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N. Friesen · R. Fritsch · K. Bachmann

Hybrid origin of some ornamentals of *Allium* subgenus *Melanocrommyum* verified with GISH and RAPD

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Abstract Random amplified polymorphic DNA (RAPD) and genomic in situ hybridization (GISH) methods have been used to verify the hybridogenic origin and to identify the parental species of some ornamental cultivars in the subgenus *Melanocrommyum* of the genus *Allium*. The cultivars had been selected from seed obtained after uncontrolled pollination in breeders' fields. The combination of GISH analysis with RAPD markers is very suitable for testing the hybridogenic origin of plants and to ascertain the parental species of the hybrids in such cases. As suspected, *A. macleanii* and *A. cristophii* are the parental species of 'Globemaster'. The parental species of cultivar 'Globus' are *A. karataviense* and *A. stipitatum*, and not *A. cristophii* and *A. giganteum* as has been assumed on morphological grounds. Cultivars 'Lucy Ball' and 'Gladiator' are of hybrid origin, though only one of the parental species, *A. hollandicum*, could be confirmed. The cultivars 'Purple Sensation', 'Mount Everest', 'White Giant', 'Michael H. Hoog' and 'Mars' are not hybrids since neither GISH nor RAPD suggest the presence of a second genome. 'Purple Sensation' belongs to *A. hollandicum*, 'Mount Everest', 'White Giant' and 'Mars' to *A. stipitatum*, 'Michael H. Hoog' to *A. rosenorum*.

Key words *Allium* · Ornamental cultivars · Hybrids · GISH · RAPD

Introduction

Genomic in situ hybridization (GISH) has proved to be very valuable for identifying chromosomes from different species in hybrid karyotypes (Schwarzacher et al. 1989, 1992; Leitch et al. 1991; Hizume 1994; Keller et al. 1996; Schwarzacher 1996; Friesen et al. 1997). Labelled total genomic DNA from one of the parental species can be used as a probe, and has often been found to be specific enough to mark the chromosomes from that parent. This method offers new opportunities in phylogenetic and taxonomic studies for determining and testing genomic relationships of wild and cultivated plant species (Heslop-Harrison and Schwarzacher 1996).

Several species of *Allium* subgenus *Melanocrommyum* have ornamental properties. About a dozen of them have been introduced from the wild into European gardens in the last century, and approximately the same number again in this century. However, most of these strains have some characters that limit their value as garden ornamentals. The introduction of more suitable natural strains from the wild is expensive if at all possible. The species are endemic to arid regions of Southwest and Central Asia. Travel in these regions is difficult, and it has not been possible to gain access to some of the countries due to the political situation. Therefore the strains available in Europe have been used for the development of new character states, especially by Dutch bulb growers. Usually, large amounts of seed from insect-pollinated plants grown together were sown, and useful new varieties were selected from among the offspring. The hybrid character of these cultivars is suspected on the basis of their character combinations, but the exact parental combination could not be determined with certainty.

In the present study we have used GISH to investigate some ornamental cultivars of *Allium* subgenus *Melanocrommyum* ('Globemaster', 'Globus', 'Gladiator', 'Lucy Ball', 'Mars', 'Michael H. Hoog', 'Mount Everest', 'Purple Sensation' and 'White Giant'). Most of these were initially selected as described above and are believed to be hybrids. However, there is no general agreement about their parental species, and different parents are proposed in the growers' catalogs (Bijl

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N. Friesen (✉) · R. Fritsch · K. Bachmann
Department of Taxonomy, Institute for Plant Genetics and Crop Plant Research, 06466 Gatersleben, Germany
E-mail: friesenn@ipk-gatersleben.de

Table 1 Decorative cultivars of the subgenus *Melanocrommyum* of spontaneous garden origin and their supposed parental species

Name of the cultivar	Parental species according to:		
	Bijl (1994)	Hoog and Dix export	Ruksãns (1997)
'Globemaster'	*A. macleanii* × *A. cristophii*	–	–
'Gladiator'	*A. macleanii* × *A. aflatunense* hort.	*A. macleanii* × *A. aflatunense* hort.	*A. macleanii* × *A. aflatunense* hort.
'Lucy Ball'	*A. macleanii* × *A. aflatunense* hort.	*A. macleanii* × *A. aflatunense* hort.	*A. macleanii* × *A. aflatunense* hort.
'Globus'	–	*A. giganteum* × *A. cristophii*	*A. karataviense* × *A. cristophii*
'Mount Everest'	–	*A. stipitatum* × *A. aflatunense* hort.	*A. stipitatum* × *A. aflatunense* hort.
'Mars'	–	*A. stipitatum*	*A. stipitatum* × *A. aflatunense* hort.
'Purple Sensation'	*A. aflatunense* hort.	*A. aflatunense* hort.	*A. hollandicum*
'White Giant'	–	*A. stipitatum*	–
'Michael H. Hoog'	–	*A. rosenbachianum*	*A. jesdianum*

1994; Hoog and Dix 1996; Rukšans 1997). The parental species suggested for these cultivars are listed in Table 1. Here, we have started in each case by testing the most likely parentage with GISH. When this did not check out, up to five other potential parents were selected on the basis of morphological characters. All of these species were then studied with random amplified polymorphic DNA (RAPD) markers to select the most probable parent for the GISH experiments. RAPD analyses (Welsh and McClelland 1990; Williams et al. 1990) are now commonly used for estimating genetic relationships among closely related populations or species and for molecular evidence concerning the hybrid origin of plants (Demeke et al. 1992; Crawford et al. 1993; Wang et al. 1994 Maaß and Klaas 1995) in spite of some incongruence in most of the RAPD data sets (Rieseberg 1996). We have used GISH to test the most likely hypotheses suggested by the RAPD data.

Materials and methods

Plant material

A total of 53 accessions of 11 species and nine cultivars from the living collection of the Department of Taxonomy of IPK, Gatersleben, belonging to the subgenus *Melanocrommyum* were investigated (Table 2).

