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Allotetraploid origin of *Allium altyncolicum* (*Alliaceae*, *Allium* sect. *Schoenoprasum*) as investigated by karyological and molecular markers*

Nikolai Friesen, Nikolai Borisjuk, Ted H. M. Mes, Manfred Klaas, and Peter Hanelt

Received August 23, 1996; in revised version December 9, 1996

Key words: *Alliaceae, Allium altyncolicum, A. ledebourianum, A. schoenoprasum.* – Allopolyploidy, C-banding, GISH, ITS sequencing, PCR-RFLP of cpDNA, RFLP mapping of rDNA, RAPD.

Abstract: The tetraploid *Allium altyncolicum* ($2n = 4x = 32$) is considered to be of hybrid origin, because most of its morphological characters are intermediate between those of its putative parents, *A. schoenoprasum* and *A. ledebourianum*. In the present work an attempt has been made to ascertain its parentage by several methods: Giemsa C-banding, genomic in situ hybridization (GISH), PCR-RFLP of cpDNA, restriction enzyme mapping of the rDNA, and RAPDs. C-banding and GISH indicates clearly that *A. altyncolicum* is a segmental allopolyploid. *Allium schoenoprasum* and *A. ledebourianum* are the most likely the parental species and the larger part of the genome of *A. altyncolicum* (26 chromosomes) is derived from *A. schoenoprasum*. The low genetic divergence between these three species was confirmed by the lack of sequence variation in the ITS sequences of nuclear rRNA genes and of the plastid rbcL-atpB intergenic spacer. Both parental species and *A. altyncolicum* could be distinguished by RFLP of the rDNA repeats. The geographic origin of the putative parental species was investigated using RAPDs.

Interspecific hybridization and polyploidy are the most distinctive and widespread modes of speciation in higher plants. Thirty to 70% of all plant species, among them a number of crop plants, have been estimated to have polyploidy in their lineages (Grant 1981). The success of polyploid species has been attributed to their ability to survive better in unstable climates compared to their diploid progenitors (Ehrendorfer 1980), presumably because of their increased heterozygo-

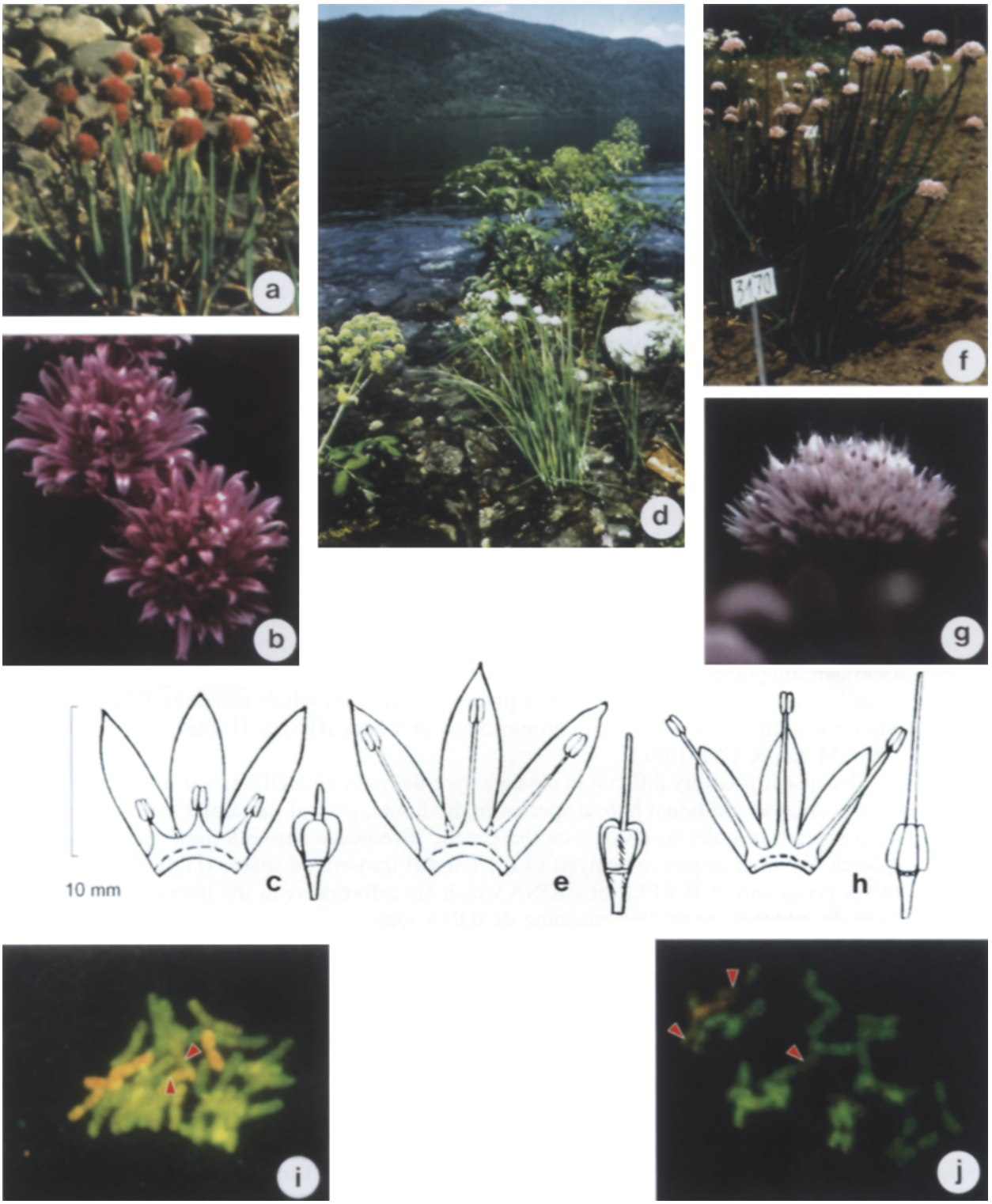
* Dedicated to emer. Univ.-Prof. Dr Friedrich Ehrendorfer on the occasion of his 70th birthday

