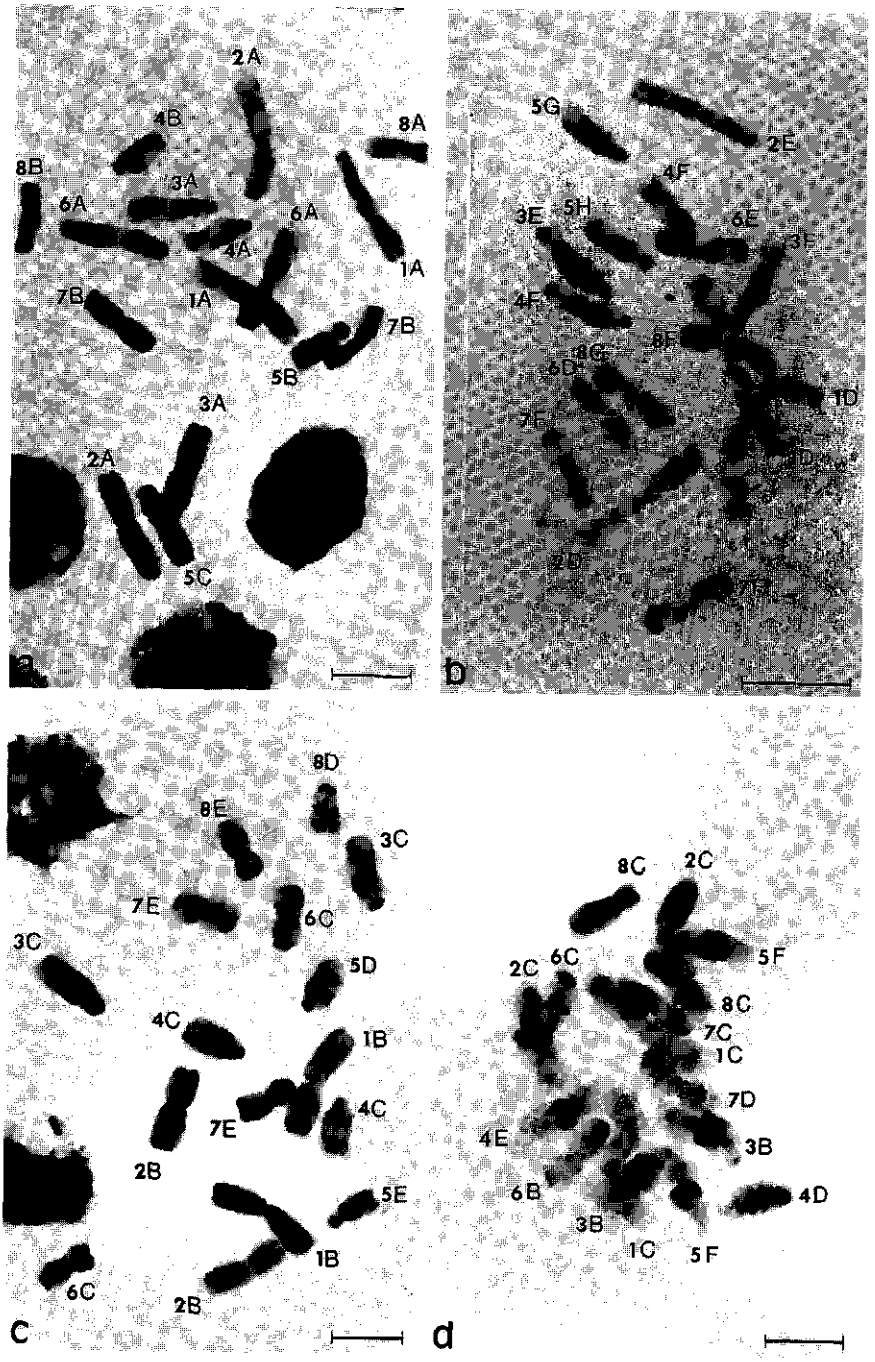

FIGURE 3. C-band polymorphism in the accessions F051-2 (a) of *A. ligtu* ssp. *ligtu*, AJ4S (b) of *A. ligtu* ssp. *incarnata* and AM3S (c) and K101-1 (d) of *A. ligtu* ssp. *simsii*. The numbers and letters correspond with the polymorphic forms as depicted in Figure 1b. Bar = 10 mm.

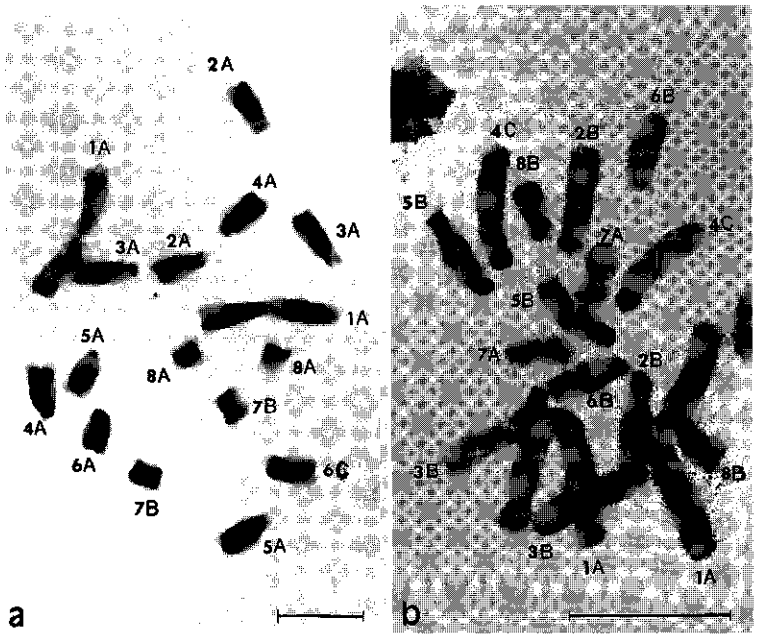

FIGURE 4. C-banded karyotypes of accessions Qw2 (a) and Q001 (b) of *A. magnifica* ssp. *magnifica*. The numbers and letters correspond with the polymorphic forms in Figure 1c. Bar = 10 mm.

The four accessions of *A. magnifica* ssp. *magnifica*, F169-3, Q001, Q008 and Qw2 that were used for C-banding, showed some differences in banding pattern and in relative chromosome length (Figures 1c and 4; Table 2). The amount of C-banded heterochromatin in these accessions ranged from 6.3 to 6.9 % of the total chromosome length (Table 2).

The lengths of the haploid chromosome complements of some species accessions as measured in Feulgen-stained preparations (Figure 5) of the diploid interspecific hybrids *A. ligtu* x *A. aurea*, *A. inodora* x *A. ligtu* and *A. aurea* x *A. magnifica* ssp. *magnifica*, are given in Table 3. Total chromosome length was much longer in accession K101-1 of *A. ligtu* ssp. *simsii* than in accessions AL1S and AL5S of *A. ligtu* ssp. *ligtu*.

The homoeology of the chromosomes 1, 2, 5, 6, 7 and 8 in the three species *A. aurea*, *A. ligtu* ssp. *ligtu* and *A. magnifica* ssp. *magnifica* was established through the analysis of metaphase I stages of male meiosis in interspecific hybrids (Figure 6). Univalents and bivalents were identified on the basis of chromosome length, centromere position and C-banding pattern and homoeologous chromosomes could be matched. Because of their close resemblance, it was not possible in all cases to distinguish between the acrocentric chromosomes 3, 4 and, in case of *A. aurea* and *A. magnifica*, chromosome 6. Chromosome 6 of *A. ligtu* was occasionally involved in a multivalent, together with the chromosome pairs 1 or 2.

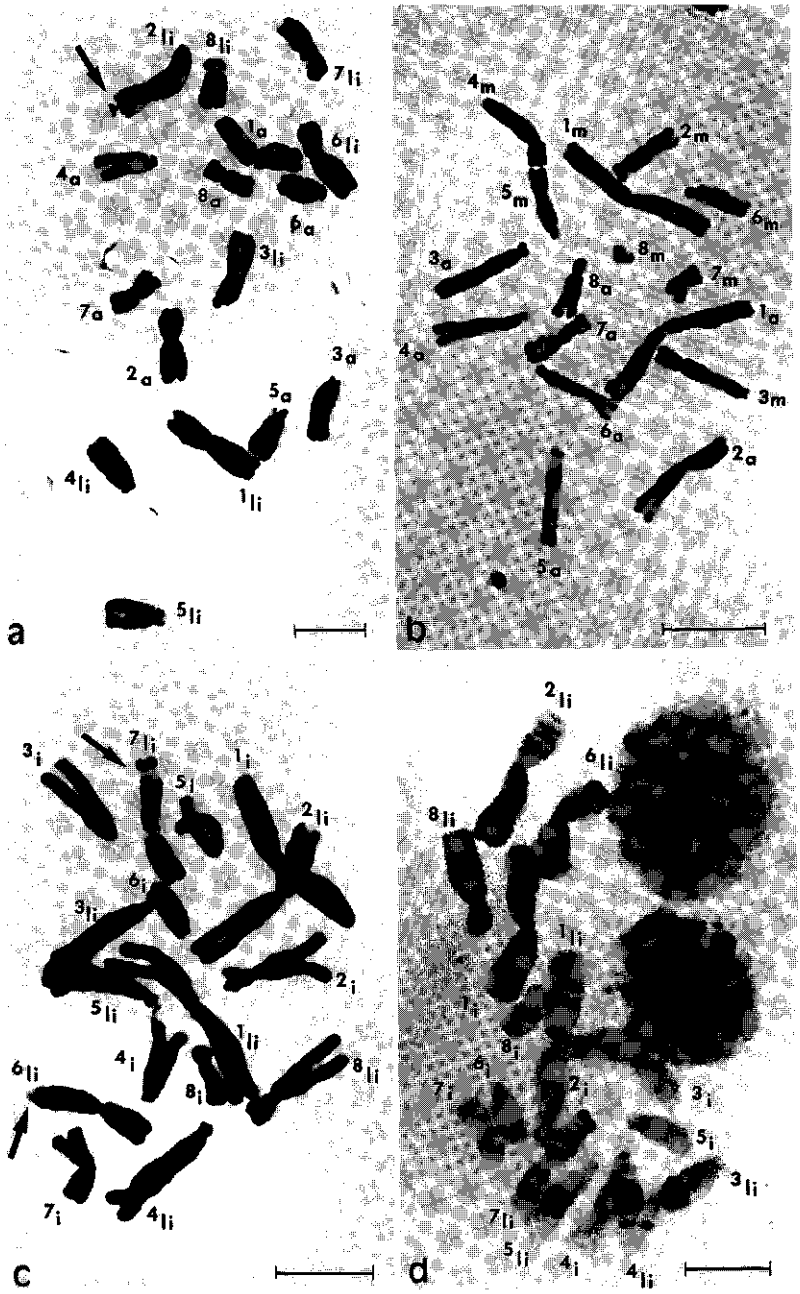

FIGURE 5. a-c Feulgen-stained karyotypes of diploid interspecific hybrids (a) between *A. ligtu* ssp. *ligtu* and *A. aurea* (AL5SA002), (b) between *A. aurea* and *A. magnifica* ssp. *magnifica* (A016Q001-3), and (c) between *A. inodora* and *A. ligtu* ssp. *simsii* (P006K101-1), and (d) the C-banded karyotype of the same hybrid (P006K101-1). For the karyotype of *A. inodora* see Buitendijk and Ramanna (1996). a= *A. aurea*; i= *A. inodora*; li= *A. ligtu*; m= *A. magnifica* ssp. *magnifica*. Arrows indicate secondary constrictions in *A. ligtu* chromosomes 2, 6 and 7. Bar = 10 mm.

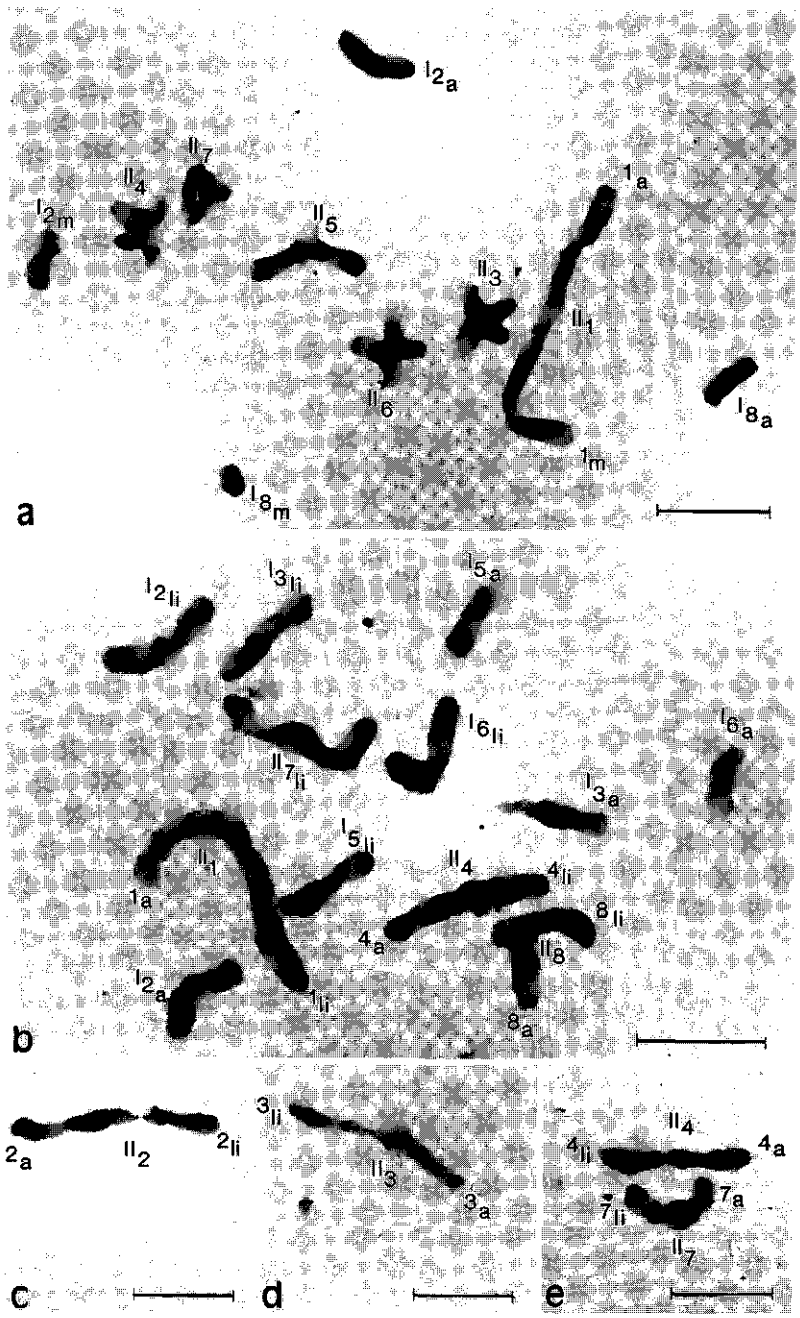

FIGURE 6. Metaphase I stages of male meiosis in diploid interspecific hybrids (a) between *A. aurea* and *A. magnifica* ssp. *magnifica* (A016Q001-3) and (b) between *A. ligtu* ssp. *ligtu* and *A. aurea* (AL5SA002), with (c-e) three individual bivalents of this hybrid (AL5SA002). Univalents (I) and bivalents (II) are identified on the basis of their C-banding pattern and morphology. a= *A. aurea*; li= *A. ligtu*; m= *A. magnifica* ssp. *magnifica*. Bar = 10 mm.