Isolation of DNA

Total DNA was isolated by the method of Shaghai-Maroof et al. (1984) with slight modifications according to Maaß and Klaas (1995). After treatment with 10 μg/ml of RNase A for 2 h at 37°C, the DNA was purified in 3-ml CsCl gradients according to standard procedures (Sambrook et al. 1989). The purified DNA was dissolved and stored in TE buffer, and the concentration was determined fluorometrically.

Chromosome preparations

All of the taxa studied here are from desert-like habitats and have obligatory ephemeral life cycles. Young roots can be obtained from bulbs only during a limited time period between October and January.

Excised roots from the putative hybrids were kept in distilled water on ice overnight. They were then transferred to room temperature for 20 min and pre-treated for 1.5 h at room temperature in an aqueous 0.05% solution of colchicine. The tissue was fixed in a freshly prepared 3 : 1 mixture of 96% ethanol/glacial acetic acid. Preparation of root-tip spreads followed essentially the methods described by Schwarzacher et al. (1989) with some modification (Friesen et al. 1997). The fixed root-tips were partially digested with cellulase and pectolyase (4% cellulase + 1% pectolyase) for 20–40 min (the exact time had to be optimised for each species) before squashing in 45% acetic acid. Cover slips were removed after freezing with dry ice and the slides were dried. The preparations were either used immediately or else kept refrigerated for up to 2 months before in situ hybridization.

Probe preparation and in situ hybridization

Total genomic DNA from *A. hollandicum*, *A. stipitatum*, *A. macleanii*, *A. aflatunense*, *A. cristophii*, *A. karataviense*, *A. giganteum*, *A. rosenorum* and *A. rosenbachianum* was sheared by sonication to 300–500-bp fragments and labelled with biotin with the ULISIS Biotin labelling kit (Kreatech Diagnostic, LK-1102-pBIO). Total genomic DNA from the same species was fragmented to 100–200-bp pieces by autoclaving for 6 min and used as blocking DNA. (Anamthawat-Jónsson et al. 1990; Heslop-Harrison et al. 1990). In situ hybridization and probe detection followed Friesen et al. (1997). The probe mix containing approximately 40 ng of biotinylated genomic DNA, 0.4–8 μg of blocking DNA, 50% de-ionized formamide, 10% dextran sulphate, 10 μg of sonicated salmon sperm DNA, and 2 × SSC was denatured at 80°C for 10 min., and then immediately put on ice for 2–5 min. Twelve microlitres were applied to each slide and covered with a coverslip. DNA-DNA in situ hybridization was carried out overnight in a moist chamber at 37°C. After the hybridization step the slides were washed for 5 min each in 50% formamide in 2 × SSC, 2 × SSC and 1 × SSC at 42°C; or, for a stringent wash, for 5 min each in 1 × SSC, 0.5 × SSC and 0.1 SSC at 60°C. Biotinylated DNA was detected with FITS-Streptavidin and amplified once with biotinylated Antistreptavidin (Boehringer Mannheim). Chromatin was counterstained using propidium iodide. All in situ hybridizations were repeated at least once with different proportions of labelled DNA and blocking DNA, for a more reliable result.

RAPD analysis

Amplification was carried out using seven arbitrary 10-bp primers (G02, G13, G19, D03, AB04, AA17, and AC02), obtained from

Table 2 The origin of the investigated accessions (BG = Botanical Garden)

Taxon	Gatersleben accession number and its origin
A. aflatunense B. Fedt.	612 (BG Budapest), 1178 (BG Strassbourg as *A. elatum*), 1211 (BG Leningrad as *A. altissimum*), 2657 (BG Dresden as *A. altissimum*), 3692 (Kazakhstan, Tian-shan)
A. cristophii Trautv.	1920 (BG Manchester), 5253 (Turkmenistan, Kopetdag), 5262 (Turkmenistan, Kopetdag).
A. giganteum Regel	1702 (From bulb shop), 2285 (Tadjikistan, Vakhsh-Karatau), 2676 (BG Chorog), 2945 (Tajikistan, Gazimaylik), 5252 (Turkmenistan, Kopetdag), 5279 (Turkmenistan, Kopetdag).
A. hollandicum R. Fritsch	1122 (Garden origin as *A. aflatunense*), 2602 (Garden origin as *A. aflatunense*), 1631 (Garden origin as *A. aflatunense*), 1801 (Garden origin as *A. aflatunense* 'Purple Sensation'), 2615 (BG Amsterdam as *A. aflatunense* 'Purple Sensation'), 2800 (BG Frankfurt/Main as *A. aflatunense*)
A. jesdianum Boiss. et Reut.	3951 (Iran, Yazd -locus classicus).
A. karataviense Regel	3678 (Kazakhstan, Karatau), 5040 (Uzbekistan, Chatkal).
A. macleanii Baker (*A. elatum* Regel)	5455 (Hoog & Dix, Holland), 0465 (BG Amsterdam as *A. loratum*), 2218 (BG Minsk), 2531 (Tadjikistan, Hissar), 1911 (Tadjikistan), 2415 (Tadjikistan, Darvaz)
A. rosenbachianum Regel	3124 (Tadjikistan, Baldzhuan - locus classicus).
A. rosenorum R. Fritsch	2938 (Tadjikistan, Gazimaylik), 3781 (Hoog & Dix, Holland as *A. rosenbachianum* 'Michael H. Hoog'), 5081 (Uzbekistan, Hissar).
A. sarawschanicum Regel	2939 (Tadzhikistan, Gazimaylik), 2946 (Uzbekistan, Hissar), 3673 (Uzbekistan, Zaravshan).
A. stipitatum Regel	1044 (BG Göteborg as *A. altissimum*), 1311 (Tadjikistan, Hissar), 1343 (Tajikistan, Hissar), 2613 (BG Amsterdam as *A. rosenbachianum*), 2614 BG Amsterdam as *A. rosenbachianum*), 2618 (BG Amsterdam as *A. hirtifolium*), 3246 (Peter Nijsen, Heemstede as *A. sp.* 'Mount Everest'), 3670 (Uzbekistan, Kugitang), 3738 (Kasakhstan, Tian-Shan), 3782 (Hoog & Dix, Holland as *A. altissimum*),3967 (Iran, Bakhtiar), 5480 'White Giant' (Hoog & Dix, Holland), 'Mars'5135 (Hoog & Dix, Holland)
'Globus'	5134 (Hoog & Dix, Holland)
'Lucy Ball'	5136 (Hoog & Dix, Holland)
'Globemaster'	5476 (J. Bijl, Limmen, Holland) and (Hoog & Dix, Holland)
'Gladiator'	5477 (Hoog & Dix, Holland)