sity and the flexibility provided by the presence of additional alleles (TAL 1980, ALLARD & al. 1993).

One of the polyploid species of *Allium* sect. *Schoenoprasum* is *A. altyncolicum* (2n = 4x = 32) which evolved after the last ice age. It is a fully fertile species with a narrow distribution on the pebbled and rocky shores at Teletskoe Lake and along the banks of the Tom river in the Altai mountains (Fig. 1d,e).

The taxonomic history of the tetraploid species *A. altyncolicum* is very complicated. Originally, the Russian botanist P. N. KRYLOV (1912) described this taxon as *A. ledebourianum* var. *intermedium* and proposed its hybrid origin, with the putative parental species *A. ledebourianum* and *A. schoenoprasum*. In 1922 the Swedish botanist TURESSON collected a tetraploid *A. schoenoprasum* in the Altai at the Teletskoe Lake (TURESSON 1931, 1966). At the same time, ALBERT LEVAN began cytological studies of *A. schoenoprasum* and investigated also the tetraploid plant from the Altai mountains (LEVAN 1936). LEVAN found that the tetraploid plants are autotetraploids, because in meiosis this species has some identical quadrivalents. Almost all European botanical gardens received seeds of this taxon from TURESSON. The exact origin "Teletskoe Lake" was generalized into "Siberia". This was the reason for the erroneous conclusion that tetraploid *A. schoenoprasum* should be widespread in Siberia (STEARN 1980, TATLIOGLU 1993). In botanical gardens, these tetraploid plants had been given the following names: *A. sibiricum, A. schoenoprasum* var. or subsp. *sibiricum, A. ledebourianum* or *A. schoenoprasum,* even though they differed from typical European specimens of *A. schoenoprasum* (FRIESEN 1996). The number of chromosomes of all other taxa of sect. *Schoenoprasum* from Siberia is 2n = 16. Tetraploid plants where found only in the material from Teletskoe Lake (FRIESEN 1985, 1987, 1988; AGAPOVA & al. 1990). These tetraploid plants were morphologically different from *A. schoenoprasum.* Most of their characters are intermediate between *A. schoenoprasum* (Fig. 1a–c) and another Altaian species, *A. ledebourianum* (Fig. 1f–h), and these plants were finally described as a new hybrid species *A. altyncolicum* (FRIESEN 1987). *Allium schoenoprasum* is widespread naturally in Eurasia and North America while *A.*

Fig. 1. *a–c Allium schoenoprasum*; *a* plants at a natural stand in Siberia (photo by N. FRIESEN), *b* inflorescences, *c* flower dissection. *d–e A. altyncolicum; d* plants in Altai at Teletskoje Lake (Photo K. PISTRICK), *e* flower dissection. *f–h A. ledebourianum*; *f* plants in the living collection in Gatersleben (photo by N. FRIESEN), *g* inflorescence, *h* flower dissection. *i* Root tip metaphase chromosome preparations of *A. altyncolicum* after GISH using biotin-labelled total genomic DNA from *A. schoenoprasum* (Tax 9/8) as a probe and DNA of *A. ledebourianum* (Tax 3173) as blocking DNA (1:40). GISH distinguished at least five parental chromosomes of *A. ledebourianum* (pink fluorescence) while the other chromosomes are more closely related to the *A. schoenoprasum* genome (yellow-green fluorescence). *j* Root tip metaphase chromosome preparations of hybrid (21b) after GISH using biotin-labelled total genomic DNA from *A. schoenoprasum* as a probe and DNA of *A. ledebourianum* as blocking DNA (1:40). GISH distinguished one satellite parental chromosome of *A. ledebourianum* (pink fluorescence) and three other chromosomes with a more faint signal. The other chromosomes probably have a high sequence similarity to the *A. schoenoprasum* genome. Probe and chromosomes were denatured together


a
b
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f
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10 mm
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ledebourianum is restricted to a narrow natural area in the West Altai mountains. Both species share a common habitat in the Altai mountains.