Discussion

In the last two decades there have been numerous reports on intraspecific variation in nuclear DNA amount in angiosperm taxa (reviewed in Bennett, 1985; Price, 1988; Bennett and Leitch, 1995). Whereas some species showed a remarkable genomic constancy (Greilhuber and Speta, 1985; Nath *et al.*, 1992; Greilhuber and Ebert, 1994; Baranyi and Greilhuber, 1995), other species revealed variation in nuclear DNA amount at a constant chromosome number (e.g. Laurie and Bennett, 1985; Rayburn *et al.*, 1989; Michaelson *et al.*, 1991; Ceccarelli *et al.*, 1993). In many cases most (but not all) of the variation could be attributed to C-band polymorphism. Intraspecific DNA variation has been associated with several growth related parameters (e.g. Biradar *et al.*, 1994), and with climatological or geographical variation (e.g. Bennett, 1976; Rayburn and Auger, 1990).

Nuclear DNA variation has mostly been detected through Feulgen microdensitometry or flow cytometry. Critical evaluation of data and comparison with already reported values have indicated, that methodological problems in both microdensitometry (Greilhuber and Ebert, 1994; Baranyi and Greilhuber, 1995) and flow cytometry (Doležel et al., 1992) can give rise to contradictionary results. Flow cytometric measurements are performed after staining nuclei with different fluorochromes, which, as a result of their staining properties, may all have advantages and disadvantages while detecting DNA variation. Since the staining of DNA with fluorochromes is dependent on chromosome structure (Darzynkiewicz and Traganos, 1988) and chromosome structure is a species specific character in plants (Nagl, 1982), some error might be introduced when determining DNA content through the indirect method of flow cytometry (and most probably also microdensitometry). For this reason there is an absolute need to compare the results of these indirect methods of determining DNA amounts with the results of chemical extraction methods.

When using flow cytometry, some research groups prefer the use of fluorochromes (e.g. propidium iodide, ethidium bromide), that intercalate between the molecules of the DNA helix and are assumed to be independent of the base pair composition (e.g. Doležel *et al.*, 1992). Other research groups (e.g. Rayburn *et al.*, 1989 and 1992), however, choose for instance DAPI, a fluorochrome that allows very sensitive measurements, but is reported to be base pair composition dependent (Manzini *et al.*, 1983). Rayburn *et al.*, (1992) argued that the intraspecific variation in *Zea mays* could best be estimated using DAPI, since this fluorochrome was shown to detect the full range of variation within this species that was observed by using microdensitometry, whereas with PI only a smaller range of variation was detected. Doležel *et al.*, (1992), who measured DNA amounts of various plant species with three different fluorochromes, however, advised not to use base dependent fluorochromes, but DNA intercalators. We think that the discrepancy in results is probably due to the plant material, and thus to the type of DNA variation that was measured. Whereas the plant material that was measured by Doležel *et al.* (1992) consisted of species from different families and varied considerably in their base pair composition, the material of

Rayburn *et al.*, (1989 and 1992) comprised populations of only one species and mainly differed in heterochromatin composition. Rayburn *et al.* (1992) found a high negative correlation between the ratio of the PI and DAPI fluorescence values and the amount of heterochromatin and indicated that this PI/DAPI ratio could be used to estimate the heterochromatin content. In *Alstroemeria* we found no such correlation when the values of 12 species were considered (Buitendijk *et al.*, 1997), nor did we find indications for such correlation within a species (this paper). The PI/DAPI ratio was fairly constant (with a few exceptions) within each of the three *Alstroemeria* species of this study, despite the occurrence of heterochromatin polymorphism. This finding shows that it remains difficult to generalize the relationship between heterochromatin and flow cytometric estimations of DNA variation without considering the effects of variation in chromosome length, chromosome structure and overall base pair composition.

The variation in nuclear DNA amount as observed in the accessions of *A. aurea* seems to be continuous, also more or less continuous (except for accession K101-1) in *A. ligtu*, and discontinuous in *A. magnifica* ssp. *magnifica*. Some of the intraspecific variation can probably be attributed to heterochromatin polymorphism, but it is not the only source of variation. Assuming a constant total chromosome length within *A. aurea* and a linear relationship between DNA amount, length of euchromatic and heterochromatic chromosome regions as described in Buitendijk *et al.*, (1997), the heterochromatin polymorphism in *A. aurea* would account for a variation in DNA amount of about 3 pg. (4.6 pg. actually observed). There is no significant correlation between heterochromatin amount and total nuclear DNA amount in *A. ligtu*, considering the data of the five accessions AL1S, AL5S, F051-2, AM3S, AJ4S (Table 2; r=0.77, n=5, P=0.131). One accession (K101-1) shows a moderate amount of heterochromatin with a high amount of DNA. The exceptional PI/DAPI ratios and 2C-values of some of the *Alstroemeria* accessions (underlined in Table 2) may, in the case of *A. aurea*, be due to artificial selection and possibly introgression (some accessions were grown in breeders' nurseries for many years) or, in case of *A. ligtu*, to chromosome length polymorphism (see Table 3). The discontinuity of the values found in *A. magnifica* ssp. *magnifica* could be due to a broad species concept (cf. Bayer, 1987: 198), but needs further study.

In *Alstroemeria* we found two kinds of differences between homologous chromosomes: differences in chromosome length (e.g. chromosome 8 of *A. aurea* and chromosome 6 of *A. ligtu*) and differences in the amount of heterochromatin (i.e. size and number of C-bands in all chromosomes that carry C-bands, except for chromosome 3 of *A. aurea*). Polymorphic C-band patterns are frequent in plants (Greilhuber and Speta, 1976; Kenton, 1991; Badaeva *et al.*, 1994). Loidl, Greilhuber and Schweizer developed a model to explain the amplification, transposition, conservation and homogenization of heterochromatic DNA sequences during the evolution of plant genomes (Loidl, 1983; Greilhuber and Loidl, 1983; Schweizer and Loidl, 1987). Following this model, the amplification and transfer of repetitive sequences takes place predominantly in mitotic interphase, when non-homologous chromosomes are oriented parallel and polarized as a result of anaphase movement (the "Rabl -

orientation"). In this position chromosome arms are very close together and repetitive sequences can be dispersed to more or less equi-distant positions in non-homologous chromosomes (Loidl, 1983), leading to a co-evolution of bands in neighbouring chromosome arms (Greilhuber and Loidl, 1983). In *Alstroemeria* we observed analogies in the C-band patterns of chromosomes 3, 4, 5 and, in case of *A. aurea* and *A. magnifica*, chromosome 6 (Figure 1). Analogies in C-band pattern are also found in chromosomes 7 and 8. Chromosomes 1 and 2 carry only few bands (except for *A. ligtu*), and seem to have evolved more independently. Besides the amplification and transposition of heterochromatic sequences as described in the model of Schweizer and Loidl (1987), chromosomal rearrangements (i.e. translocations and pericentric inversions) and crossing-over between heteromorphic homologous chromosomes will undoubtedly cause a further diversification in C-band pattern. According to the model of Schweizer and Loidl (1987), the mechanisms for conservation and homogenization of heterochromatin act predominantly in the first meiotic prophase. Kenton *et al.*, (1987) reported that in permanent hybrids of *Gibasis pulchella* chromosomal interchanges had occurred close to major C-bands, probably at euchromatin-heterochromatin boundaries. Loidl (1979) observed that in *Allium flavum* chiasmata occurred much more frequently at positions very close to the heterochromatic bands than in chromosome arms or regions that lacked those bands, but no uniform effect of heterochromatin on crossing-over distribution was found in *Scilla* (Berger and Greilhuber, 1991 and 1993). Thus, the conservation and homogenization of repetitive sequences are probably determined by mechanisms, acting, possibly in a species-specific manner, through localization of chiasmata and breakpoints, and allowing the plant to generate a variability that may be of adaptive significance in changing habitats. The structural differences between homologous chromosomes in the three species as depicted in Figure 1 are the result of a survey of a limited number of species accessions, that were available for our studies. Because of this limited number of accessions, and because no precise data on the geographic origin of the material were available, as for instance in the studies of Badaeva *et al.*, (1994) on natural populations of *Triticum araraticum*, it remains difficult to discover directions in species divergence and in the evolution of C-band patterns within the *Alstroemeria* species.

Through the analysis of meiotic configurations in male meiosis we could establish the homoeology of the chromosomes 1 to 8 of the three *Alstroemeria* species as presented in Figure 1. Homoeologous chromosomes possessed in most cases a similar morphology, except for chromosome 2 of *A. magnifica* and chromosome 6 of *A. ligtu*, which showed a centromere position and C-band pattern that was clearly dissimilar. The fact that no bivalents were found between the chromosomes 6 in the hybrids between *A. ligtu* ssp. *ligtu* and *A. aurea*, probably illustrates a lack of homology. The occasional observation of multivalent formation between chromosomes 1 and 6, and 2 and 6 in these hybrids indicate a certain degree of homology in chromosome arms.

The next step in exploring the nature of inter- and intraspecific nuclear DNA variation could be to specify the type, amount and location of the repetitive sequences

through molecular and cytological hybridization with known probes, as for instance was done for Fok1 repeated sequences in *Vicia faba* (Fuchs *et al.*, 1994; Maggini *et al.*, 1995). Recently a start was made to unravel the molecular background of genomic differentiation in *Alstroemeria* (Kuipers *et al.*, 1997). This resulted in, for instance, discriminative hybridization between *A. aurea* and *A. ligtu* chromosomes through GISH-bands (Genomic In Situ Hybridization) that coincided with C-bands. The resolution is, however, not yet of a level that allows to study molecular differences within species.

Acknowledgements

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Chapter 6

Embryo rescue by half-ovule culture for the production of interspecific hybrids in *Alstroemeria*

J.H. Buitendijk, N. Pinsonneaux, A.C. van Donk, M.S. Ramanna and A.A.M. van Lammeren. 1995. Scientia Horticulturae 64: 65-75.

Abstract

Interspecific hybridization in the genus *Alstroemeria* is hindered by post-fertilization barriers. Histological analysis revealed poor endosperm development from 18 days after pollination onwards, followed by malformation and abortion of embryos. To create interspecific hybrids between *A. aurea*, *A. pelegrina*, *A. magnifica*, *A. inodora* and *A. psittacina* in diallel combinations, an ovule culture technique was developed. Influence of age of ovules, sucrose concentration of medium and temperature and light during culture were tested. Harvesting ovules before the onset of endosperm degeneration, i.e., at 14 days after pollination, cutting them into halves and culturing the micropylar halves in a rotating liquid culture medium containing 6% sucrose at 21°C in the dark, led to successful embryo rescue. Germinated embryos were subcultured *in vitro* until rhizomes were formed, a prerequisite for successful transfer to the greenhouse. Full grown plants all showed interspecific morphological traits and analysis of chromosome complement confirmed their hybrid nature. Diploid hybrid plants were obtained in all the twenty interspecific 2x-2x combinations. A total of 260 interspecific hybrid plants were produced. Half-ovule culture of 2x-4x and 4x-2x crosses resulted in 43 triploid hybrid plants. Because interspecific hybrids were obtained in 100% of the interspecific combinations, it is expected that the described technique can be applied to overcome post-fertilization barriers in most crosses within the genus *Alstroemeria*.

Key words: *Alstroemeria*; embryo histology; interspecific hybridization; ovule culture; ovule histology; post-fertilization barriers.

Abbreviations: BAP=6-benzylaminopurine; DAP=days after pollination; MS=Murashige and Skoog; NAA=naphthaleneacetic acid.

Introduction

Embryo rescue techniques have been developed for numerous crops (Williams *et al.*, 1987). Depending on the requirements of the crop and the size of the embryo, either ovaries, ovules or isolated embryos may be cultured. In certain cases a combination of ovule culture and embryo culture was required (Fernandez *et al.*, 1990). Harberd (1969)

cut the ovules of *Brassica* into halves, cultured the micropylar halves in liquid medium, subcultured the germinated embryos on solid medium and obtained interspecific and intergeneric hybrids. In many monocotyledonous crops, e.g., orchids (Sagawa, 1990), grasses (Nitsche and Hennig, 1976) and bulbous or cormous crops (Ohsumi *et al.*, 1991; Van Tuyl *et al.*, 1991), interspecific and intergeneric hybrids have been produced by employing ovule culture.

Alstroemeria species are rhizomatous monocotyledonous perennials. Cultivars have originated through crossing species (Goemans, 1962; Hang and Tsuchiya, 1988; Ramanna, 1992) and through irradiation treatments (Broertjes and Verboom, 1974). They are mainly cultivated for the production of cut flowers. In some interspecific combinations seed set can be accomplished (Hannibal, 1941; Foster, 1948; Duncan, 1977, 1982), but in a majority of other interspecific combinations seed set fails (Stinson, 1942; Traub, 1943). As a consequence of such interspecific barriers, the progress in breeding has been limited.

This study started with the primary objective to systematically create diploid interspecific hybrids that could be used in studies on the inheritance of micropropagation ability. To reach this goal, it was necessary to obtain successful embryo rescue in all the interspecific combinations of a diallel crossing scheme using the species *A. aurea*, *A. pelegrina*, *A. magnifica*, *A. inodora* and *A. psittacina*. These species have been used most in *Alstroemeria* breeding (Goemans, 1962; Hang and Tsuchiya, 1988). Because an earlier described embryo rescue technique (Winski and Bridgen, 1988) proved unsatisfactory in our laboratory, further attempts were made to produce interspecific hybrids (Buitendijk *et al.*, 1992).

The present paper shows through histological studies that embryos of most interspecific hybrids do not mature, due to a failure of the endosperm to develop properly. These studies indicate the stage of development at which the endosperm is no longer capable of nourishing the embryo. Based on this knowledge a technique of culturing half-ovules is developed and applied to diallel crosses using the above mentioned *Alstroemeria* species and to several 2x-4x and 4x-2x crosses.