Operon Technologies, Alameda, California. The amplification conditions were optimized according to Maaß and Klaas (1995). One-third of the reaction mixtures was separated on 1.5% agarose gels in 0.5 × TBE, followed by staining with ethidium bromide. The presence and absence of RAPDs was assessed only among samples on the same gel. The DNA profiles were scored manually, directly from photographs of the gels, by assigning a value of 1 for band presence and 0 for band absence. The scores of band presence or absence were then used to calculate a pairwise genetic distance matrix using different coefficients. Finally, a phenogram based on UPGMA cluster analysis of the genetic distance matrix was prepared with help of the NTSYS-pc program (Applied Biostatistic Inc. New York, 1993, 1.8 version). Similarity of RAPD-patterns was determined by the calculation of *F*-values [twice the number of shared bands in two plants, divided by the total number of bands in the two plants (Kearsey and Pooni 1996)].

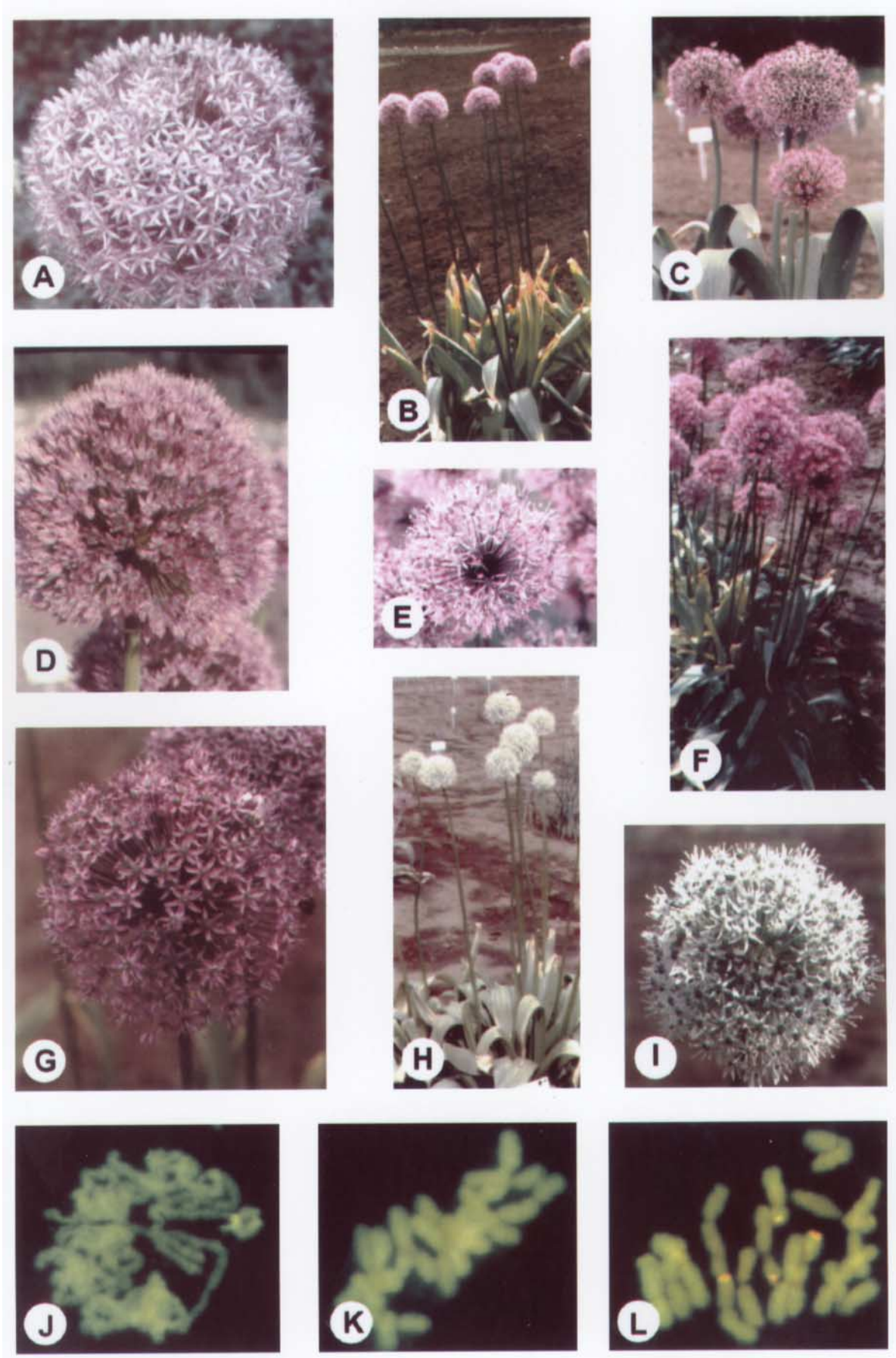
Morphological description of the putative hybrids

'Globemaster' grows into impressive plants with a thick, smooth, glossy scape 1–2-cm in diameter and 70–100-cm long. The leaves are smooth, glossy, basally somewhat narrowed, 40–60-cm long and 5–12-cm broad, somewhat yellowish green. The broadly-orbicular inflorescence is very dense, 10–15-cm in diameter, with flattened star-like, lilac to purplish flowers 2.5–3-cm in diameter. The petals are lanceolate, rather weak in consistency, with a prominent brownish-green midvein and a somewhat rounded apex. After bloom they are folded lengthwise, somewhat crumpled, and obliquely forward directed. This completely sterile cultivar combines the vegetative appearance of a stout *A. macleanii* with some inflorescence and flower characters of *A. cristophii* (Fig. 1 A).