On the basis of morphology, geography and RAPD variation, accessions of *A. oliganthum*, *A. maximowiczi* and *A. karelinii* were rather distantly related to the accessions of the other three species from the Altai mountains (unpubl. results). Therefore, we have not included these species in our more detailed investigations.

In the present work an attempt has been made to test the hybrid origin of *A. altyncolicum* assuming the very closely related species *A. schoenoprasum* and *A. ledebourianum* as its parents. Primarily cytological methods, Giemsa C-banding and genomic in situ hybridization (GISH), were used to solve this problem.

Giemsa C-banding is established as a cytological technique which can be of considerable value in providing evidence for species relationships (GREILHUBER & SPETA 1976, 1978; VOSA 1976; GREILHUBER 1984) and for evidence of the hybrid origin of some *Allium* species (FISKESJÖ 1975, ADANIYA & al. 1978, SCHUBERT & al. 1983, PUIZINA & PAPES 1996). C-banding patterns in *A. schoenoprasum* were described by VOSA (1976) for European plants, by CAI & CHINNAPPA (1987) and TARDIF & MORISSET (1991) from North American plants. These studies produced very different degrees of staining, without producing well distinguished patterns (TARDIF & MORISSET 1991).

Genomic in situ hybridization (GISH) is a powerful tool for investigating genome homology between polyploid species and their diploid progenitors. In the field of plant molecular cytogenetics, this technique was used to detect parental genomes in artificial hybrids (SCHWARZACHER & al. 1989, 1992; HESLOP-HARRISON & al. 1990; LEITCH & al. 1991; PAROKONNY & al. 1992; HIZUME 1994; KELLER & al. 1996), in allopolyploids (BENNETT & al. 1992, MUKAI & al. 1993) and in a wild grass hybrid (BAILEY & al. 1993). As a physical marker for whole genomes, GISH has been used to identify alien chromosomes in wheat (HESLOP-HARRISON & al. 1990; MUKAI & GILL 1991).

Because it was very difficult to obtain a specific result with GISH, and since we plan to analyse additional hybrid species in the future, several molecular methods were applied in order to investigate the genetic divergence between these *Allium* species. We used sequence analysis of the internal transcribed spacer (ITS) of the rRNA genes and PCR-RFLP of cpDNA which are informative at the interspecific level. In addition, restriction mapping of rDNA repeats and RAPD analysis was carried out for characterization at the "population" level to determine the genetic divergence of our accessions.

Material and methods

Plant material. A total of 21 accessions of three species from sect. *Schoenoprasum*, *A. altyncolicum*, *A. ledebourianum* and *A. schoenoprasum* from the living collection of the Department of Taxonomy of IPK Gatersleben and three triploid artificial hybrids between *A. schoenoprasum* and *A. altyncolicum* from Dr P. HAVRANEK (Olomouc, Czechia) were investigated (Table 1).

Isolation of DNA. Total DNA was isolated according to SAGHAI-MAROOF & al. (1984) with slight modifications after MAAß & KLAAS (1995). After treatment with 10 µg/ml RNase A for 2 h at 37 °C, the DNA was purified in 3 ml CsCl gradients according to standard

Table 1. Origin of the *Allium* accessions used in this study

Species	Tax No.	Origin
Allium altyncolicum	0433	Altai, Russia
FRIESEN	0679	Altai, Russia
	2170 (Olomouc)	Altai, Russia
	3157	Altai, Russia
A. karelinii POLJ.	2592	Mongolian Altai, Mongolia
A. ledebourianum	3170	Altai, Russia
SCHULT. & SCHULT. f.	3173	Altai, Russia
A. maximowiczi REGEL	2040	Far East, Russia
	2772	Far East, Russia
A. oliganthum KAR. & KIR.	3201	Kasachstan
A. schoenoprasum L.	0138	Far East, Russia
	0192	Romania
	0390	Finland
	0508	Canada
	0702	Karpaten, Ukraine
	0934	Turkey
	1215	Hungary
	1256	Bulgaria
	1712	Japan
	3174	Altai, Russia
	3176	Altai, Russia
	3446	Chukotka, Russia
	3892	Altai, Russia
	3897	Iran
	9/8 (Olomouc)	
Artificial hybrids		
between	7a	
A. schoenoprasum and	13d	
A. altyncolicum	21b	

procedures (SAMBROOK & al. 1989). The purified DNA was dissolved and stored in TE buffer, and the concentration was determined fluorometrically.