Materials and methods

Plant material

Five diploid *Alstroemeria* species (2n=2x=16) and five tetraploid hybrids (2n=4x=32) were used. Among the species, three were Chilean: *A. aurea* Graham (syn. *A. aurantiaca* D. Don.), *A. pelegrina* L. and *A. magnifica* Herb. ssp. *magnifica* (syn. *A. sierrae* Muñoz Pizarro, syn. *A. gayana* Philippi) (Bayer, 1987). Two species were Brazilian: *A. inodora* Herb. and *A. psittacina* Lehm. (syn. *A. pulchella* Sims) (Uphof, 1952). The plants were grown in a greenhouse at 15 to 25°C, with summer peaks of about 30°C. Of each species three or more genotypes were used. Self- and cross-pollinations were performed on emasculated flowers. Three ovaries were left on the mother plant, to check seed set under greenhouse conditions.

Histological methods

Ovaries of self-pollinated *A. pelegrina* (control) and of cross-pollinations of *A. pelegrina*, *A. aurea* and *A. psittacina* were collected from 4-56 days after pollination (DAP) at three and four day intervals. Ovules were dissected, fixed in 5% glutaraldehyde in 0.1M phosphate buffer (pH 7.0) for one hour, transferred to phosphate buffer, dehydrated in a graded series of ethanol and embedded in Technovit 7100. Sections (4-6 µm, Leitz rotation microtome) were stained with Toluidine blue (1%, aqueous solution) and analysed with a light microscope. When seeds from self-pollinations were collected later than 28 DAP, they contained very hard endosperm and were sectioned with a sliding microtome (Reichert) without embedding.

Ovule culture

Ovaries were harvested at 14 DAP, washed in 70% ethanol for one minute, disinfected in 1.5% NaOCl for 20 min and rinsed in water. Ovules were dissected and cut into halves as shown in Figure 1c. The culture medium contained 1/4 strength Murashige and Skoog (1962) macronutrients, full strength MS micronutrients and vitamins, 400 $mg.l^{-1}$ casein hydrolysate and 60 $g.l^{-1}$ sucrose (pH 5.8 prior to autoclaving for 20 min. at 121°C). Each treatment consisted of four petri dishes (Falcon; diameter 10 cm; height 2 cm) with 25 ml of culture medium and ten half-ovules each incubated in a climate room at 21°C in the dark on a rotary shaker at 50 rpm. Analysis of variance and Chi-square tests were applied to some of the data (Steel and Torrie, 1982). The number of flowers that were crossed and the number of ovules, that were cultured for the production of interspecific and interploidy hybrids, was different for each genotype combination. These numbers depended on the availability of plants and on the chance of simultaneous flowering. In several genotype combinations only one or two flowers were crossed.

Rhizome induction, rooting and transfer to greenhouse

To induce rhizomes, the germinated embryos were transferred to solid culture medium in a climate room at 18°C in the light (Philips 32 W, 84HF, approx. 4000 Lux; photoperiod 12 hrs.). Two culture media were tested, one containing 1 $mg.l^{-1}$ BAP and the other containing 0.5 $mg.l^{-1}$ NAA + 3 $mg.l^{-1}$ BAP. The basal media contained MS nutrients and vitamins, 400 $mg.l^{-1}$ casein hydrolysate, 40 $g.l^{-1}$ sucrose and 2.1 $g.l^{-1}$ gelrite.

As soon as rhizomes were formed, plants were transferred to a rooting medium, containing MS medium, 0.5 $mg.l^{-1}$ NAA, 45 $g.l^{-1}$ sucrose and 2.1 $g.l^{-1}$ gelrite (pH 6.1 prior to autoclaving for 20 minutes at 121°C). When the plants had formed roots of about 1 cm length, they were transferred to a greenhouse, where they were gradually acclimatized to greenhouse conditions.

Confirmation of hybridity

The hybrid character of the plants raised after interspecific pollination was checked using more than one criterion in each case. These criteria included morphological traits

of shoots, leaves and rhizomes, chromosome number, chromosome arm ratios, chromosome C-banding patterns, chromosome pairing behaviour during meiosis, pollen fertility and seed set after self-pollination.

For chromosome studies, shoot and root tips of parental species and putative hybrid plants were pre-treated in an aqueous solution of 2 mM 8-hydroxyquinoline on ice for 7-8 hours and fixed in Carnoy solution (3:1 ethanol:acetic acid). Before Feulgen staining the meristematic tissue was hydrolysed in 1 N HCl at 60°C for 8 minutes. The maceration before the Giemsa C-banding was enzymatic, using 15% (v/v) pectinase and 1.5% (w/v) cellulase in a 0.1 M citrate buffer (pH 4.8) at 38°C for 30-65 minutes. The macerated meristematic tissue was squashed and the slides were processed for Giemsa C-banding according to Ramachandran and Ramanna (1985).

Results

Histological observations

The development of seeds derived from self-pollination of *A. pelegrina* was followed histologically. At anthesis the anatropous ovules were 1.6 mm long and 700 µm wide (Figure 1a).

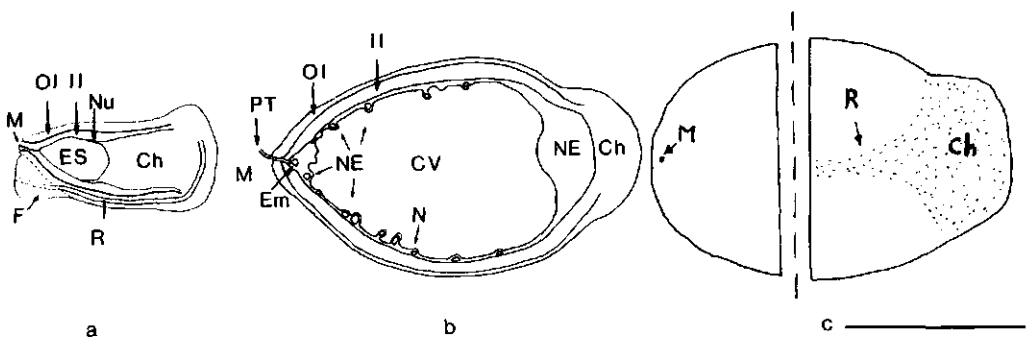

FIGURE 1. (a), Topographic representation of median section through *A. pelegrina* ovule at anthesis (0 DAP). (b), Topographic representation of longitudinal section through developing seed obtained by crossing *A. pelegrina* with *A. aurea* (14 DAP). (c), Dissection of the ovule (14 DAP). The micropylar half of the ovule was cultured. Bar is 1 mm.
abbreviations: AE= alveolar endosperm; CE= cellular endosperm; Ch= chalaza; Co= cotyledon; CV= central vacuole; Em= embryo; ES= embryo sac; F= funiculus; II= inner integument; M= micropyle; N= nucleus; NE= nuclear endosperm; Nu= nucellus; OI= outer integument; R= raphe

Two integuments covered the nucellus that contained a large embryo sac. Remnants of nucellus cells were visible at the micropylar side of the embryo sac. The nucellus between chalaza and antipodal side consisted of cells without cytoplasm near the

embryo sac and living cells near the chalaza. At seven DAP, ovules contained a small globular embryo surrounded by nuclear endosperm. At 14 DAP, the pro-embryo was 110 µm long. The endosperm was still nuclear (Figure 1b and 2a), but within four days cellularization had started (Figure 2b). The embryo was still globular at that stage. It had a suspensor and was surrounded by cellular endosperm. The endosperm had an alveolar phase towards the central vacuole (Figure 2b). In the first 14 days of development the size of the ovule increased from 1.5 to 3 mm. Between 14 and 28 DAP, cell walls formed in the endosperm, which hardened gradually. The shape of the embryo changed from globular (7 DAP) to ovoid (35 DAP) and eventually cylindrical (56 DAP). When seed and embryo development had completed at 56 DAP, the seed had a diameter of 3.5 mm and the embryo was 1.1 mm long and 390 µm wide (Figure 2c). Root and shoot meristems and a cotyledon were present.

The early stages of embryo and endosperm development (4-14 DAP) in the ovules of cross-pollinated *A. pelegrina*, *A. aurea* and *A. psittacina* were similar to those observed in the seeds obtained after self-pollination of *A. pelegrina* (Figure 2d). The later developmental stages in the cross-pollinations were, however, retarded with respect to the size of the ovule and the number of divisions in the nuclear endosperm (from 18 DAP onwards). The transition from nuclear to cellular endosperm did not occur (Figure 2e). At 18 DAP the embryo still exhibited normal shape and size in the cross *A. psittacina* x *A. aurea* (Figure 2e). In general, all cross-pollinated combinations were anatomically similar. Both malformed and retarded embryos were observed from 25 DAP onwards (Figure 2f). The ovule collapsed completely in the period between 21 and 35 DAP.

Ovule culture

Five weeks after the start of the culture, germinating embryos were either floating freely in the culture medium or they were still attached to the half-ovules. Seven DAP half-ovules from a self-pollinated tetraploid showed poor germination on any sucrose concentration (Table 1). In seven and 14 DAP half-ovules, the rate of germination was maximal when sucrose concentration was between 6 and 12%. 21 DAP half-ovules yielded 100% germination on sucrose concentration between 4 and 10%. The numbers of germinated embryos from half-ovule cultures after reciprocal cross-pollination between diploid and tetraploid plants, incubated at 15, 21 and 24°C in the light or in darkness, showed a highly significant effect (F= 14.54**) of crosses (Table 2).

TABLE 1 The numbers and percentages of germinated embryos obtained from self-pollinated half-ovules of a tetraploid hybrid, Bt204, that were cultured at 7,14,21 and 28 DAP in a culture medium with 2,4,6,8,10 and 12% sucrose concentrations. Per treatment 40 half-ovules were cultured.

	number and percentage of germinated embryos in medium with sucrose concentration of:											
DAP	2%		4%		6%		8%		10%		12%	
7	0	(0)	1	(3)	9	(23)	22	(55)	24	(60)	9	(23)
14	9	(23)	20	(50)	30	(75)	32	(80)	36	(90)	26	(65)
21	16	(40)	40	(100)	40	(100)	40	(100)	32	(80)	33	(83)
28	12	(30)	40	(100)	24	(60)	36	(90)	35	(88)	22	(55)

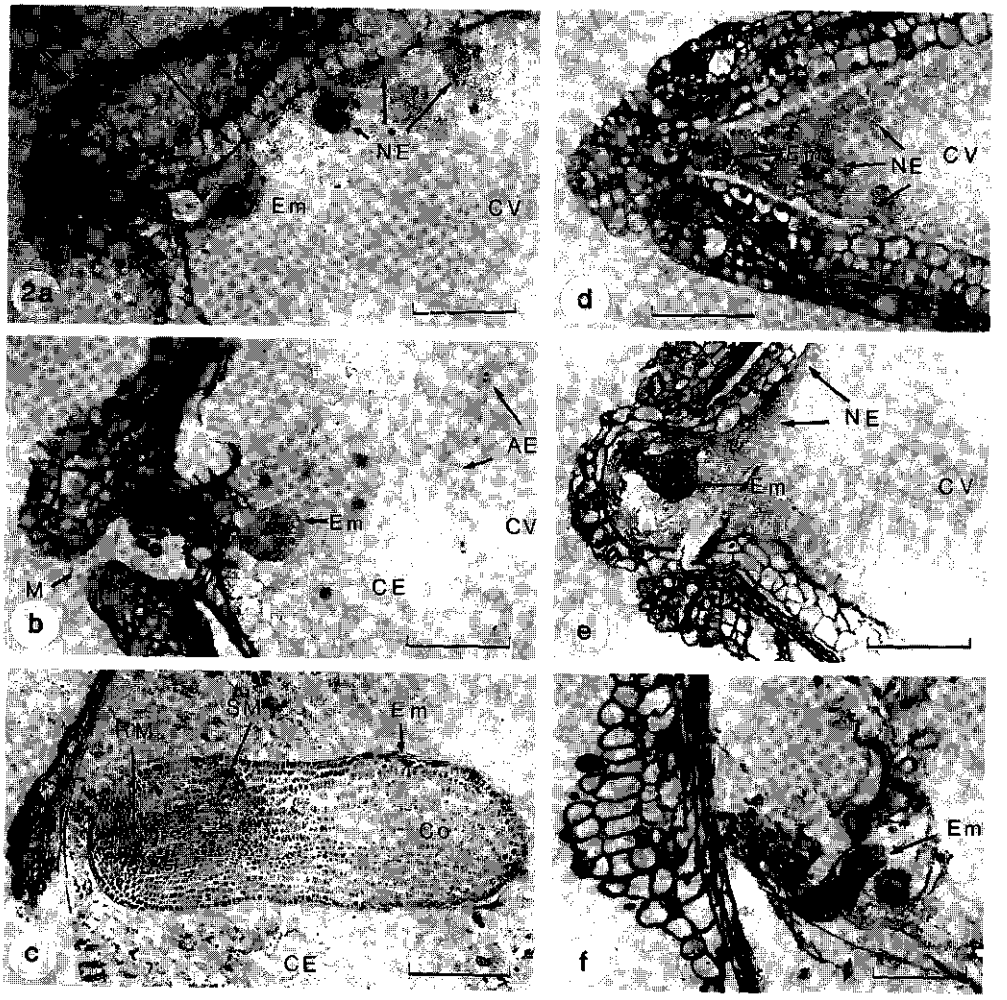

FIGURE 2. (for abbreviations see legend of Figure 1). Longitudinal semi thin sections (4-6μm) through developing seeds of *A. pelegrina* after self-pollination (a-c) and of *A. psittacina* after cross-pollination with *A. aurea* (d-f).

a. At 14 DAP the endosperm is nuclear and the embryo globular. Bar is 140 μm.

b. At 18 DAP the endosperm is cellular and exhibits alveoli near the central vacuole. The globular embryo has increased in size. Bar is 140 μm.

c. At 56 DAP the cellular endosperm has replaced the central vacuole. The cylindrical embryo is mature, exhibiting cotyledon, root and shoot meristem. Bar is 235 μm.

d. At 14 DAP the endosperm is nuclear, the embryo globular. Bar is 140 μm.

e. At 18 DAP the endosperm has remained nuclear, but the embryo has developed further. Bar is 140 μm.

f. At 25 DAP the endosperm and the embryo have degenerated. Bar is 140 μm.

TABLE 2 The numbers and percentages (in parentheses) of germinated embryos obtained from half-ovules of 4x - 2x and 2x - 4x crosses, cultured at 15, 21 and 24°C in light or darkness.