'Globus' plants are compact with smooth, glaucous scapes 40–50-cm long and 1–1.5-cm in diameter. The lanceolate, completely smooth leaves are 35–50-cm long, 3–8-cm broad, and very glaucous. The rather dense inflorescence is initially flat und becomes orbicular only towards the end of bloom. Its pinkish flowers are cup-shaped with rather broadly lanceolate petals which are more or less reflexed and crumpled after bloom. The shape and position of the leaves, the shape of the inflorescence, and the shape as well as the structure of the flowers correspond very much better to *A. stipitatum* than to *A. giganteum* as parental species. It seems much more probable that *A. karataviense*, rather than *A. cristophii*, was the second parent donating the broad, glaucous and smooth leaves, the short scape, and the cup-shaped flowers with short petals of this sterile cultivar (Fig. 1 C).

'Gladiator' plants have a smooth scape up to 120-cm long, somewhat hairy, glaucous leaves 50–75-cm long and 4–9-cm broad, and a very dense, broadly orbicular inflorescence 10–15-cm in diameter with purplish flowers. The lanceolate petals are slightly bent inwards; after bloom they are rolled up spirally and crumpled. This sterile cultivar shows several typical characters of *A. stipitatum*, though the inflorescence is very dense, and the leaves as well as the petals are shorter and broader. These characters could have been contributed by *A. giganteum* or *A. macleanii* as the possible second parent (Fig. 1 B).

'Lucy Ball' also displays a rather compact general shape even though the (basally) slightly ribbed scape is 70–100-cm high. The glossy, yellowish-green leaves are 50–70-cm long and 4–12-cm broad. Their lower two-thirds are rather straight and stiff, whereas the upper third is flaccid and hangs down. The orbicular, initially basally flattened inflorescence is rather dense with pinkish-carmine flowers on stiff pedicels of equal length. After bloom the broadly lanceolate petals are crumpled, but only slightly reflexed and not rolled in. The general appearance is similar to *A. hollandicum*, but with a bigger and denser flower head, cup-shaped flowers, and more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prominent leaves of different colour. These characters could well have been contributed by *A. macleanii* as the second parent. This cultivar is also sterile (Fig. 1 D).

'Mars' shows most characters very similar to 'Gladiator' but has 5–15-cm broad and more-erect leaves together with lanceolate, strikingly longer and somewhat broader petals. These characters could have been provided by the crossing of *A. stipitatum* with the very closely related *A. aflatunense* s. str. Both taxa are known to cross easily and to give fertile offspring. 'Mars' sets seed easily (Fig. 1 G).

'White Giant' (Fig. 1 H) and 'Mount Everest' (Fig. 1 I) are extremely similar to one another and share most of the above-mentioned vegetative and generative characters with 'Gladiator', with the exceptions of a stronger leaf indumentum, a somewhat smaller inflorescence, and greenish-white flowers with narrower petals. Both cultivars are fertile and cannot be distinguished from naturally occurring albinotic forms of *A. stipitatum*.

'Michael H. Hoog' plants are rather slender with a 100–120-cm long, densely ribbed scape and canaliculate, smooth, arcuately recurved, 45–60-cm long, but only 1.5–3-cm broad, leaves. The inflorescence is moderately dense and orbiculate with pinkish-lilac, star-like flowers and very narrow, slightly incurved petals. This fertile cultivar belongs clearly to *A. rosenorum* and seems specially to have been selected for tall plants with an intensive flower colour (Fig. 1 E).

'Purple Sensation' plants show a compact habit with a 50–70-cm long, basally slightly ribbed scape and rather straight leaves pointing foreward, which are 25–35-cm long and 2–4-cm broad. These characters are typical for *A. hollandicum*. This fertile cultivar impresses by its cup-shaped, deep-purple flowers arranged in moderately dense, orbiculate heads. Other forms of *A. hollandicum* show somewhat denser and basally flattened inflorescences and more broadly funnel-shaped flowers of different pink shades. A more loose inflorescence and the rather incurved shape, as well as the purple colour of the petals, could have been introduced by crossing with *A. altissimum* (Fig. 1 F).

Results

'Globemaster'

GISH distinguished eight chromosomes of *A. cristophii* as the parental genome (yellow fluorescence, Fig. 2 A, B) using biotin-labelled genomic DNA of *A. cristophii* (Tax 5253 and Tax 2005) as a probe and DNA of *A. macleanii* (Tax 5455) as the blocking DNA. The same was true for the genome of *A. macleanii* (yellow fluorescence, Fig. 2 C) using biotin-labelled genomic DNA from *A. macleanii* (Tax 5455) as a probe and DNA of *A. cristophii* (Tax 5253) as blocking DNA. This confirms the contributions of both proposed parents.

Fig. 1A–L **A** 'Globemaster'; **B** 'Gladiator; **C** 'Globus'; **D** 'Lucy Ball'; **E** 'Michael H. Hoog'; **F** 'Purple Sensation'; **G** 'Mars'; **H** 'White Giant'; **I** 'Mount Everest'; **J–L** Fluorescent photomicrographs of root-tip metaphase spread: 'Mars'; 'Mount Everest' and White Giant' after GISH using biotin-labelled genomic DNA from *A. stipitatum* (Tax 1044) as a probe and a mixture of DNA of three different species *A. aflatunense* (Tax 612), *A. hollandicum* (Tax 1122) and *A. rosenorum* (Tax 5132), as blocking DNA

'Globus'

