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

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

Keywords: Alstroemeriaceae, *Bomarea*, classification, Inca lily, *Leontochir*, Monocotyledonae.

Introduction

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

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

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

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

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Alstroemeria spp., which are characterized by a large genome size (2C-value: 37–79 pg) (Han *et al*., 1999).

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

Materials and methods

Plant material

Seeds and plants of 22 *Alstroemeria* species were obtained from botanical gardens and commercial breeders. The collection has been maintained for many years in the greenhouse of Unifarm at the Wageningen Agricultural University. When available, three accessions were selected for each *Alstroemeria* species, and both *B. salsilla* and *L. ovallei* were chosen as outgroups. One interspecific hybrid (*A. aurea* × *A. inodora*) was obtained from earlier research (Buitendijk *et al*., 1995) (Table 1). All accessions were identified according to their morphological traits (Uphof, 1952; Bayer, 1987).

AFLP protocol

Genomic DNA was isolated from young leaves of greenhouse-grown plants using the cetyltrimethylammonium bromide (CTAB) method according to Rogers & Bendich (1988). The AFLP technique followed the method of Vos *et al*. (1995) with modifications of selective bases of pre- and final amplifications

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

Code	Plant material	Accession†	Distribution/altitude‡
	Chilean species		
C1	*A. andina* Phil.	IX-2	Chile 26°–31°S.L., 2900–3700 m(1)
C2	*A. angustifolia* Herb. ssp. *angustifolia*	AN1S, AN2S, AN7K	Chile, 33°S.L., <1000 m(1)
C3	*A. aurea* Grah.	A001, A002, A003	Chile, 36°–42°/47°S.L., 200–1800 m(1)
C4	*A. diluta* Bayer	AD2W, AD4K, AD5K	Chile, 29°–31°S.L., 0–100 m(1)
C5	*A. exserens* Meyen	AO2S, AO5S, AO7Z	Chile, 34°–36°S.L., 1500–2100 m(1)
C6	*A. garaventae* Bayer	AH6Z, AH8K	Chile, 33°S.L., 2000 m(1)
C7	*A. gayana* Phil.	XIII-2	Chile 29°–32°S.L., 0–200 m(1)
C8	*A. haemantha* Ruiz and Pav.	J091–1. J091–4	Chile, 33°–35°S.L., 0–1800 m(1)
C9	*A. hookeri* Lodd. ssp. *cumminghiana*	AQ5S, AQ6Z, AQ7Z	Chile, 32°–34°S.L., 0–500 m(1)
C10	*A. hookeri* Lodd. ssp. *hookeri*	AP2S, AP3S, AP8K	Chile, 35°–37°S.L., 0–300 m(1)
C11	*A. ligtu* L. ssp. *incarnata*	AJ7S, AJ12K	Chile, 35°S.L., 1100–1400 m(1)
C12	*A. ligtu* L. ssp. *ligtu*	AL4S, AL6K, AL11K	Chile, 33°–38°S.L., 0–800 m(1)
C13	*A. ligtu* L. ssp. *simsii*	AM6K, AM7K, K101–1	Chile, 33°–35°S.L., 0–1800 m(1)
C14	*A. magnifica* Herb. ssp. *magnifica*	Q001–4, Q001–5, Q007	Chile, 29°–32°S.L., 0–200 m(1)
C15	*A. modesta* Phil.	AK2 W, AK3 W	Chile 29°–31°S.L., 200–1500 m(1)
C16	*A. pallida* Grah.	AG4Z, AG7K, AG8K	Chile 33°–34°S.L., 1500–2800 m(1)
C17	*A. pelegrina* L.	AR4S, C057–1, C100–1	Chile, 32°–33°S.L., 0–50 m(1)
C18	*A. pulchra* Sims. ssp. *pulchra*	AB3 W, AB7S, AB8S	Chile, 32°–34°S.L., 0–1000 m(1)
C19	*A. umbellata* Meyen	AU2Z	Chile, 33°–34°S.L., 2000–3000 m(1)
	Brazilian species		
B1	*A. brasiliensis* Sprengel	BA1K, BA2K, R001–1, R001–2	Central Brazil(2)
B2	*A. inodora* Herb.	P002, P004–6, P008–3	Central and Southern Brazil(2)
B3	*A. pstittacina* (D) Lehm.	D031, D032, D92–02–1	Northern Brazil(2)
B4	*A. pstittacina* (Z) Lehm.	93Z390–2, 93Z390–4, 96Z390–6	Northern Brazil(2)
O1	*Bomarea salsilla* Mirbel.	M121	Central and Southern South America(3)
O2	*Leontochir ovallei* Phil.	U001	Central Chile(4)
	Interspecific hybrid		
F1	A1P2–2	(A001 × P002)-2	Buitendijk *et al*. (1995)