Temperature (°C)	Light	D362 x A003 (4x - 2x)	A003 x D362 (2x - 4x)	D662 x A003 (4x - 2x)	Total
15°C	dark	24 (60)	5 (13)	11 (28)	40 (33)
21°C	light	38 (95)	7 (18)	27 (68)	72 (60)
21°C	dark	34 (85)	1 (3)	31 (78)	66 (55)
24°C	dark	32 (80)	7 (18)	26 (65)	65 (54)
Total		128	20	95	243 (51)
Percentage		80	13	59	51

Analysis of variance

Source of variation	df	SS	MS	*F*
Crosses	2	1531.50	765.75	14.54**
Treatments	3	200.92	66.97	2.55 ns
Error	6	157.83	26.31	
Total	11	1890.25		

Per treatment 40 half-ovules were cultured at 14 DAP in a culture medium with 6% sucrose.
NS, not significant; ** $F = 14.54$, $P(F_6^2 > 14.50) = 0.005$; $F = 2.55$, $P(F_6^3 > 3.29) = 0.1$

When *A. aurea* A003 (diploid) was used as the male parent in a cross with D362 (tetraploid), the number of germinated embryos was higher than when *A. aurea* was used as a female parent. A significant effect of treatments was found (χ^2= 10.83*), when Chi-square test criterion was applied on totals of treatments, after having tested for homogeneity of the data of Table 2. After independent comparisons between pairs of treatments it was concluded that (1) both incubation at 21°C and 24°C was better than incubation at 15°C (χ^2= 17.07*** and 10.41***), (2) there was no significant difference between incubation at 21°C and at 24°C (χ^2= 0.80 n.s.) and (3) there was no significant effect of light during culture at 21°C (χ^2= 0.43 n.s.).

Outcome of interspecific hybridization

All interspecific crosses and interploidy crosses gave rise to plants after culturing 14 DAP half-ovules in 6% sucrose liquid culture medium at 21°C in the dark. A total of 50 parental genotype combinations was attempted and 260 hybrid plants were obtained (Table 3). Half-ovule culture in 2x-4x and 4x-2x crosses resulted in 43 hybrids.

Besides half-ovule culture, normal seed set occurred after interspecific pollinations between *A. inodora* and *A. pelegrina* and between *A. inodora* x *A. psittacina* and its reciprocal (Table 3). In all other combinations there was no seed set.

Rhizome induction, rooting and transfer to the greenhouse

The germinated embryos, that were present five weeks after the start of the half-ovule cultures, were transferred to a solid culture medium. Usually the germinated embryo exhibited the organs, that could also be recognized in germinating embryos of normal seeds, i.e., cotyledon, shoot and root. The *in vitro* seedlings attained a swollen habit and shoots were generally stunted. After one or more subcultures such stunted shoots elongated and subsequently rhizomes were formed. The first hybrid plants containing

TABLE 3. Seed set in interspecific crosses of five *Alstroemeria* species and the numbers of hybrids obtained through half-ovule culture.

Species crosses	Seed set*	Half-ovule culture	
		No. of cross combinations	No. of true hybrids**
A. aurea x *A. pelegrina*	-	3	4
A. aurea x *A. magnifica*	-	2	11
A. aurea x *A. inodora*	-	2	4
A. aurea x *A. psittacina*	-	1	3
A. pelegrina x *A. aurea*	-	8	82
A. pelegrina x *A. magnifica*	-	2	13
A. pelegrina x *A. inodora*	-	9	20
A. pelegrina x *A. psittacina*	-	1	2
A. magnifica x *A. aurea*	-	2	7
A. magnifica x *A. pelegrina*	-	1	4
A. magnifica x *A. inodora*	-	1	10
A. magnifica x *A. psittacina*	-	1	4
A. inodora x *A. aurea*	-	2	11
A. inodora x *A. pelegrina*	+	5	41
A. inodora x *A. magnifica*	-	1	7
A. inodora x *A. psittacina*	+	1	12
A. psittacina x *A. aurea*	-	1	4
A. psittacina x *A. pelegrina*	-	3	12
A. psittacina x *A. magnifica*	-	2	4
A. psittacina x *A. inodora*	+	2	5
Total		50	260

* - = no seed set after interspecific pollination
\+ = seeds were obtained after interspecific pollination

** confirmed as true hybrids based on morphology, karyotypes, chromosome pairing and fertility. Plants are maintained as *in vitro* cultures.

evident rhizomes were established eight weeks after pollination, the last ones 22 weeks after pollination. In some cases, the swollen embryo tissue formed callus from which plants were regenerated. Rhizomes were formed on both BAP and BAP+NAA media (data not presented). Rhizome formation occurred directly after subculture of hybrids when *A. magnifica* was the female parent. Hybrids of which *A. inodora* was the female parent had to be subcultured repeatedly before rhizomes were formed. In crosses involving *A. aurea* as a female parent there was considerable blackening of the embryo, especially of the cotyledon. About 50% of the germinated embryos formed a rhizome.

All plants rooted well and were transferred to the greenhouse. The rate of survival after weaning depended on the hybrid combination and ranged from 40%, in the hybrids between *A. pelegrina* and *A. magnifica*, to about 90% in the other hybrids.

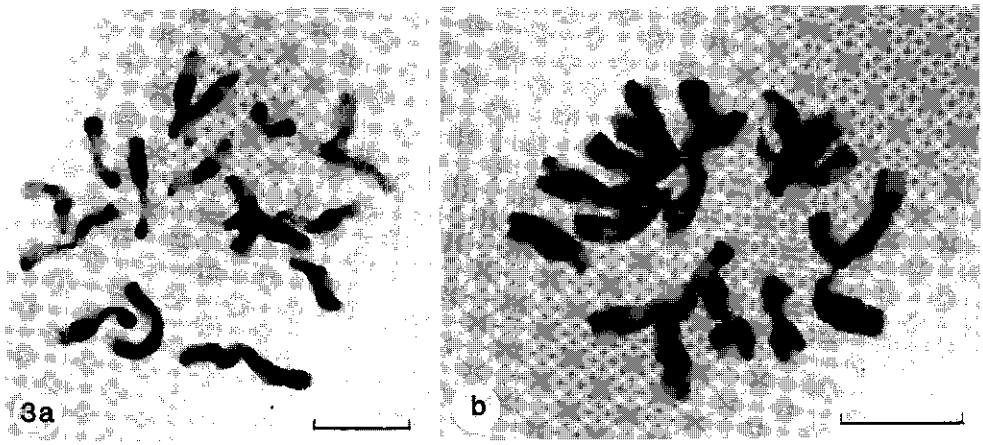

FIGURE 3. (a), Chromosomes of *A. aurea* (2n=2x=16) after Giemsa C-banding. 14 Chromosomes exhibit C-bands. Bar is 10 µm. (b), Chromosomes of *A. aurea* x *A. inodora* hybrid (2n=2x=16) after Giemsa C-banding. Only seven *A. aurea* chromosomes show clear bands. The other chromosomes have no or very narrow bands. Bar is 10 µm.

Confirmation of hybridity

All 260 plants, that were obtained from interspecific crosses (Table 3), proved to be diploid hybrids (2n=2x=16, Figure 3). The 2x-4x and 4x-2x crosses resulted in 43 triploid hybrid plants (2n=3x=24). Especially the karyotype characteristics based on both Feulgen staining and Giemsa C-banding were useful for confirming hybridity of young plantlets, that were growing *in vitro*. All flowering hybrid plants obtained so far, showed intermediate flower characters as compared to the two parents (data not presented). Subsequent observations on meiosis indicated a failure of chromosome pairing and almost total pollen sterility.

Discussion

The optimal time for culture of half-ovules from self-pollination appeared 21 DAP (Table 1). However, when the technique is applied to obtain hybrid embryos from interspecific crosses, the culture should be started at 14 DAP. Histological analysis revealed that during the first 14 days after interspecific pollination, embryo and endosperm development was normal. Stagnation in endosperm development started between 14 and 18 DAP with the failure of cellularization. Because malformed or retarded embryos were observed from 18 DAP onwards, it is concluded that the degeneration of the embryo is caused by the failure of endosperm development and thus a lack of nourishment. When ovules are cultured at an early stage of development of two or seven DAP (De Jeu and Jacobsen, 1995; Winski and Bridgen, 1988) instead

of 14 DAP, the embryo is subjected to *in vitro* conditions for an unnecessarily large part of its development. Nonetheless, it cannot be excluded that embryo development in interspecific combinations other than studied here, stagnates in earlier stages. In such cases early ovule culture may be preferred.

It has been shown that it was essential to subculture hybrid plants until a rhizome was formed, because only plants with rhizomes could be transferred to the greenhouse successfully (Hakkaart and Versluijs, 1985). Results of rhizome formation on hybrids from reciprocal crosses suggest a maternal influence. Delay in rhizome formation is related to poor development of axillary meristems and may likely be caused by strong apical dominance of the first shoot (Bond and Alderson, 1993).

Transferability of plantlets to greenhouse conditions was generally satisfactory, but depended on the parental species of the hybrid. The poor transferability of *A. pelegrina* x *A. magnifica* hybrids might be explained by the growth characteristics of the parental species, since in our greenhouse *A. magnifica* was dormant during summer and both parental species always recovered poorly after repotting.

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Chapter 7

Genetic variability for rhizome multiplication rate in micropropagation of *Alstroemeria*

Abstract

A total of 88 genotypes of *Alstroemeria* was micropropagated, using *in vitro* grown rhizomes as explants. The material included accessions of five species and their interspecific hybrids. The number of propagules, obtained after three successive culture periods, varied considerably. Rhizome multiplication rate (RMR), defined as the mean multiplication factor of standardized rhizome explants that are cultured *in vitro* for a period of four weeks, varied significantly among species as well as among hybrids. Among the five species, RMR ranged from 1.21 to 3.10 in accessions of *A. psittacina* and *A. aurea*, respectively. Among their diploid interspecific hybrids RMR ranged from 1.11 to 2.23. Two diploid interspecific combinations, i.e. the hybrids *A. inodora* × *A. magnifica* and *A. aurea* × *A. magnifica*, exhibited positive heterosis for RMR. Seven other hybrid combinations showed RMRs that were more or less intermediate to those of their parental species. A genetic basis for RMR was evident. RMR correlated well with the number rhizome nodes ($r=0.91$, $P<0.001$), to a lesser extent with the rhizome diameter ($r=0.58$, $P<0.001$), the degree of branching ($r=0.53$, $P<0.001$) and the length of internodes ($r=0.31$, $P<0.01$) and poorly with the percentage of rooted rhizomes ($r=0.06$, ns). The results indicate that it is worthwhile to incorporate RMR as a selection criterion in *Alstroemeria* breeding programmes in order to produce cultivars with acceptable multiplication rates. The choice of parental species and parental genotypes, to be used in interspecific crosses, is crucial, and should be based on the capacity to produce a fair number of nodes in both parents. Other morphological traits of the rhizomes that favour micropropagation are a high degree of branching and long internodes. Since one of these traits is often sub-optimal in the parental species, it may be worthwhile to pursue complementation of these favourable traits in the hybrids.

Key words: *Alstroemeria*, genotype, heterosis, micropropagation, rhizome morphology.

Introduction

The genus *Alstroemeria* (Family Alstroemeriaceae; Order Liliales) consists of species and hybrids that are highly appreciated for their flowers. The species originate from South America where they thrive in very diverse habitats (Aker and Healy, 1990). They spread through the growth of rhizomes and the dispersal of seeds. The natural populations of species are diploid, and the plants are expected to be heterozygous as a result of their outcrossing nature. There are large differences between species with respect to the morphology of their rhizomes (Chapter 1). Hybrids have been produced

by crossing species, and these can be diploid, triploid or tetraploid. Many of these interspecific hybrids have been commercialized and are cultivated for the production of cut flowers. The propagation of cultivars is practised by splitting of the rhizomes. When plants are propagated in soil, multiplication is slow. Propagation *in vitro* allows higher multiplication rates.

When *Alstroemeria* rhizomes are micropropagated in a continuous propagation system, they are cut into explants and subcultured onto a fresh culture medium (Pierik *et al.*, 1988; Monette, 1992). At each subculture the shoots are decapitated. Both the removal of shoot apices and rhizome apices (Bond and Alderson, 1993b), and the addition of cytokinins to the culture medium (Gabryszewska and Hempel, 1985; Pierik *et al.*, 1988) stimulate the growth and the branching of the rhizome. Since only explants that contain rhizome tips can be rooted and transferred to soil successfully (Hakkaart and Versluijs, 1985), it is important that many new rhizome tips are formed during micropropagation.

Pierik *et al.* (1988) and Bond and Alderson (1993a and b) have shown that there are genotypical differences in multiplication rate *in vitro*. Some hybrids multiply with a factor 2.9 in four weeks, whereas others have a multiplication factor of only 1.1 (Pierik *et al.*, 1988). These data concern *Alstroemeria* hybrids of which the genetic background is unknown. Therefore it is not possible to assess the influence of the parental (species) genomes on the multiplication rate of these interspecific hybrids. An understanding of the influence of the parentage of hybrids and of rhizome morphology on multiplication rate would help breeders to develop strategies for breeding cultivars with acceptable multiplication rates.

The aim of this investigation was to explore the micropropagation potential in *Alstroemeria*. More specifically, it was aimed to trace the influence of the genetic background, and of the rhizome morphology of the parental species, on rhizome multiplication rate (RMR) in hybrids. For the purpose of this study, five species, that have frequently been used in breeding programmes and that have a divers rhizome morphology, were intercrossed, and with the help of ovule culture, interspecific hybrids were produced (Buitendijk *et al.*, 1995). Hence, these five species, their diploid hybrids, and some triploid and tetraploid hybrids, were micropropagated in a continuous propagation system and monitored during three propagation cycli. Multiplication rates and morphological features of the rhizomes were determined and the performance of genotypes and groups of genotypes was compared.

Material and methods

Species

Twenty-three accessions of the species *A. aurea*, *A. pelegrina*, *A. magnifica* ssp. *magnifica*, *A. inodora* and *A. psittacina* were obtained from breeding companies and botanical gardens (Table 1). Five accessions were obtained as *in vitro* clones. The other 18 accessions were obtained as mature plants. To initiate *in vitro* cultures, plants

were selfed, and seeds were surface sterilized and germinated *in vitro* on an MS medium (Murashige and Skoog, 1962) at half strength, with 20 $g.l^{-1}$ sucrose. To buffer the effect of a possible segregation for multiplication rate, 10 randomly chosen seedlings were used in the experiment. Together, they were considered to represent the mother plant. It was assumed that inbreeding during one generation had no negative effect on the performance of the progeny. When the seedling rhizomes consisted of five nodes, they were cloned on MS medium with 0.5 $mg.l^{-1}$ BAP (6-benzylaminopurine) and 40 $g.l^{-1}$ sucrose, until enough material was available to start the experiment.