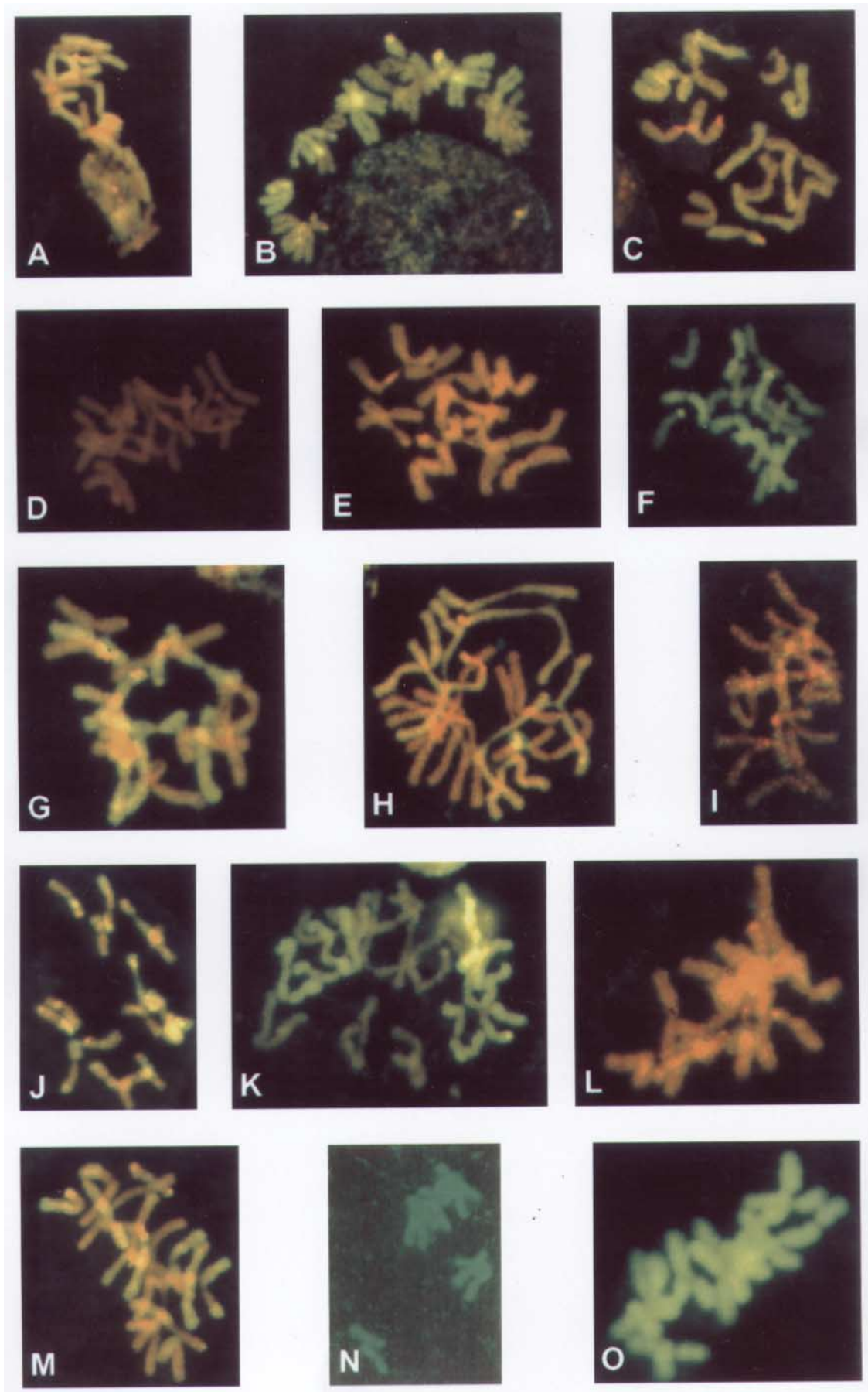
GISH showed no hybridization signals (Fig. 2 D, E) using biotin-labelled genomic DNA from *A. giganteum* (Tax 5279) as a probe and DNA of *A. cristophii* as blocking DNA, nor when using biotin-labelled genomic DNA from *A. cristophii* (Tax 5253) as a probe and DNA of *A. giganteum* (Tax 5279) as blocking DNA. Thus, *A. giganteum* and *A. cristophii* were not the parental species of this cultivar. RAPD products of *A. aflatunense*, *A. altissimum*, *A. stipitatum*, *A. macleanii*, *A. hollandicum*, *A. rosenorum*, *A. karataviense*, and of 'Globus' with five Operon primers (G13, G19, D03, AB04, and AC02) showed *A. stipitatum* and *A. karataviense* to have the most similar amplification fragments with 'Globus'. These species were included in the GISH study, which distinguished eight parental chromosomes from the *A. stipitatum* genome (Fig. 2 F) using biotin-labelled genomic DNA from *A. stipitatum* (Tax 5279) as a probe and DNA of *A. karataviense* (Tax 5040) as the blocking DNA. Also the nine parental chromosomes from *A. karataviense* were distinguished using biotin-labelled genomic DNA from *A. karataviense* (Tax 5040) as a probe and DNA of *A. stipitatum* (Tax 1044) as blocking DNA.

'Gladiator'

Eight parental chromosomes from *A. hollandicum* (yellow fluorescence) could be distinguished using biotin-labelled genomic DNA from *A. hollandicum* (Tax 1122) as a probe and DNA of *A. macleanii* (Tax 5455) as blocking DNA (Fig. 2 H). However, GISH did not distinguish any chromosome if biotin-labelled genomic DNA from *A. macleanii* (Tax 5455, as a probe) and DNA of *A. hollandicum* (Tax 1122, as blocking DNA) are used (Fig. 2 I). GISH was only able to distinguish some chromosomes to be related to but not fully homologous with the *A. macleanii* genome when biotin-labelled genomic DNA from another accession of *A. macleanii* (Tax 465) was used as a probe and DNA of *A. hollandicum* (Tax 1122) as blocking DNA (Fig. 2 J).

'Lucy Ball'

We obtained similar results as with 'Gladiator', for which the same parental species were proposed. Eight parental chromosomes from *A. hollandicum* (yellow fluorescence) were distinguished (Fig. 2 K) using biotin-labelled genomic DNA from *A. hollandicum* (Tax 1122) as a probe and DNA of *A. macleanii* (Tax 5455) as blocking DNA. GISH showed only sporadic hybridization signals and did not distinguish any chromosomes (Fig. 2 L) when biotin-labelled genomic DNA from *A. macleanii* (Tax 5455) was used as a probe with DNA of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A. hollandicum as the blocking DNA. Some chromosomes and fragments related to, but not fully homologous with the *A. macleanii* genome were distinguished using biotin-labelled genomic DNA from another accession of *A. macleanii* (Tax465) as a probe and DNA of *A. hollandicum* (Tax 1122) as blocking DNA (Fig. 2 M).