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

(Han *et al*., 1999). To assess interspecific variation, autoradiograms comprising the AFLP fingerprints of a mixture of three accessions per species were analysed by pooling 5 μL of the final selective amplification products according to Mhameed *et al*. (1997). The low level of variation between individual samples showed that pooling accessions was justified. Three primer combinations (E + ACCA/M + CATG, E + ACCT/M + CATC and E + AGCC/M + CACC) were selected from a test of 96 primer combinations, and these produced 272, 211 and 233 bands, respectively (Table 2). The choice of the primers used in the study was based upon the visual clarity of banding patterns generated and a preferably low fingerprint complexity. The complexity of the banding pattern is a major limiting factor for scoring AFLP fingerprints of large-size genomes.

Data analysis

Positions of unequivocally visible and polymorphic AFLP markers were transformed into a binary matrix, with '1' for the presence, and '0' for the absence of a band at a particular position. The genetic distance (GD) between species was based on pair-wise comparisons and calculated according to the equation: $GD_{xy} = 1 - [2N_{xy}/(N_x + N_y)]$, where N_x and N_y are the numbers of fragments to individuals x and y, respectively, and N_{xy} is the number of fragments shared by both (Nei & Li, 1979). Genetic distances were computed by the software package TREECON (v. 1.3b) (Van De Peer & De Wachter, 1993). The dendrogram of the 22 *Alstroemeria* species, the interspecific hybrid, *Bomarea* and *Leontochir* was generated based on the GD matrix by using cluster analysis, the UPGMA (unweighted pair group method using arithmetic averages) method with 1000 bootstraps (Sneath & Sokal, 1973; Felsenstein, 1985) (Fig. 1). Principal co-ordinate analysis was performed to access interspecies relationships based on the Nei & Li (1979) coefficient $[2N_{xy}/(N_x + N_y)]$ using the NTSYS-PC program (Rohlf, 1989).

Results and discussion

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

Buitendijk & Ramanna (1996) suggested that the Chilean and Brazilian species form distinct lineages. The genetic diversification of *Alstroemeria* species as detected by the AFLP technique revealed three main clusters with 99% bootstrap values: the Chilean species, the Brazilian species and the outgroup (Fig. 1). This finding would support an early divergence of these groups and is consistent with the occurrence of interspecific crossing barriers between the Chilean and Brazilian species (De Jeu & Jacobsen, 1995; Lu & Bridgen, 1997). The variance of the first three principal co-ordinates accounted for 34.9% of the total variation, differentiated effectively among the species and reflected the main clustering of the dendrogram. From the principal co-ordinate plot, four groups were clearly demarcated:

Table 2 Sequences of adaptors and primers used

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

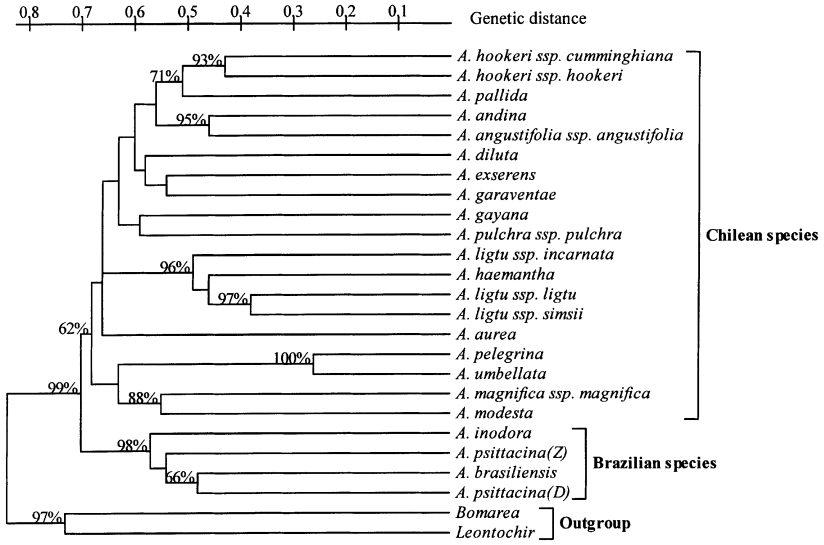

Fig. 1 Dendrogram of 22 *Alstroemeria* species, *Bomarea salsilla* and *Leontochir ovallei* resulting from a UPGMA cluster analysis based on Nei's genetic distances obtained from 716 AFLP bands. The bootstrap analysis was conducted using TREECON (v. 1.3b) with 1000 bootstrap subsamples of the data matrix.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

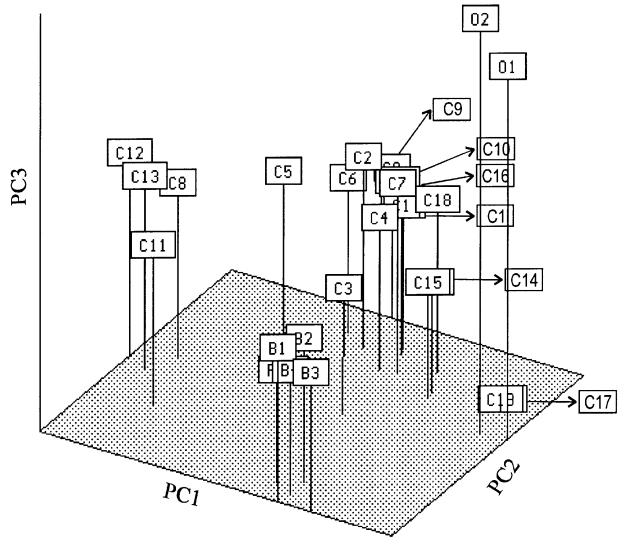

Fig. 2 Relationships among 22 *Alstroemeria* species, the F_1 hybrid, *Bomarea salsilla* and *Leontochir ovallei* by principal co-ordinate analysis using Nei and Li coefficients. The three principal co-ordinates accounted for 34.9% of the total variation. PC1, PC2 and PC3: first, second and third principal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

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

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

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

Alstroemeria pelegrina and *A. umbellata* were assigned as sister species with a GD of 0.26 showing a remarkable genetic similarity (data available on request). The species we coded under the name *A. umbellata* actually seemed to be an *A. pelegrina* species that did not flower for many years. *Alstroemeria haemantha* was assigned to a group together with *A. ligtu* ssp. *ligtu*, *A. ligtu* ssp.

incarnata and *A. ligtu* ssp. *simsii* (Figs 1 and 2) (Aker & Healy, 1990; Ishikawa *et al.*, 1997). Bayer (1987) suggested the synonymous name of *A. ligtu* ssp. *ligtu* for *A. haemantha* Ruiz and Pavon. Our results support this hypothesis. *Alstroemeria exserens* was positioned between the Chilean group and the *A. ligtu* group (Fig. 2). *Alstroemeria andina* and *A. angustifolia* ssp. *angustifolia*, and *A. hookeri* ssp. *cumminghiana* and *A. hookeri* ssp. *hookeri* were clustered together with 95% and 93% bootstrap values, respectively.

The interspecific hybrid (A1P2–2) was included in our study in order to investigate the possibility of the identification of the parental genotypes. The F_1 hybrid A1P2–2 showed a 0.45-GD value with *A. inodora* and 0.59 GD value with *A. aurea* showing genomic contribution of both parents (data available on request). It indicated the feasibility of the AFLP technique as a tool for the identification of parental genotypes (Sharma *et al.*, 1996; Marsan *et al.*, 1998). *Bomarea* and *Leontochir* showed the mean GD value of 0.83 as the outgroup, thus showing large genetic distances within the Alstroemeriaceae family.

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

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