Interspecific hybrids

A total of 65 hybrids was used in the experiment (Table 1). Thirty-four diploid interspecific hybrids originated from crosses between individual genotypes of the five species mentioned above (Buitendijk *et al.*, 1995). The other diploid, triploid and tetraploid hybrids were obtained from breeding companies. The parentage of the hybrids, with respect to the species that were used, is indicated with the genome formula (Table 1). The letter A represents the genome of *A. aurea*, C the genome of *A. pelegrina*, Q the genome of *A. magnifica*, P the genome of *A. inodora*, D the genome of *A. psittacina*, L the genome of *A. ligtu*, and ? represents the genome of an unidentified species. The hybrids included the cultivars 'Orchid', 'Toledo', 'Victoria', 'Marina', 'Libelle' and 'Cinderella'. The material was cloned as described at the end of the previous section.

Structure of the rhizome (see also chapter 2)

The rhizome of *Alstroemeria* has a sympodial growth habit. The first shoot of a seedling terminates with a leaf, the apical meristem stops to grow (Figure 2a, chapter 2). Growth is continued by the axillary bud, which is positioned in the axil of the first (scaly) leaf. (L1) The bud develops into the rhizome tip and then gives rise to the second shoot (Figure 2b-c, chapter 2). Again an axillary bud in the axil of the first leaf of this shoot is pushed forward and the process repeats itself. Thus a rhizome is formed by the chain of basal stem internodes of successively developed shoots (Figure 2c, chapter 2). Each shoot also has a bud in the axil of the second leaf. The higher leaves have no visible axillary buds. The axillary bud of the second leaf can either remain dormant or it can develop into a lateral rhizome. In the latter case, the rhizome starts to branch (Figure 2d, chapter 2).

Experimental conditions

Prior to the experiment, all clones were pre-cultured under identical conditions as in the experiment during two culture periods of four weeks, in order to impose an adaptation of the plant material to these conditions. The propagation medium contained MS medium at full strength, with 40 $g.l^{-1}$ sucrose. The pH of all culture media was adjusted at 6.1, and BAP (1 $mg.l^{-1}$)and gelrite (2.2 $g.l^{-1}$) were added. Culture media were autoclaved at 121°C for 20 minutes and dispensed into polypropylene containers (Vitro

Vent Plant Tissue containers, Duchefa), 200 ml in each container. Initial explants were standardized, and of each genotype 20 explants were cultured, five explants in a container. Five extra explants served, if necessary, to replace contaminated rhizomes. The containers were placed in a climate room at 18°C with fluorescent tube illumination for 12 hrs daily (Philips 32W, 84HF, approximately 4000 Lux).

Experiment

At the start of the experiment, all explants consisted of a rhizome with one rhizome tip and two shoots (Figure 1). The shoots were decapitated at 5 mm distance from the rhizome. After four weeks of culture the rhizomes were dissected into new rhizome explants, now with a minimum size of one rhizome tip and two shoots.

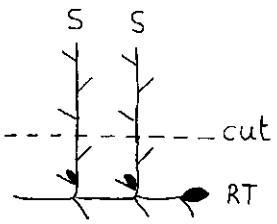

FIGURE. 1. A standardized rhizome explant, with two shoots (S) and one rhizome tip (RT).

The actual size of the explants depended on the size of the rhizomes after the first propagation cycle. Thus the new explants were no longer identical in size. All rhizome material was used as starting material for the second propagation cycle. The number of new explants was recorded (n4w) and subcultured onto fresh propagation medium. This procedure was repeated after eight weeks and the number of new explants was recorded again (n8w). After three propagation cycli, morphological traits were scored and the total number of propagules was recorded (n12w). Rhizome multiplication rate (RMR), defined as the mean multiplication factor of standardized rhizome explants, that are cultured *in vitro* for a period of four weeks, was determined. The morphological traits of the rhizome concerned total rhizome length, diameter, presence of roots, and the number of shoots, nodes, rhizome tips, and aberrant rhizome tips. The number of rhizome tips per node, the internodal length, RMR, and the multiplication factor in each of the three propagation cycli (mf1, mf2, mf3) were calculated. For an explanation of definitions is referred to Table 1. Rhizome tips were recorded aberrant, when they displayed either fasciation or vitrification. Some genotypes produce more aberrations than others. Such genotypes are often more sensitive to BAP, and a concentration of 1 $mg.l^{-1}$ may be too high. In such cases the whole clone can be physiologically and morphologically distorted. Therefore it was decided that the data of a particular replicate were not included in the analyses, when the percentage of aberrant rhizome tips exceeded 10%.

Experimental design and statistical analysis

An experimental unit consisted of 20 standardized explants in four plastic containers, that were dissected and subcultured after 4, 8 and 12 weeks. The plant material of each genotype was grouped and placed on a random position in the climate room. The experiment was replicated for the species accessions and for some of the hybrid genotypes (Table 1). It was assumed that the experimental error for hybrids was equal to the experimental error for species. Statistical analysis was conducted using the Statistical Package SAS. In order to analyse the observed variation in the n12w and the RMR, two models were used.

$\underline{y}_{ij} = \mu + \varepsilon_i + \underline{e}_{ij}$ (1) and

$\underline{y}_{ijklmnop} = \mu + \alpha_i + \beta_{ij} + \gamma_{ijk} + \delta_{ijkl} + \underline{b}_{ijklm} + \underline{c}_{ijklmn} + \underline{d}_{ijklmno} + \underline{e}_{ijklmnop}$ (2), with

μ = general mean,

ε_i = fixed effect of genotype i

$\underline{e}_{ij}$ = stochastic effect of independent, normally distributed random variables, with zero expectation and equal variance for each of the i genotypes and j replicates,

α_i = fixed effect of type i,

β_{ij} = fixed effect of species j,

γ_{ijk} = fixed effect of ploidy level k,

δ_{ijkl} = fixed effect of genome formula l,

$\underline{b}_{ijklm}$ = stochastic effect of accession m,

$\underline{c}_{ijklmn}$ = stochastic effect of parental genotype combination n,

$\underline{d}_{ijklmno}$ = stochastic effect of full sib o, and

$\underline{e}_{ijklmnop}$ = stochastic effect of independent, normally distributed random variables, with zero expectation and equal variance for each of the genotypes and the p replicates.

With the first model it was aimed to compare all the genotypes, irrespective of the group it belonged to. The effect of the genotype was considered to be fixed. The distribution of residuals of the n12w and the RMR in model 1 was monitored and compared to a normal distribution (test of Shapiro-Wilk). Analysis of variance was performed with the General Linear Models Procedure. With the second model it was aimed to compare groups of genotypes. The genotypes were regarded as random samples from groups of genotypes. The nested classification of the plant material into types (wild material or hybrid), species, ploidy levels, genome formulae, accessions, parental genotype combinations and full sibs (Table 1) was used to judge on the significance of the genetic variance at the different levels of classification. The effects of the type, the species, the ploidy level and the genome formula were considered to be fixed, the effects of the species accession, the parental genotype combination and the full sib were considered to be stochastic. Analysis of variance was conducted with the MIXED procedure and the method of REML (REstricted Maximum Likelihood). Least squares means and their standard errors were obtained for groups of genotypes. Fisher's F-test and Student's t-test were used to determine significance of fixed and stochastic effects, and contrasts. Comparison-wise significance levels were utilized. Mid

parent values (average of both parental species) were calculated for nine diploid interspecific combinations and compared with the actually observed values. Pearson's correlation analyses were conducted for the correlation between RMR and other traits.

Results

The numbers of propagules, RMR, and morphological traits were averaged for each genotype and presented in Table 1. The number of propagules after 12 weeks *in vitro* culture (n12w) varied considerably among the 88 genotypes. Rhizome multiplication rate (RMR) ranged from 1.11 to 3.10, with most values between 1.20 and 2.00. The coefficient of variation among replicated estimations of RMR was 10%. The distribution of residuals of RMR in model 1 was approximately normal, which was not the case with the residuals of n12w. All analyses were run with and without genotype A006. The exceptionally high multiplication rate of this genotype strongly influenced mean values and correlation coefficients. Because A006 was not used in interspecific crosses, it was decided to present the results of the analyses in which A006 was excluded. Analysis of variance using model 1 showed that there was significant genetic variation for RMR ($P<0.001$). When RMR was fitted to model 2 the genetic variation at the level of type, species, ploidy level and genome formulae was not significant. Most variation was found at the level of the parental genotype combination in the hybrids ($P=0.087$) and at the level of species ($P=0.125$), and subsequently at the level of species accessions ($P=0.176$) and genome formulae ($P=0.185$).

Least squares means of RMR were computed for groups of genotypes (Table 2) and it was tested which of the groups were significantly ($P<0.05$) different from each other. There was no significant difference in RMR between species and hybrids. Among the five species, RMR ranged from 1.31 to 1.65. *Alstroemeria inodora* had a significantly higher multiplication rate than *A. pelegrina* and *A. psittacina*. *Alstroemeria magnifica* had a significantly higher multiplication rate than *A. psittacina*. The tetraploid hybrids had lower RMRs than the triploid and the diploid hybrids. Among nine diploid interspecific combinations, RMR ranged from 1.30 to 2.18 (Table 3), whereas a much smaller range of 1.35 to 1.62 was expected on the basis of mid parent values. Positive heterosis for micropropagation rate was observed in the hybrids *A. aurea* × *A. magnifica* (AQ) and *A. magnifica* × *A. inodora* (QP). Micropropagation of the hybrids *A. aurea* × *A. psittacina* (AD) and *A. pelegrina* × *A. psittacina* (CD) was expected to be poor on the basis of mid parent values, but the observed RMRs were even lower. The ranking of the observed values for the genome formulae was nearly the same as for the mid parent values ($r_{Spearman} = 0.89$, $P= 0.001$). The coefficient of correlation $r_{Pearson}$ was 0.80 ($n=9$, $P=0.009$). The effect of the parental genotypes was evident among the hybrids *A. aurea* × *A. pelegrina* (AC) (Table 1), where RMRs ranged from 1.11 to 2.04. Individual full sibs also differed considerably in their RMR, for example C049A001-28 and C049A001-2 had RMRs of 1.32 and 2.03, respectively.

The morphological features of the *in vitro* grown rhizomes of species and of hybrids were highly variable (Figure 2a-i, Table 1). Whereas the rhizomes of *A. pelegrina* and *A. magnifica* were compact, those of *A. aurea* and *A. inodora* were elongated and shoots were farther apart. The rhizomes of *A. psittacina* were slender. The number of nodes produced during 12 weeks *in vitro* culture (nnod) varied from 212 to more than 1000 and in the outstanding accession A006 it exceeded 3000 (Table 1). The degree of branching of the rhizome, i.e. the number of rhizome tips per node (nrtpn), ranged from 0.16 to 0.27 among species accessions, and from 0.15 to 0.34 among hybrids. Rhizome internodes were short in the tetraploid hybrids, and long in the triploid hybrids. The rhizome diameter was considerably. The percentage of aberrant rhizome tips (part) was generally low (Table 1). Only in the species *A. magnifica*, *A. inodora* and in one of their F1 hybrids, it occasionally exceeded 10%. Table 4 shows the average values of morphological observations in the five species and nine of their diploid F1 interspecific combinations. The species *A. magnifica* and *A. inodora* produced many nodes, as did the hybrids with genome formulae AQ, AP and QP. The rhizomes of *A. aurea*, *A. inodora* and *A. psittacina*, and those of the *A. aurea* × *A. inodora* (AP) hybrids, generally showed a low degree of branching, whereas the rhizomes of *A. magnifica* and the other diploid hybrids branched more frequently. Internodes were short in *A. pelegrina* and *A. magnifica* and in the hybrids with the genome formulae CQ, CP, CD and QD. Long internodes were observed in *A. aurea*, *A. inodora*, in their F1 hybrid (AP) and in the hybrid *A. magnifica* × *A. inodora* (QP). The rhizome diameter was small in *A. pelegrina* and *A. psittacina*, slightly larger in *A. aurea*, *A. magnifica*, *A. inodora*, and very large in the hybrid *A. magnifica* × *A. inodora* (QP). The percentage of rooted rhizomes was low in *A. aurea*, *A. pelegrina* and *A. magnifica* and in the *A. aurea* × *A. inodora* hybrids. The rhizomes of *A. inodora*, *A. psittacina* and the hybrids with genome formulae AD and QP rooted frequently.

The heterotic RMR values of the AQ and QP hybrids could probably be attributed to a heterotic number of nodes (cf. Tables 3 and 4). The degree of branching in these hybrids only slightly exceeded the mid parent values. The internodes of the QP hybrids were longer than expected, but this was not the case in the AQ hybrids. The rhizome diameter of these hybrids exceeded the mid parent values to a great extent. When all genotypes except A006 were taken into account n=87, the number of nodes correlated well with RMR ($r=0.91$, $P<0.001$). The degree of branching of the rhizome ($r=0.53$, $P<0.001$), the rhizome diameter ($r=0.58$, $P<0.001$), and the internodal length ($r=0.31$, $P<0.01$) showed lower, but significant coefficients of correlation with RMR. The frequency of rooting correlated poorly with RMR ($r=0.06$, ns). A fairly good correlation was observed between the multiplication factor in the first culture period and RMR ($r=0.86$, $P<0.001$). The multiplication factor in the second ($r=0.68$, $P<0.001$) and third culture period ($r=0.70$, $P<0.001$) correlated less well with RMR.

TABLE 1. Classification of 88 *Alstroemeria* genotypes and the average values of observations after three cycli of micropropagation.
Reps. = number of replicate experiments; n12w = number of propagules after 12 weeks; RMR = rhizome multiplication rate = $(n12w/20)^{1/3}$; nnod = number of nodes after 12 weeks; nrtpn = number of rhizome tips per node; lint = internodal length (10^{-1} mm); diam = diameter of rhizome (10^{-1} mm); proot = percentage of rooted rhizomes (%); part = percentage of aberrant rhizome tips (%). * All species accessions were diploid, except for A017 which was triploid. ** Plant material was obtained as mature plant (1), as *in vitro* clone from ovule culture (2), or as *in vitro* clone from breeding companies (3).