GISH did not give any hybridization signal when another possible parent, *A. giganteum*, was tested. RAPD screening of several species which could also be suggested as the second parental species of these cultivars, using more than 20 randomly selected 10-mer primers, showed that, apart from *A. hollandicum*, only *A. macleanii* is more or less related to these cultivars.

All available accessions of *A. macleanii*, six selected accessions of *A. hollandicum*, and single accessions of 'Purple Sensation', 'Gladiator' and 'Lucy Ball' were investigated with seven Operon primers for more precise elucidation of possible parental accessions. A total of 122 DNA fragments were amplified, 97 of which (80.1%) were polymorphic. Relative to 'Lucy Ball' and 'Gladiator', RAPD fragments of *A. hollandicum* accessions showed between 43.3% and 56.4% similarity, whereas our *A. macleanii* accessions gave only between 21.3% and 34.9% similarity (Table 3). A dendrogram based on UPGMA cluster analysis of the RAPD data showed three clearly distinct groups: *A. macleanii*, *A. hollandicum*, and the cultivars (Fig. 3). All *A. hollandicum* accessions, including 'Purple Sensation', are more homogeneous than our accessions of *A. macleanii*. 'Lucy Ball' and 'Gladiator' are placed between *A. hollandicum* and *A. macleanii*, but closer to *A. hollandicum.*

Fig. 2A–O **A–C** Fluorescent photomicrographs of the 'Globemaster' chromosomes after GISH: **A**, **B** using biotin-labelled genomic DNA from *A. cristophii* (A -Tax 5253 and B Tax 2005) as a probe and DNA of *A. macleanii* (Tax 5455) as blocking DNA. GISH distinguishes parental genomes from *A. cristophii* (8 yellow chromosomes). **C** using biotin-labelled genomic DNA from *A. macleanii* (Tax 5455) as a probe and DNA of *A. cristophii* (Tax 5253) as blocking DNA. GISH distinguishes parental genomes from *A. macleanii* (8 yellow chromosomes); **D–G** Fluorescent photomicrographs of the 'Globus' chromosomes after GISH: **D** using biotin-labelled genomic DNA from *A. giganteum* (Tax 5279) as a probe and DNA of *A. cristophii* as blocking DNA; GISH shows no hybridization. **E** using biotin-labelled genomic DNA from *A. cristophii* (Tax 5253) as a probe and DNA of *A. giganteum* (Tax 5279) as blocking DNA. GISH shows no hybridization. **F** using biotin-labelled genomic DNA from *A. stipitatum* (Tax 5279) as a probe and DNA of *A. karataviense* (Tax 5040) as blocking DNA; GISH distinguishes parental genomes from *A. stipitatum* (8 yellow chromosomes). **G** using biotin-labelled genomic DNA from *A. karataviense* (Tax 5040) as a probe and DNA of *A. stipitatum* (Tax 1044) as blocking DNA; GISH distinguishes parental genomes from *A. karataviense* (9 yellow chromosomes); **H–J** Fluorescent photomicrographs of 'Gladiator'(Tax 5477) chromosomes after GISH: **H** using biotin-labelled genomic DNA from *A. hollandicum* (Tax 1122) as a probe and DNA of *A. macleanii* (Tax 5455) as blocking DNA; GISH distinguishes parental genomes from *A. hollandicum* (8 yellow chromosomes). **I** using biotin-labelled genomic DNA from *A. macleanii* (Tax 5455) as a probe and DNA of *A. hollandicum* (Tax 1122) as blocking DNA; GISH does not distinguish any chromosomes. **J** using biotin-labelled genomic DNA from another accession of *A. macleanii* (Tax 465) as a probe and DNA of *A. hollandicum* (Tax 1122) as blocking DNA; GISH distinguishes some chromosomes related to the *A. macleanii* genome, which are not fully homologous; **K–L** Fluorescent photomicrographs of the 'Lucy Ball' (Tax 5136) chromosomes after GISH. **K** using biotin-labelled genomic DNA from *A. hollandicum* (Tax 1122) as a probe and DNA of *A. macleanii* (Tax 5455) as blocking DNA; GISH distinguishes parental genomes from *A. hollandicum* (8 yellow chromosomes). **L** using biotin-labelled genomic DNA from *A. macleanii* (Tax 5455) as a probe and DNA of *A. hollandicum* as blocking DNA; GISH shows only spoard hybridization signals and does not distinguish any chromosomes. **M** using biotin-labelled genomic DNA from other accession of *A. macleanii* (Tax 465) as a probe and DNA of *A. hollandicum* (Tax 1122) as blocking DNA; GISH distinguishes some chromosomes and fragments related to the *A. macleanii* genome, which are not fully homologous. **N** Fluorescent-photomicrographs of a root-tip metaphase spread of the 'Purple Sensation' after GISH using biotin-labelled genomic DNA from *A. hollandicum* (Tax 2800) as a probe and mixture of DNA of three different species *A. macleanii* (Tax 5455), *A. stipitatum* (Tax 1044) and *A. rosenbachianum* (Tax 3126), as blocking DNA; **O** Fluorescent photomicrograph of a root-tip metaphase spread of the 'Michael H. Hoog' after GISH using biotin-labelled genomic DNA from *A. rosenorum* (Tax 5232) as a probe and mixture of DNA of two different species, *A. rosenbachianum* (Tax 3124) and *A. jesdianum*, (Tax 3951) as blocking DNA

'Purple Sensation'

All chromosomes gave strong hybridization signals using biotin-labelled genomic DNA from *A. hollandicum* (Tax 2800) as a probe and a DNA-mixture of *A. macleanii* (Tax 5455), *A. stipitatum* (Tax 1044), and *A. rosenbachianum* (Tax 3126) as blocking DNA (Fig. 2 N). Using biotin-labelled genomic DNA from *A. macleanii*, *A. stipitatum* or *A. rosenbachianum* as a probe and DNA of *A. hollandicum* as blocking DNA did not produce any hybridization signals. According to GISH, 'Purple Sensation' has no other genome than that of *A. hollandicum.* RAPD data confirm this conclusion (Fig. 3).

