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Published in:
Theoretical and Applied Genetics

DOI:
10.1007/s001220050686

Citation for published version (APA):

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N. Friesen · R. Fritsch · K. Bachmann

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

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...
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.

### 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-Maroo 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

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<th>Parental species according to:</th>
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<td>‘Michael H. Hoog’</td>
<td>–</td>
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#### 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. rosenorm* 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. (Anam-thawat-Jönsson 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
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 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 absence of RAPDs was assessed only among samples on the same gel.

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-ovulose 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 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. hollanicum, but with a bigger and denser flower head, cup-shaped flowers, and more opercula.
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 albinoic forms of *A. stipitatum*.

‘Michael H. Hoog’ plants are rather slender with a 100–120-cm long, densely ribbed scape and canaliculate, smooth, arcately 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 forward, 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. christophii* as the parental genome (yellow fluorescence, Fig. 2 A, B) using biotin-labelled genomic DNA of *A. christophii* (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. christophii* (Tax 5253) as blocking DNA. This confirms the contributions of both proposed parents.

‘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. christophii* as blocking DNA, nor when using biotin-labelled genomic DNA from *A. christophii* (Tax 5253) as a probe and DNA of *A. giganteum* (Tax 5279) as blocking DNA. Thus, *A. giganteum* and *A. christophii* were not the parental species of this cultivar. RAPD products of *A. aflatunense, A. altissimum, A. stipitatum, A. macleanii, A. hollandicum, A. rosenorum, A. karataviensis*, and of ‘Globus’ with five Operon primers (G13, G19, D03, AB04, and AC02) showed *A. stipitatum* and *A. karataviensis* 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. karataviensis* (Tax 5040) as the blocking DNA. Also the nine parental chromosomes from *A. karataviensis* were distinguished using biotin-labelled genomic DNA from *A. stipitatum* (Tax 1044) 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
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.

‘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. rosenorum, A. stipitatum or A. rosenbachianum as a probe 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-patterns of 14 accessions of *A. macleanii*, *A. hollandicum* and hybrids, calculated in terms of *F*-values

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

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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

*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
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* occurs in the Northeast and the Pamir and Hindukush ranges, or in the 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 genetic 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 ‘Globemaster’ and ‘Lucy Ball’). If the genetic 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 apparent 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 (Ruksans 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 hybrid with a third species that we have not yet been able to trace. 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, *Allium hollancicum*, 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. hollancicum*, ‘Mount Everest’, ‘White Giant’ and ‘Mars’ to *A. stipitatum*, and ‘Michael H. Hoog’ to *A. rosenorum*.

Acknowledgements We thank P. Hanelt and F. Blattner for their valuable comments on the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (DFG) projekt HA 1848/3–1 for N. Friesen.

References