TYPE Species*/ploidy Genotype number	Genotype code	Genome formula	Source**	Reps.	n12w	RMR	nnod	nrtpn	lint	diam	proot	part
SPECIES												
A. aurea												
1	A001	AA	1	4	43	1.29	296	0.16	32	30	2	1
2	A002	AA	1	2	77	1.57	434	0.20	28	31	25	0
3	A003	AA	1	3	44	1.30	287	0.17	26	26	8	1
4	A006	AA	1	2	594	3.10	3697	0.26	37	50	0	0
5	A011	AA	3	2	101	1.71	645	0.21	25	26	0	0
6	A016	AA	1	2	50	1.36	319	0.18	21	28	4	0
7	A017	AAA	3	2	145	1.93	922	0.18	40	43	31	1
A. pelegrina												
8	C024	CC	1	3	78	1.58	571	0.22	8	19	2	1
9	C025	CC	1	5	57	1.42	422	0.20	8	20	12	2
10	C030	CC	1	2	50	1.36	577	0.19	7	23	8	0
11	C042	CC	1	3	42	1.28	328	0.19	7	18	10	0
12	C049	CC	1	3	52	1.38	466	0.18	8	21	9	0
13	C055	CC	1	2	69	1.51	472	0.24	8	19	3	3
14	C423	CC	1	2	36	1.22	314	0.20	6	15	3	21
A. magnifica												
15	Q001	QQ	3	3	110	1.77	837	0.27	7	24	0	9
16	Q005	QQ	1	3	72	1.53	477	0.27	11	22	6	38
17	Q008	QQ	1	2	61	1.45	440	0.23	8	29	2	38
A. inodora												
18	P002	PP	1	3	72	1.53	422	0.19	19	23	81	3
19	P003	PP	3	2	150	1.96	1154	0.20	22	20	69	5
20	P004	PP	3	2	72	1.53	384	0.18	29	26	62	5

TABLE 1. Continued

TYPE Species*/ploidy Genotype number	Genotype code	Genome formula	Source**	Reps.	n12w	RMR	nnod	nrtpn	lint	diam	proot	part
A. psittacina												
21	D031	DD	1	4	52	1.37	324	0.20	19	19	50	0
22	D033	DD	1	2	35	1.21	277	0.21	13	19	89	0
23	D032	DD	1	2	51	1.36	309	0.17	29	19	77	0
HYBRIDS												
Diploids												
24	A001C024-10	AC	2	1	42	1.28	213	0.23	11	22	57	0
25	A003C030-1	AC	2	1	44	1.30	244	0.18	21	27	0	0
26	A200C100	AC	3	2	29	1.13	222	0.16	11	21	38	0
27	C049A001-1	AC	2	3	109	1.76	594	0.21	15	25	0	0
28	C049A001-2	AC	2	1	167	2.03	1076	0.32	19	40	30	0
29	C049A001-5	AC	2	2	60	1.44	341	0.22	12	21	3	0
30	C049A001-10	AC	2	1	85	1.62	499	0.24	13	26	18	1
31	C049A001-12	AC	2	1	128	1.86	729	0.24	17	27	1	2
32	C049A001-14	AC	2	1	116	1.80	819	0.24	17	31	15	0
33	C049A001-16	AC	2	1	141	1.92	938	0.34	15	30	0	0
34	C049A001-19	AC	2	1	93	1.67	485	0.22	16	21	2	0
35	C049A001-21	AC	2	1	61	1.45	352	0.21	13	21	29	0
36	C049A001-25	AC	2	1	84	1.61	464	0.20	18	26	4	0
37	C049A001-28	AC	2	1	46	1.32	296	0.20	10	18	21	0
38	C039A001-10	AC	2	1	107	1.75	711	0.21	17	29	4	2
39	C045A001-1	AC	2	1	61	1.45	317	0.22	13	22	10	0
40	C057A002-1	AC	2	1	139	1.91	719	0.25	14	24	36	1
41	C057A003-2	AC	2	1	40	1.26	234	0.18	11	24	14	0
42	C063A001-3	AC	2	1	51	1.37	297	0.22	10	25	4	0
43	C055A002-8	AC	2	1	126	1.85	635	0.24	12	25	6	2
44	C055A003-3	AC	2	1	171	2.04	1087	0.23	4	15	13	0
45	C100A100-1	AC	3	2	63	1.46	363	0.19	13	24	5	2
46	C100A100-2	AC	3	2	58	1.43	302	0.21	20	34	6	1

TABLE 1. Continued

TYPE Species*/ploidy Genotype number	Genotype code	Genome formula	Source**	Reps.	n12w	RMR	nnod	nrtpn	lint	diam	proot	part
47	C100A100-3	AC	3	1	39	1.25	275	0.16	10	19	21	0
48	C100A200-4	AC	3	1	27	1.11	212	0.20	12	19	32	0
49	A016Q001-1	AQ	2	1	223	2.23	1080	0.25	16	33	27	5
50	A016Q001-2	AQ	2	2	218	2.22	865	0.25	17	33	27	3
51	A016Q001-3	AQ	2	2	150	1.96	782	0.23	12	26	4	1
52	Q010A001-3	AQ	2	1	215	2.21	958	0.25	29	43	26	2
53	A001P004-1	AP	2	2	131	1.87	755	0.18	53	42	11	2
54	A011P004-1	AP	3	2	129	1.86	783	0.17	48	34	0	4
55	P004A300	AP	3	2	45	1.31	328	0.15	24	22	7	1
56	D100A400	AD	3	1	44	1.30	326	0.21	16	20	88	0
57	C042Q001-2	CQ	2	2	52	1.37	350	0.19	9	25	19	1
58	C042Q001-3	CQ	2	3	52	1.38	351	0.23	9	25	12	5
59	C042Q001-6	CQ	2	3	86	1.63	574	0.23	10	27	16	2
60	C042Q001-9	CQ	2	1	81	1.59	436	0.24	12	28	10	2
61	C042Q001-10	CQ	2	1	75	1.55	483	0.20	11	29	24	1
62	C042Q001-12	CQ	2	1	54	1.39	336	0.22	11	27	10	0
63	C042Q001-13	CQ	2	1	90	1.65	580	0.23	12	30	26	1
64	P004C100-1	CP	3	1	68	1.50	483	0.21	10	25	37	0
65	D032C025-4	CD	2	1	45	1.31	288	0.26	7	20	24	0
66	Q008P004-2	QP	2	1	213	2.20	1124	0.22	28	44	84	2
67	P004Q001	QP	3	2	209	2.19	1273	0.26	21	42	88	2
68	P002Q014	QP	2	1	160	2.00	868	0.22	19	32	0	15
69	Q001D100	QD	3	1	60	1.44	327	0.24	12	24	50	0
70	D100Q001	QD	3	2	69	1.51	481	0.21	9	25	42	2
71	A200L100	AL	3	1	74	1.55	400	0.22	30	29	22	1
72	'Orchid'	A?	3	1	122	1.83	684	0.25	14	25	0	0
73	'Toledo'	A?	3	1	127	1.85	697	0.27	18	30	5	0

TABLE 1. Continued

TYPE Species*/ploidy Genotype number	Genotype code	Genome formula	Source**	Reps.	n12w	RMR	nnod	nrtpn	lint	diam	proot	part
Triploids												
74	A600A200C200-3	AAC	3	2	47	1.33	290	0.21	15	27	17	0
75	A005A001P004	AAP	3	1	95	1.68	566	0.17	42	37	0	0
76	D100Q001A011-1	DQA	3	1	74	1.55	433	0.24	15	27	4	0
77	D100Q001A011-2	DQA	3	1	67	1.50	967	0.23	17	27	9	0
78	'Libelle'	???	3	1	49	1.35	304	0.23	19	35	5	0
79	'Marina'	???	3	1	88	1.64	468	0.22	19	36	42	0
80	'Victoria'	???	3	1	123	1.83	685	0.22	22	35	21	0
Tetraploids												
81	C100C100A100A100-1	CCAA	3	1	108	1.75	548	0.22	16	28	2	2
82	C100C100A100A100-2	CCAA	3	1	77	1.57	365	0.21	19	32	0	0
83	D132	CCDD	1	1	49	1.35	290	0.20	11	27	42	0
84	D362	CCDD	1	1	65	1.48	417	0.18	13	29	60	0
85	D662	CCDD	1	1	37	1.23	237	0.17	13	28	69	2
86	D100D100Q001Q001	DDQQ	3	2	36	1.21	230	0.21	12	24	14	0
87	D583	????	1	1	41	1.27	242	0.20	14	34	96	3
88	'Cinderella'	????	3	1	92	1.66	513	0.24	14	34	61	2

FIGURE. 2. Propagules of *Alstroemeria aurea* (a), *A. pelegrina* (b), *A. magnifica* (c), *A. inodora* (d) and *A. psittacina* (e), propagule with fasciated rhizomes (f) and propagules of the diploid hybrids *A. aurea* × *A. magnifica* (g), *A. inodora* × *A. aurea* (h) and *A. pelegrina* × *A. magnifica* (i).

TABLE 2. Least Squares Means estimates and standard errors for rhizome multiplication rate (RMR) of species and hybrids

Type	RMR ± standard error *
species (excl. A006)	1.48 ± 0.23a
hybrids	1.55 ± 0.26a
Species	
A. aurea (excl. A006)	1.44 ± 0.07 abc
A. pelegrina	1.40 ± 0.06 ab
A. magnifica	1.60 ± 0.10 bc
A. inodora	1.65 ± 0.10 c
A. psittacina	1.31 ± 0.09 a
Group	
species (excl. A006)	1.48 ± 0.23 ab
2x-hybrids	1.63 ± 0.13 b
3x-hybrids	1.59 ± 0.16 ab
4x-hybrids	1.43 ± 0.16 a

* Letters denote whether or not species (or groups of plants) are significantly different at the P=0.05 level. Species (or groups) with the same letters do not differ significantly.

TABLE 3. Least Squares Means and standard errors for rhizome multiplication rate (RMR) of hybrids, with nine different genome formulae, their mid parent values (mpv) and proportional divergence from the mid parent values (%).

Genome formula*	Number of genotypes	RMR ± std error**	mpv	prop. div. (%)
AC	25	1.50 ± 0.14 a	1.42	+6
AQ	4	2.14 ± 0.21 bcd	1.52	+41
AP	3	1.68 ± 0.19 ac	1.54	+8
AD	1	1.30 ± 0.30 a	1.37	-5
CQ	7	1.50 ± 0.24 a	1.50	0
CP	1	1.50 ± 0.30 ab	1.52	-1
CD	1	1.31 ± 0.30 a	1.35	-4
QP	2	2.18 ± 0.22 bd	1.62	+45
QD	2	1.47 ± 0.22 a	1.46	+1

* A= *A. aurea*, C= *A. pelegrina*, Q= *A. magnifica*, P= *A. inodora*, D= *A. psittacina*

** Letters denote whether or not interspecific combinations are significantly different at the 0.05 level. Interspecific combinations with the same letters do not differ significantly.

TABLE 4. Average values of morphological observations on five species and on diploid hybrids, with nine different genome formulae, after 12 weeks of micropropagation, and in parenthesis the proportional divergence from the mid parent value (%). For abbreviations see Table 1.

Species/hybrid	Genome formula*	Number of genotypes	nnod		nrtpn		lint		diam		proot		part
A. aurea (excl. A006, A017)	AA	5	396		.18		26		28		6		<1
A. pelegrina	CC	7	450		.20		7		19		6		4
A. magnifica	QQ	3	585		.26		9		25		2		27
A. inodora	PP	3	653		.19		23		23		72		5
A. psittacina	DD	3	303		.19		20		19		74		0
2x-hybrid	AC	25	497	(+17%)	.22	(+15%)	14	(-18%)	25	(+3%)	15	(+150%)	<1
2x-hybrid	AQ	4	921	(+88%)	.24	(+11%)	19	(+7%)	34	(+26%)	20	(+400%)	3
2x-hybrid	AP	3	622	(+18%)	.17	(-11%)	42	(+69%)	33	(+27%)	6	(-85%)	3
2x-hybrid	AD	1	326	(-7%)	.21	(+11%)	16	(-33%)	20	(-16%)	88	(+120%)	0
2x-hybrid	CQ	7	444	(-14%)	.22	(-4%)	10	(+31%)	27	(+24%)	17	(+325%)	2
2x-hybrid	CP	1	483	(-12%)	.21	(+8%)	10	(-32%)	25	(+16%)	37	(-5%)	0
2x-hybrid	CD	1	288	(-24%)	.26	(+29%)	7	(-47%)	20	(+6%)	24	(-40%)	0
2x-hybrid	QP	2	1199	(+94%)	.24	(+7%)	25	(+56%)	43	(+79%)	78	(+111%)	5
2x-hybrid	QD	2	404	(-9%)	.22	(-2%)	10	(-28%)	25	(+12%)	45	(+18%)	<1

* A= *A. aurea*, C= *A. pelegrina*, Q= *A. magnifica*, P= *A. inodora*, D= *A. psittacina*

Discussion

The survey of the variability for rhizome multiplication rate (RMR) in *Alstroemeria* demonstrated that there was significant genetic variation among species and hybrids. Also within species there were large differences in RMR. Comparison of the performance of interspecific hybrids with that of their parental species displayed additive inheritance of RMR in some hybrids and non-additive inheritance in others. A genetic basis for RMR was evident. Two diploid interspecific combinations exhibited positive heterosis for RMR. It should however be realized that (1) the multiplication rate was monitored for only three propagation cycli, and (2) for most interspecific combinations only a small number of hybrids was tested. The number of parental genotype combinations and the progeny size is too low to allow general conclusions.

Many papers report the presence of a genotypic effect on multiplication rates *in vitro*, e.g. Pierik *et al.* 1988 and Bond and Alderson 1993a and b (*Alstroemeria*), Zens and Zimmer 1988 *(Anthurium)*, Gavidia *et al.* 1991 (*Digitalis*), Pierik *et al.* 1982 (*Gerbera*), Allavena and Rossetti 1986 (*Phaseolus*), Jona and Vigliocco 1987 (*Prunus*), Valles and Boxus 1987 (*Rosa*). Reports on the inheritance of multiplication rate are more scarce. Custers and Bergervoet (1992) explored the micropropagation potential of the progeny of interspecific crosses in *Nerine* and concluded that there was considerable variation and scope for selection. In strawberry, Simpson and Bell (1989) compared the multiplication rates of six genotypes and with that of their F1-progenies. These researchers showed that it was not possible to predict the response of a particular genotype from the performance of their parents. They observed negative heterosis in crosses between the two fastest proliferating genotypes.