'Michael H. Hoog'

All chromosomes had strong hybridization signals after GISH using biotin-labelled genomic DNA of *A. rosenorum* (Tax 5232) as a probe and a mixture of DNA of *A. rosenbachianum* (Tax 3124) and *A. jesdianum* (Tax 3951) as blocking DNA (Fig. 2 O). Using biotin-labelled genomic DNA from *A. rosenbachianum* or of *A. jesdianum* s. str as probes and DNA of *A. rosenorum* as blocking DNA gave no hybridization signals. 'Michael H. Hoog' seems to be derived directly from *A. rosenorum.*

'Mars', 'Mount Everest' and 'White Giant'

All chromosomes gave strong hybridization signals using biotin-labelled genomic DNA from *A. stipitatum* (Tax 1044) as a probe and a mixture of DNA of *A. aflatunense* (Tax 612*)*, *A. hollandicum* (Tax 1122), and

Table 3 Similarity of RAPD-paterns of 14 accessions of *A. macleanii*, *A. hollandicum* and hybrids, calculated in terms of *F*-values

Acc.	0465	1911	2218	2415	2531	5455	5136	5477	1122	1801	2602	1631	2615	2800
0465	100													
1911	48.9	100												
2218	47.3	59.1	100											
2415	49.1	54.3	58.0	100										
2531	50.9	59.7	59.6	64.7	100									
5455	76.6	53.2	53.8	52.8	54.5	100								
5136	26.9	27.4	30.7	34.9	33.3	25.8	100							
5477	25.0	22.2	24.4	21.3	28.7	23.8	63.6	100						
1122	13.9	15.8	14.3	11.5	16.1	13.9	54.6	56.2	100					
1801	10.5	09.1	08.2	05.8	08.9	09.3	43.3	45.8	69.2	100				
2602	07.8	10.2	09.3	06.7	10.0	07.9	45.6	48.2	69.7	77.1	100			
1631	09.8	11.1	11.4	08.7	11.9	09.9	53.4	54.8	78.5	77.8	80.9	100		
2615	09.2	10.5	09.5	06.9	10.2	08.1	46.6	49.4	71.8	82.7	89.5	86.7	100	
2800	10.9	11.1	12.8	09.9	12.1	09.8	50.6	50.0	68.3	75.8	73.3	77.1	78.9	100

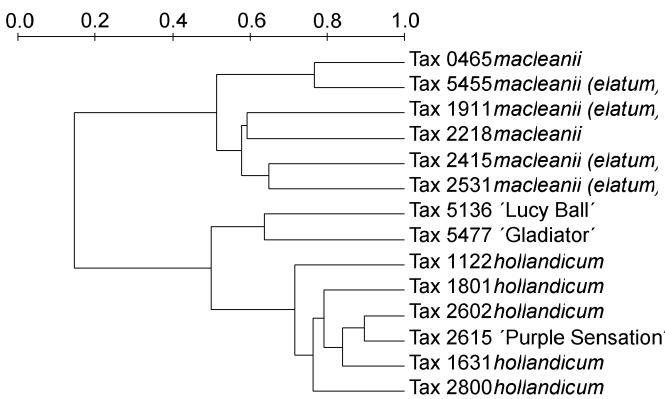

Fig. 3 UPGMA dendrogram (Jaccard coefficient) of six accessions of *A. macleanii*, five accessions of *A. hollandicum* and three cultivars, based on 122 polymorphic RAPD markers

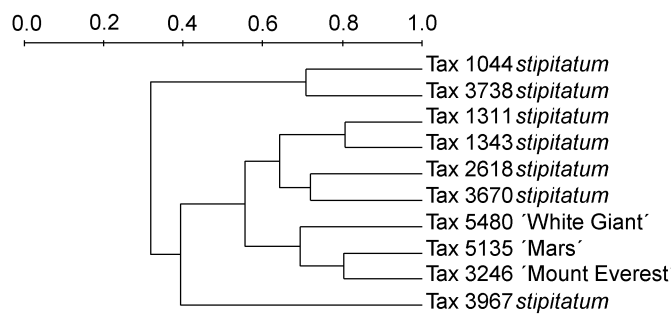

Fig. 4 UPGMA dendrogram (Jaccard coefficient) of seven accessions of *A. stipitatum* and three cultivars, based on 124 polymorphic RAPD markers

A. rosenorum (Tax 5132) as blocking DNA (Figs. 1 J–L). Using biotin-labelled genomic DNA from *A. hollandicum*, *A. aflatunense* or *A. rosenorum* as probes and DNA of *A. stipitatum* as blocking DNA, produced only scattered and weak signals. Thus, these three cultivars belong to *A. stipitatum*.

RAPD data showed the same result. DNA of eight different accessions of *A. stipitatum* and of single accessions of 'Mars', 'Mount Everest', and 'White Giant' were amplified with seven operon primers of arbitrary sequences. A total of 124 DNA fragments were amplified. The similarity of RAPD fragments between *A. stipitatum* accessions and these cultivars was between 30.3% and 57.8%, whereas the similarity between *A. stipitatum* accessions was much lower (Table 4). Thus the cultivars falls inside the general variability of the species. In the UPGMA dendrogram, based on 124 polymorphic RAPD markers, the cultivars were placed among the other *A. stipitatum* accessions (Fig. 4). In an

Table 4 Similarity of RAPD-paterns of seven accessions of *A. stipitatum* and three cultivars calculated in terms of *F*-values

Acc.	1044	1311	1343	2618	3670	3738	3967	5480	5135	3246	5134
1044	100										
1311	21.7	100									
1343	23.3	80.8	100								
2618	22.4	61.7	67.2	100							
3670	24.4	61.0	66.7	71.9	100						
3738	70.8	30.0	31.6	32.1	36.4	100					
3967	30.4	36.9	40.8	37.3	40.2	32.5	100				
5480	30.4	57.4	54.8	57.1	56.5	35.9	39.7	100			
5135	34.1	47.1	51.5	63.1	52.9	46.7	39.7	64.1	100		
3246	35.0	55.3	57.8	60.0	54.5	44.2	40.8	74.6	80.3	100	
5134	20.7	15.0	19.5	21.8	20.8	24.4	15.3	23.7	23.2	23.7	100

analysis including *A. hollandicum* as an outgroup, the topology of the groups remains unchanged.

Discussion

The parental species of the cultivars investigated in this paper have their natural habitats in Southwest to Central Asia. Among the species discussed, *A. stipitatum* covers nearly the whole area from the Tian-Shan range in the Northeast and the Pamir and Hindukush ranges in the East, to the high mountainous areas in eastern and central Iran. It is therefore not surprising that this taxon displays great morphological diversity. Similarly, *A. karataviense* is known to have a scattered distribution over an area roughly between 38–46° N and 68–76°E. Morphologically, this taxon is no less variable. *A. giganteum* is distributed from the eastern shelf of the Caspian Sea along the Kopetdag and Paropamis ridges and along the classical 'Oxus depression' to sub-montane parts of the Hissar, Pamir and Hindukush ridges. It is to be expected that the morphological variation of these species finds its counterpart in molecular variation of the nuclear DNA. Other species such as *A. macleanii* (including its broad-leafed variant *A. elatum*), which grows in montane areas of the Pamir-Alaian and Hindukush ridges, or *A. rosenorum*, from sub-montane slopes of the Hissar ridge, show a much smaller area of distribution. However, even here we cannot be sure that the accessions we used for GISH and the parental accessions of the breeders have an identical genetical background. Our data strongly underline that GISH alone works selectively enough to discriminate the genome of distantly related taxa (see below under 'Globemaster' and 'Globus'). GISH also successfully discriminates between more closely related taxa if accessions genetically identical to the parental ones can be used (see below under 'Gladiator' and 'Lucy Ball'). If the genetical identity of the tester strains and the parental taxa remains questionable, GISH in combination with RAPD data proved to be a more secure basis for decisions because the two methods complement each other.

'Globemaster' has been correctly interpreted as a hybrid by its breeder. This hybrid cultivar approaches the ideal of the breeder: nearly all favorable characters of both parental species can be combined in one plant, which in addition shows positive heterosis for plant stature and the shape of the petals. The cultivar is also ideal for cytogenetics: both parental genomes are apparently still complete and have remained karyotypically unchanged.

'Globus', in contrast, appears to have been initially misinterpreted, and it took karyological analysis to suggest the correct interpretation by pointing to *A. karataviense* as a probable parental species (Rukšans 1996). The original choice of *A. giganteum* as a possible parent species may have been the result of wrong taxonomic identification because this name is sometimes erroneously used for *A. stipitatum* both in the trade and by gardens. However, the general acceptance of *A. christophii* as the other parent is difficult to explain since there is no trace of the flower and petal characters of *A. christophii* in this cultivar.

Until now the second parental species of 'Gladiator' could not be satisfactorily identified by GISH. It remains difficult to explain the origin of the hairy leaves if *A. hollandicum* and a species closely related to *A. macleanii*, both with smooth leaf margins, were the parents of this cultivar. One possible reason is that the accession of *A. macleanii* that we used here differs in some essential intraspecific character polymorphisms from the true parental plant of this cultivar. On the other hand, we cannot exclude that the parental plant of *A. macleanii* was itself a hybrid with a third species that we have not yet been able to trace. GISH only indicated homology of several chromosome sections of 'Gladiator' with *A. macleanii* (Fig. 2 J), and we obtained similar results with 'Lucy Ball' for which the same parental species were proposed (Fig. 2 M).

The genetical inclusion of 'Purple Sensation' among other strains of *A. hollandicum*, despite its clear morphological differences, was an unexpected result. Most probably this cultivar is really a variety of that species, because our accession of *A. stipitatum*, which represents "*A. altissimum*" of the Dutch bulb trade, gave weak GISH signals with all 16 chromosomes and therefore excludes that species as one of the parents of 'Purple Sensation' unless a very different Dutch accession of *A. altissimum* has taken part in the breeding of 'Purple Sensation'.

'Mount Everest' and 'White Giant' were verified as belonging to *A. stipitatum* by both GISH (Figs. 1 K, L) and RAPD data (Fig. 4). The same is true with 'Mars', for which no hybridisation with another taxon could be traced (Fig. 1 J). Our tested accessions of *A. hollandicum* and *A. aflatunense* showed only scattered and weak signals with all of its chromosomes. However, it is not impossible that 'Mars' goes back to an introgression at least two or three generations earlier.

Conclusions

The combination of GISH analysis with RAPD markers is very suitable for providing evidence on the hybridogenic origin of plants and to ascertain the parental species of hybrids. Employing this approach we conclude that:

(1) 'Globemaster': *A. macleanii* and *A. cristophii* are the supposed parental species of this cultivar.

(2) 'Globus': the real parental species are *A. karataviense* and *A. stipitatum*, and not *A. cristophii* and *A. giganteum* as has been assumed.

(3) 'Lucy Ball' and 'Gladiator': both cultivars are of hybrid origin, though only one of the supposed parental species, *A. hollandicum*, could be confirmed. It remains questionable whether the second supposed parental species, *A. macleanii*, has been involved.

(4) 'Purple Sensation', 'Mount Everest', 'White Giant', 'Michael H. Hoog' and 'Mars': these cultivars are not hybrids as has been supposed by some. GISH and RAPD was not able to indicate any second genome. 'Purple Sensation' belongs to *A. hollandicum*, 'Mount Everest', 'White Giant' and 'Mars' to *A. stipitatum*, and 'Michael H. Hoog' to *A. rosenorum*.

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