In the present investigation an attempt was made to identify morphological traits of the *Alstroemeria* rhizome that favour rapid micropropagation and might be used to select indirectly for RMR. In our view, the number of nodes should be considered as the trait that contributes most to RMR and should have a moderate to high level in both parents. The inheritance of RMR in diploid hybrids (Table 3) showed additivity in seven species combinations and non-additivity in two other species combinations. If the number of nodes was low in one of the parents, RMR was also low in the hybrid. The branching capacity and the internodal length, however, also played an important role. We suspect, that the heterotic performance of the hybrids *A. aurea* × *A. magnifica* (AQ) and *A. magnifica* × *A. inodora* (QP) partly resulted from the complementation of internodal length in one parent and branching capacity in the other, while the number of nodes was reasonable to good in both parents. A direct causal relationship between RMR and the number of rhizome nodes, the degree of branching and the internodal length is plausible, because RMR is determined by the number of separable rhizome explants. When a rhizome produces only a few new nodes, as a direct consequence, RMR will remain low. And, when it develops only very few lateral rhizomes, RMR will be limited by the number of rhizome tips. Because a standardized rhizome explant contains three nodes (Figure 2), the degree of branching is expected to be optimal when a rhizome tip (which itself contains one visible node) is produced at every second

node of the main rhizome (nrtpn=0.33). When the internodal length drops below a certain threshold value (probably about 1 to 2 mm) RMR is reduced, because the rhizome parts cannot be separated without damaging axillary buds. Longer internodes probably do not particularly favour micropropagation, nor affect it adversely. Whereas the number of nodes may limit RMR in the hybrids *A. aurea* × *A. psittacina* (AD), *A. pelegrina* × *A. inodora* (CP) and *A. pelegrina* × *A. psittacina* (CD), the degree of branching may be the limiting factor in the hybrids *A. aurea* × *A. inodora* (AP), and internodal length may limit RMR in the hybrids *A. pelegrina* × *A. magnifica* (CQ), *A. pelegrina* × *A. inodora* (CP), and *A. pelegrina* × *A. psittacina* (CD) (Tables 3 and 4). Although a large diameter is associated with rapid multiplication in many genotypes, this trait is apparently not strictly linked with RMR, since, for example, several tetraploid hybrids exhibit large rhizome diameters, but multiply rather slowly (Table 1).

In this investigation all genotypes were cultured under one particular set of experimental conditions. Each of these conditions, such as the concentration of BAP, the temperature, dissectioning of rhizome explants, etcetera, may have been sub-optimal for some genotypes and supra-optimal for others (cf. Pierik *et al.* 1988, Bond and Alderson 1993a and b). Therefore, the best results can only be obtained by 'tailoring' the experimental conditions to the genotype. Vitrification and fasciation of shoots and meristems, as observed in *Alstroemeria*, are general phenomena in many crops (e.g. Kataeva *et al.* 1991). It appeared that some species (*A. magnifica*), and some genotypes within species (C423 of *A. pelegrina*) and within hybrids (C042Q001-3, P002Q014) are more susceptible to physiological stress than others (Table 1).

In conclusion, the variability for rhizome multiplication rate in *Alstroemeria* offers scope for selection at several stages of a breeding programme. Particularly the choice of the parental species and of the genotypes within species, that are used in interspecific crosses, has a profound effect on the performance of a hybrid. This choice should be based on the capacity to produce a fair number of nodes in both parents. Furthermore, it is suspected that micropropagation in a hybrid might be favoured by the complementation of morphological traits of the rhizome in the parental species, such as branching capacity of the rhizome and internodal length. Selection among full sibs, using the multiplication factor of standardized explants in four weeks as a selection criterion, may also be worthwhile.

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Chapter 8

General discussion

In the biological investigations of the early part of the 20th century, ornamental plants were mostly considered as the objects of botanical curiosity. From about 1950 onwards, some ornamental species became more widely known because of their commercial value, and others because they were used in basic biological research. In many of the earlier investigations on plant chromosome cytology, ornamental plants predominated (Darlington, 1963, e.g. *Fritillaria*, *Gasteria*, *Scilla*) and contributed greatly for an understanding of biological phenomena such as chromosome evolution, speciation, polyploidy, and the evolution of crop plants (Stebbins, 1971; Grant, 1971). Although *Alstroemeria* was cytologically investigated at an early stage (Strasburger, 1882, in Taylor 1926), this genus had almost escaped the attention of plant cytologists until recently. Valuable cytological work on the chromosomes of species and hybrids of *Alstroemeria* was carried out during the 1970's (Koornneef, 1972; Koolstra, 1973a,b; van Eijk-Bos, 1974), but remained unpublished. In the 1980's, when *Alstroemeria* became popular as a cut flower crop, the knowledge regarding the chromosomes and genomes of this crop increased (Tsuchiya *et al.*, 1987; Tsuchiya and Hang, 1989).

Several features of *Alstroemeria* chromosomes and genomes render them attractive for cytological investigations. The basic number is relatively small (x=8), the chromosomes are large, easily identifiable, and distinctly differentiated among species with respect to chromosome morphology and C-banding patterns (Chapter 3). Concurrent with chromosome size and C-banding patterns, the genomes possess huge amounts of DNA, the 2C values ranging from 36.5 to 78.9 pg. in their diploid cells (Chapter 4). This is undoubtedly a remarkable range of DNA variation between species, that has occurred without the alteration of the ploidy status. Besides the interspecific variation, a considerable amount of intraspecific variation with respect to DNA values and C-banding pattern is present as well (Chapter 5). Obviously the increase in DNA has resulted from amplification of repetitive DNA fractions of the genomes, of which only some can be visualized through C-banding of chromosomes.

It is remarkable that it is possible to create interspecific hybrids between species that differ nearly two-fold in genome size. With the help of an embryo rescue technique, more than 250 interspecific hybrids have been produced between five species that are mostly used breeding programmes (Chapter 6). These hybrids, their parental species and their progeny after selfing or back-crossing, can be useful when unravelling breeding-related or fundamental problems. The plant material is particularly suitable for an investigation of the molecular organization of the chromosomes with the recently developed techniques of 'chromosome painting' (Kuipers *et al.* 1997). In this context it should be remarked, however, that the parents involved in these hybrids were collected from botanical gardens and from the breeders. This means that a certain amount of artificial selection and 'genetic drift' may have occurred. The degree of heterozygosity may have decreased, because species accessions have probably been selfed in order to maintain them.

'Chromosome painting' has opened the possibility to study the similarities and differences between the species, and to assess the evolutionary events that moulded the genomes of the *Alstroemeria* species. Furthermore, fluorescent (FISH) and genomic *in situ* hybridization (GISH) provide the potential for relating the molecular organization of chromosomes to phenomena such as chromosome pairing, crossing-over, recombination and introgression. Because of the tremendous diversity and differentiation among *Alstroemeria* species, that are spread out in a wide range of geographic areas of South America (Aker and Healy 1990), it is expected that the species possess distinct types of repetitive DNA sequences. Some of the so-called species-specific repetitive DNA sequences have indeed been isolated from the Chilean and Brazilian *Alstroemeria* species (De Jeu *et al.* 1997). Species-specific repetitive sequences can also be used as DNA probes for the identification of individual chromosomes, or chromosome segments in species, interspecific hybrids and back-cross progenies (Kamstra *et al.* 1997). A surprising finding is that some probes were localized in genomes that lack C-bands, at similar positions where in other genomes C-bands were found. This once again demonstrates that certain repetitive DNA sequences tend to accumulate at particular sites on the chromosomes (see also Chapters 3 and 5). Through FISH and GISH the extent of homeologous pairing and recombination in some distant interspecific hybrids and back-cross products has recently been determined (Kamstra *et al.* unpublished), and a further elucidation of the process of introgression is likely to be realized in the near future. Comparison with the results obtained in other genera, e.g. *Allium* (Friessen *et al.*, 1997), *Gasteria* × *Aloe* (Takahashi *et al.*, 1997) and *Saccharum* (D'Hont *et al.*, 1996) is most challenging.

Although 'chromosome painting' can be a powerful technique for the physical localization of certain types of DNA sequences on the genomes of plants and animals, it cannot give an answer to the intriguing question of what might be the significance of the huge amounts of DNA in certain organisms. In the search for an explanation of the evolutionary or ecological implications of large genomes it is for example surprising that both geophytic plants (see introduction of Chapter 4) and amphibians have large genomes and share analogous adaptations, such as dormancy, to escape from the harsh conditions in their habitats.

The diversity within the genus *Alstroemeria* is also prevalent with respect to rhizome morphology (Chapter 2). The rhizomes of species differ considerably in length, diameter and branching pattern, and some of these features are of importance when rhizomes are being multiplied *in vitro* (Chapter 7). A high number of rhizome nodes produced in each subculture, a high branching capacity, and long internodes favour a rapid multiplication *in vitro.* It was demonstrated that rhizome multiplication rate in *Alstroemeria* is a heritable trait, and that the choice of parental species and genotypes, to be used in interspecific crosses, is crucial. This choice should primarily be based on the capacity to produce a fair number of nodes in both parents. The performance during micropropagation of two interspecific hybrid combinations was considerably better than the performance of both parents. This heterotic performance was suspected to be due to complementation of morphological traits of the rhizome, that favour rapid

multiplication. Although no instances of negative heterosis were observed among nine diploid hybrid combinations, the occurrence of negative heterosis in these or other combinations cannot be ruled out. *Alstroemeria* is one of many rhizomatous plants that are being micropropagated commercially. Among these, there are numerous plants with ornamental and medicinal value, but also some food crops, e.g. *Cordyline*, *Dracena* (Agavaceae); *Canna* (Cannaceae), *Hosta*, *Zantedeschia* (Liliaceae); *Musa*, *Strelitzia* (Musaceae); *Nymphaea* (Nymphaeaceae); *Cattleya*, *Cymbidium*, *Dendrobium*, *Eulophia*, *Vanilla* (Orchidaceaea); *Zingiber*, *Cardomum*, *Costus*, *Curcuma*, *Hedichium*, *Kaempferia*, (Zingiberales). It is likely that there are analogies between these crops and *Alstroemeria*, especially where it concerns rhizome morphology and its influence on micropropagation.

A significant feature of the interspecific hybrids that were produced was that all of them were highly sterile due to disturbances of meiosis (Ramanna and Buitendijk, unpublished). The only instances where seed set was obtained after selfing, were on hybrids that produced numerically unreduced gametes. Remarkably, 2n-gamete formation was very rare among inter Chilean species hybrids, and frequently found in the hybrids between Chilean and Brazilian species. From the latter hybrids, numerous progenies could be obtained after selfing. Such progenies were invariably tetraploid, as determined through flow cytometric estimation and chromosome counts (Chapter 4). Thus, it was a demonstration of how polyploid cultivars had spontaneously originated in the breeder's nurseries, and it vindicates the hypothesis of Harlan and De Wet (1975) that most of the natural polyploids have originated through the functioning of 2n-gametes.

Summarizing the research underlying this thesis: Species material has been collected, taxonomically identified, morphologically, cytologically and genomically characterized, interspecific hybrids have been produced and analysed, rhizome multiplication rate *in vitro* has been assessed and the genetic differences for the latter trait have been pointed out. This work has contributed to basic research by providing defined plant material and basic information on the chromosomes and genomes of a number of *Alstroemeria* species and hybrids. The plant material was made available to breeders, and was used in new research projects.

Summary

The vegetatively propagated ornamental *Alstroemeria* has become highly popular in a relatively short period. During the last 40-50 years botanists and breeders realized that the genus *Alstroemeria* has tremendous potential as a cut flower crop, a bedding plant and a potted plant. They also became aware of the limited knowledge regarding the species and the interspecific hybrids that in several cases occurred spontaneously in their nurseries. There was a growing need for species descriptions and background information on their natural habitats. During the last 25 years, and especially during the last ten years, much information became available on the cultivation methods, tissue culture techniques and on the application of mutation breeding. Biosystematic studies and cytological investigations on chromosomes were, however, scarce in comparison to other major cut flowers.

In the biosystematic work on *Alstroemeria* frequent use was made of characteristics of the flowers. The underground organs, i.e. the rhizome and the root system, have not been described extensively. With the assumption that the morphological features of the rhizomes may affect micropropagation, the underground organs of several species and hybrids were studied. This investigation resulted in descriptions and illustrations of the general morphological structure of the rhizome, and of the underground organs of nine species and three hybrids in particular. The rhizomes varied considerably in length, diameter, internodal length, and the number of lateral rhizomes and visible axillary buds, giving more substance to the idea that the different species and hybrids respond differently when propagated *in vitro*.

A large part of the research that is described in this thesis concerned the chromosomes and genomes of *Alstroemeria* species and hybrids. The chromosomes of eight species were analysed after staining with Feulgen's reagent. The morphology of the chromosomes, i.e. the length, arm length ratio and the secondary constrictions, was clearly visible and chromosome arms could be measured. The application of Giemsa C-banding displayed unique banding patterns on the chromosomes of each of the eight species that were investigated. These banding patterns have cytotaxonomic value, and, once a larger number of species and species accessions has been studied, can throw light on the evolutionary events that occurred within the genus. The length of the chromosome complement already indicated that the species of *Alstroemeria* possess large genomes. This finding was confirmed through flow cytometric analysis of nuclear DNA amounts. The amount of nuclear DNA in the diploid cells (2C-values), as determined through flow cytometric measurement of the relative fluorescence of isolated nuclei that were stained with propidium iodide (PI), ranged from 36.5 to 78.9 pg among the accessions of 12 species. When nuclei were stained with DAPI, a fluorescent dye with different staining properties, different values were obtained. The ratio of PI and DAPI fluorescence varied from 1.60 to 1.88. The Brazilian species had lower PI/DAPI ratios (1.60-1.67) than the Chilean species (1.68-1.88). The 2C-values together with the PI/DAPI ratio of the twelve species enabled the separation of species

into four groups. These groups were (1) *A. magnifica* ssp. *magnifica*, *A. pelegrina*, *A. philippii* and *A. pulchra* ssp. *pulchra*, (2) *A. angustifolia* ssp. *angustifolia*, *A. aurea* and *A. hookeri* ssp. *hookeri*, (3) *A. ligtu* ssp. *ligtu* and *A. ligtu* ssp. *simsii*, and (4) *A. brasiliensis*, *A. caryophyllaea*, *A. inodora* and *A. psittacina*. There were remarkable karyotype similarities within each of these groups, with regard to total chromosome length and C-banding pattern. A survey of the extent of intraspecific variation in genome size of three Chilean species revealed up to 1.21 fold variation in *A. ligtu*. The variation in genome size could be attributed to C-band and chromosome length polymorphism. The intraspecific variation in *A. magnifica* ssp. *magnifica* was discontinuous, and might be due to a broad taxonomic species concept of this particular taxon [as, according to Bayer (1987), the taxa *A. gayana* and *A. sierrae* are conspecific with *A. magnifica* ssp. *magnifica*]. Giemsa C-banding and flow cytometry could both be used to check the hybrid nature of the plants that were produced in the interspecific hybridization programmes. Individual chromosomes of the parental species could be recognized in the C-banded karyotypes of most hybrids, and 2C-values as determined through flow cytometry were intermediate between those of the two parents. With the technique of flow cytometry it was possible to distinguish between aneuploid (2n=4x+1=33) and euploid (2n=4x=32) plants.

In order to obtain well defined plant material, species were collected from breeders, botanical gardens and research institutes, and interspecific crosses were made between five species, that are often used in the development of cultivars. These were the Chilean species *A. aurea*, *A. pelegrina* and *A. magnifica* ssp. *magnifica* and the Brazilian species *A. inodora* and *A. psittacina*. Seed set in interspecific crosses, however, was poor. Histological observations of the fertilized ovules revealed a poor development of the endosperm, and a reasonable to good development of the embryo during the first two to three weeks after pollination. Through *in vitro* culture of dissected ovules, it was possible to produce more than 250 hybrids.

Conventionally, *Alstroemeria* plants are vegetatively propagated by division of greenhouse grown rhizomes, usually once a year. The multiplication rate with this practice of propagation is rather low (about two to six new plants), because it is restricted by the number of lateral rhizomes that are present on the mother plant. The development of micropropagation methods, using in vitro grown rhizomes, has enabled the mass propagation of plant material. However, the rhizome multiplication rate of hybrids is mostly unpredictable, and for groups of cultivars it is extremely low. An analysis of the rhizome multiplication rate of defined plant material, consisting of species and their interspecific hybrids, demonstrated that there is a genetic base for the performance during micropropagation. Thus, it is worthwhile to incorporate rhizome multiplication rate as a selection criterion in *Alstroemeria* breeding programmes. The choice of parental species and genotypes, to be used in interspecific crosses, is crucial. Because of the high degree of heterozygosity of the parental genotypes, selection among full sibs can be profitable. Morphological features of the rhizomes, such as the number of rhizome nodes, the degree of branching, and internodal length, may serve as criteria for indirect selection. It is suspected that the complementation of these

morphological traits in the parental genotypes might lead to a superior micropropagation performance of the hybrid.

The research that is described in this thesis has contributed by providing defined plant material and basic information on species and hybrids of *Alstroemeria*. The results are beneficial for the breeding of new cultivars, and for further applied and fundamental research in the genus *Alstroemeria*.

J.H. Buitendijk. 1998. A cytological characterization of genomes of *Alstroemeria*, the production of interspecific hybrids, and their performance during micropropagation.

Samenvatting

Het vegetatief vermeerderde siergewas *Alstroemeria* is in korte tijd erg populair geworden. In de afgelopen 40 à 50 jaar ontdekten plantenliefhebbers, plantentelers en veredelaars dat *Alstroemeria* zeer veel mogelijkheden heeft voor het gebruik als snijbloemgewas, als tuinplant en ook als potplant. Tegelijkertijd werd men zich bewust van de beperkte beschikbare kennis van de botanische soorten en de soortshybriden, die herhaaldelijk bij toeval gevonden werden, maar waarvan de ontstaanswijze dikwijls onbekend was. Er was in toenemende mate behoefte aan soortbeschrijvingen en aan informatie over de natuurlijke groeiplaatsen van de diverse soorten. Gedurende de laatste 25 jaar, en vooral de laatste 10 jaar, kwam er veel kennis beschikbaar over teeltmethoden, over weefselkweektechnieken en over het gebruik van mutatieveredeling. De kennis over de chromosomen en over de verwantschapsrelaties tussen de soorten is echter nog steeds gering in vergelijking tot die bij andere belangrijke snijbloemgewassen.

In hun plantbeschrijvingen maakten taxonomen veelvuldig gebruik van bijzonderheden van de bloem. De ondergrondse organen, zoals het rhizoom (de wortelstok) en het wortelstelsel, worden zelden beschreven. In het hier beschreven promotieonderzoek werden de ondergrondse delen van diverse soorten en soortshybriden bestudeerd, in de veronderstelling dat er een verband bestaat tussen rhizoomhabitus en vermeerderingssnelheid van plantmateriaal met behulp van weefselkweektechnieken, waarbij rhizoomweefsel onder geconditioneerde omstandigheden wordt gekweekt. Deze studie resulteerde in beschrijvingen en illustraties van de algemene structuur van het rhizoom, en van de verschillende verschijningsvormen ervan in soorten en soortshybriden. Er werden grote verschillen in lengte, diameter en internodiumlengte van de rhizomen waargenomen. Ook de mate van vertakking en het aantal zichtbare okselknoppen varieerde aanzienlijk, hetgeen het vermoeden versterkte dat de soorten een verschillende vermeerderingssnelheid hebben in weefselkweek.

Een groot deel van het onderzoek beschreven in dit proefschrift gaat over de chromosomen en genomen van de botanische soorten en soortshybriden. De lengte van de chromosomen, de lengteverhouding tussen de chromosoomarmen, en de secundaire chromosoominsnoeringen konden duidelijk worden waargenomen en gemeten. Na een chemische behandeling van chromosoompreparaten door middel van Giemsa C-bandering, konden karakteristieke bandenpatronen op de chromosomen van acht onderzochte soorten worden waargenomen. Deze bandenpatronen hebben cytotaxonomische waarde, en zodra een groot aantal soorten en soortsaccessies is bestudeerd, kunnen zij licht meer werpen op de evolutionaire processen die hebben plaatsgevonden binnen het geslacht *Alstroemeria*. De grote lengte van de chromosomen wees erop dat de soorten in het bezit zijn van grote hoeveelheden DNA. Dit werd bevestigd door metingen met behulp van een flowcytometer. De hoeveelheid DNA in de kernen van diploide cellen, de zogenaamde 2C-waarde, werd vastgesteld

door de relatieve fluorescentie te meten van geïsoleerde celkernen die gekleurd waren met de fluorescerende DNA kleurstof propidium iodide (PI). Deze 2C-waarden in 12 onderzochte soorten liepen uiteen van 36.5 to 78.9 pg DNA. Na kleuring van de celkernen met DAPI (4',6-diamidino-2-phenylindole), een fluorescerende kleurstof die een andere binding aangaat met het DNA dan PI, werden lagere waarden verkregen. De verhouding tussen de PI en de DAPI fluorescentie in de Braziliaanse soorten was lager (1.60-1.67) dan in de Chileense soorten (1.68-1.88). Op basis van de 2C-waarden èn de verhouding tussen de PI en de DAPI fluorescentie konden de 12 onderzochte soorten in vier groepen worden ingedeeld. Dit waren (1) *A. magnifica* ssp. *magnifica*, *A. pelegrina*, *A. philippii* en *A. pulchra* ssp. *pulchra*, (2) *A. angustifolia* ssp. *angustifolia*, *A. aurea* en *A. hookeri* ssp. *hookeri*, (3) *A. ligtu* ssp. *ligtu* en *A. ligtu* ssp. *simsii*, en (4) *A. brasiliensis*, *A. caryophyllaea*, *A. inodora* en *A. psittacina*. Binnen deze groepen was er een opmerkelijke gelijkenis in karyotypen ten aanzien van de totale chromosoomlengte en het C-bandenpatroon. De intraspecifieke variatie in genoomgrootte (d.w.z. de variatie tussen de accessies van een soort) werd in drie Chileense soorten nader onderzocht. In *A. ligtu* had de accessie met de hoogste 2C-waarde 21% meer DNA dan de accessie met de laagste waarde. Deze variatie binnen de soort werd toegeschreven aan verschillen in het aantal en de grootte van de C-banden en aan verschillen in chromosoomlengte. De onderzochte accessies van *A. magnifica* ssp. *magnifica* vertoonden een discontinue variatie. Een mogelijke verklaring hiervoor is dat een breed soortsbegrip is gehanteerd [de taxa *A. gayana* en *A. sierraea* worden door Bayer (1987) tot één en dezelfde soort, *A. magnifica* ssp. *magnifica*, gerekend]. Met de Giemsa C-bandering en met flowcytometrie was het ook mogelijk om het hybride karakter van nieuw verkregen soortskruisingsprodukten aan te tonen. Na Giemsa C-bandering van chromosoompreparaten van de soortshybriden, waren de individuele chromosomen van de oudersoorten te herkennen, terwijl fluorescentiemetingen aan celkernen met de flowcytometer 2C-waarden opleverden die intermediair waren tussen die van de beide oudersoorten. Daarnaast konden met behulp van flowcytometrie aneuploïde planten (met één extra chromosoom) worden onderscheiden van planten met het euploïde chromosoomaantal.

Om goed gedefinieerd plantmateriaal te verkrijgen werden de soorten betrokken van veredelaars, botanische tuinen en onderzoeksinstellingen. Er werden kruisingen gemaakt tussen vijf soorten die veelvuldig zijn gebruikt bij het tot stand komen van het huidige cultivarsortiment. Dit waren de Chileense soorten *A. aurea*, *A. pelegrina* en *A. magnifica* ssp. *magnifica* en de Braziliaanse soorten *A. inodora* en *A. psittacina*. De zaadzetting in de soortskruisingen was echter slecht. Een histologisch onderzoek toonde aan dat het endosperm in de jonge zaden zich slecht ontwikkelde, terwijl het embryo nog in redelijk goede conditie verkeerde gedurende de eerste twee à drie weken na bestuiving. De ontwikkeling en de toepassing van een methode waarbij het jonge zaad uit de zaaddoos werd gehaald en onder geconditioneerde omstandigheden verder werd gekweekt, leverde uiteindelijk een groot aantal soortshybriden op.

De conventionele vermeerderingswijze van plantgoed van *Alstroemeria* bestaat uit het scheuren van het rhizoomstelsel, doorgaans éénmaal per jaar. De

vermeerderingsfactor is hierbij echter laag (ongeveer twee tot zes nieuwe planten), en wordt bepaald door het aantal zijrhizomen aan de moederplant. Wanneer rhizomen onder geconditioneerde omstandigheden *in vitro* worden gekweekt, is een efficiëntere vermeerdering mogelijk. Echter, de snelheid van vermeerdering van hybriden is vaak moeilijk te voorspellen en sommige groepen van cultivars vermeerderen uiterst traag in weefselkweek. Met het hier beschreven onderzoek werd aangetoond dat de vermeerderingssnelheid in weefselkweek genetisch bepaald is. Het is dan ook lonend om de vermeerderingssnelheid van het rhizoom op te nemen als selectiecriterium bij de veredeling van nieuwe *Alstroemeria* rassen. De keuze van de kruisingsouders in soortskruisingen is hierbij zeer belangrijk. Aangezien de soorten genetisch behoorlijk heterogeen zijn, is het belangrijk om ook binnen de oudersoorten te selecteren. Daarnaast is het zinvol om te selecteren binnen de nakomelingschap van de kruisingen. Morfologische kenmerken van de rhizomen, zoals het aantal knopen in een rhizoom, de mate van vertakking en de internodiumlengte lijken te kunnen dienen als indirecte selectiecriteria. Er zijn aanwijzingen dat het mogelijk is om cultivars met een superieure vermeerderingssnelheid te veredelen door bij de keuze van de kruisingsouders te streven naar complementatie van een sterke vertakking in de ene ouder en een redelijke internodiumlengte in de andere ouder. De productie van een redelijk tot hoog aantal scheuten in beide ouders lijkt eveneens van belang.

Het onderzoek dat in dit proefschrift werd beschreven heeft bijgedragen tot een uitbreiding van de kennis van de soorten en soortshybriden binnen het geslacht *Alstroemeria*, met name betreffende de morfologie van de rhizomen, de chromosomen, de DNA hoeveelheden in diploïde cellen, de ontwikkeling en toepassing van een 'embryo rescue' methode voor het doorbreken van soortskruisingsbarrieres, en de genetische variatie voor de vermeerderingssnelheid van rhizomen in weefselkweek. Tevens is gedefinieerd plantmateriaal beschikbaar gekomen voor veredelingsdoeleinden en voor nieuw onderzoek.

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Nawoord

Het experimentele onderzoek dat in dit proefschrift is beschreven, heeft plaatsgevonden tussen november 1989 en oktober 1993 aan de vakgroep voor Plantenveredeling van de Landbouwuniversiteit te Wageningen. In deze periode zijn veel mensen betrokken geweest bij het onderzoek en zij hebben elk op hun eigen wijze bijgedragen aan dit proefschrift. In de periode 1994 tot en met 1997 werden de resultaten verwerkt en beschreven. De volgende personen en hun bijdrage wil ik bij name noemen.

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Curriculum vitae

De auteur van dit proefschrift, Joska Hendrina Buitendijk, werd geboren op 17 april 1959 in Eefde. In 1977 begon zij aan haar studie aan de Landbouwuniversiteit te Wageningen (LUW). Daar koos zij de studierichting Plantenveredeling, met als doctoraalvakken plantenveredeling, genetica en nematologie, en een stage aan het Welsh Plant Breeding Station in Aberystwyth, Wales. In 1984 rondde zij haar studie af en werkte een half jaar mee aan een onderzoek naar zaadkieming bij tomaat aan de vakgroep Plantenfysiologie van de LUW, en vervolgens drie maanden aan een onderzoek naar resistentiemechanismen tegen het aardappelcystenaaltje aan de Stichting voor Plantenveredeling (nu CPRO-DLO) te Wageningen. In januari 1986 kwam zij in dienst van het bedrijf Zaadunie (nu Novartis Seeds) in Enkhuizen, waar zij leiding gaf aan de laboranten van de afdeling weefselkweek. Zij was verantwoordelijk voor de ontwikkeling van protocollen voor de vermeerdering, 'embryo rescue', en haploïdisatie van diverse groente en bloemgewassen, alsmede voor de productie van plantmateriaal ten behoeve van de veredeling. In november 1989 keerde zij terug naar Wageningen om te starten met een promotie-onderzoek in *Alstroemeria*, waarbij zij tot en met 1993 als assistent-in-opleiding werd aangesteld bij de vakgroep Plantenveredeling van de LUW. De resultaten van dit onderzoek staan beschreven in dit proefschrift.

The author of this thesis, Joska Hendrina Buitendijk, was born in Eefde on 17 April 1959. In 1977 she started her studies in Plant Breeding at Wageningen Agricultural University (WAU). With Plant Breeding and Genetics as main subjects, Nematology as minor subject, and a practical training at the Welsh Plant Breeding Station in Aberystwyth, Wales, she graduated in 1984. She collaborated for half a year in a research project on the germination of tomato seeds at the Department of Plant Physiology of WAU, and for three months in a research project on resistance against potato cyst nematode at the Stichting voor Plantenveredeling (currently CPRO-DLO) in Wageningen. In January 1986 she joined the seed company, Zaadunie (currently Novartis Seeds), in Enkhuizen, where she was Head of the tissue culture group. She was responsible for the development of protocols for micropropagation, embryo rescue and haploidization of several vegetable and flower crops, and for the production of plant material for breeding purposes. In 1989 she returned to Wageningen and started research on *Alstroemeria* at the Department of Plant Breeding of WAU until the end of 1993. The results of this research are described in this thesis.