Chromosome preparation. Excised roots were pre-treated for 3 h at room temperature in an aqueous 0.05% solution of colchicine. They were fixed in a freshly prepared solution of 96% ethanol/glacial acetic acid (3:1). For C-banding, meristems were hydrolyzed in 0.1 N HCl for 8 min at 60 °C, dissected on a slide in 45% acetic acid and squashed under a coverslip (VOSA 1973). The coverslip was removed with the dry ice method, and the slide was dried at room temperature for 7–10 days. Slides were then immersed in a supersaturated $Ba(OH)_2$ solution for 6–7 min at room temperature, washed 20–30 min in running tap water, incubated in 2 × SSC at 60 °C (90 min), and washed in distilled water (10 min). They were then stained in a 3% Giemsa (Merck) solution in a phosphate pH 6.8 buffer, washed in distilled water (2–5 min), dried one day at room temperature, and mounted in Euparal. For in situ hybridization: Root-tip spread preparations followed essentially the methods described previously by SCHWARZACHER & al. (1989) with some modification. Briefly, fixed root-tips were partially digested with cellulase and pectolyase (4% cellulase + 1% pectolyase) for 25 min before squashing in 45% acetic acid. Coverslips

were removed after freezing with dry ice and the slides dried. The preparations were used immediately or kept refrigerated for up to 2 months before in situ hybridization.

Analysis of C-banding. Chromosome spreads were photographed. Chromosomes were outlined and C-bands were localized on large prints, observing at the same time the cell under the microscope to make sure that there were no misinterpretations. Chromosomes were identified and paired from measurements made on these drawings, using total length and relative position of centromere.

A band was considered to be present on a given chromosome even if it appeared absent or could not be seen in some of the cells of that plant (KENTON 1978, TARDIF & MORISSET 1991).

C-banding was investigated for six accessions from the Altai mountains (Tax 3176, 3174, 3170, 3173, 433 and 3157; Table 1).

Genomic in situ hybridization (GISH). Total genomic DNA from the putative parental species *A. schoenoprasum* (Tax 3176, 9/8) and *A. ledebourianum* (Tax 3170, 3173) was used as probe, together with excess amounts of unlabelled blocking DNA from *A. ledebourianum* and *A. schoenoprasum* for higher consistency of the DNA : DNA in-situ hybridizations.

Probe preparation and in situ hybridization. Total genomic DNA from *A. schoenoprasum*, *A. ledebourianum* and *A. altyncolicum* was sheared by sonication to 300–500 bp fragments and labeled with biotin with the ULISIS biotin labeling kit (Kreatech Diagnostic, LK-1102-pBIO). Total genomic DNA from the same species was used as blocking DNA after fragmentation to 200 bp pieces by autoclaving for 6 min (HESLOP-HARRISON & al. 1988, 1990; ANAMTHAWAT-JÓNSSON & al. 1990, 1996). In situ hybridization and probe detection followed FUCHS & al. (1995) with minor modifications of the denaturation and washing steps. The probe mix contained c. 40 ng biotinylated genomic DNA, 0.8-4 µg blocking DNA, 50% de-ionized formamide, 10% dextran sulfate, 10 µg sonicated salmon sperm DNA, and 2xSSC, was denatured at 80 °C for 10 min and then immediately put on ice for 2–5 min or was cooled down slowly. Twelve µl 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 hybridization the slides were washed in 50% formamide in 2 × SSC, 2 × SSC and 1 × SSC at 42 °C, 5 min each, or for a stringent wash in 1 × SSC, 0.5 × SSC and 0.1 × SSC at 60 °C 5 min each. Biotinylated DNA was detected with FITS-streptavidin and amplified once with biotinylated antistreptavidin (Boehringer Mannheim). Chromatin was counterstained using propidium iodide.

Sequence analysis of the ITS of rDNA. The entire region was amplified according WHITE & al. (1990) with the primers ITS5 and a biotinylated ITS4 in a 50 µl PCR reaction. The PCR products were purified from a TAE agarose gel with a geneclean II kit, then separated into ssDNA strands with magnetic streptavidine coated beads according to manufacturer's instructions (Bio 101, Dynal). The entire region of ITS1, 5.8sRNA, ITS2 was sequenced in both directions. Sequencing primers were ITS4, ITS5, and the interior primers MK23 (5.8 sRNA into ITS2) and MK29 (5.8sRNA into ITS1). The ssDNA templates were sequenced with a T7-DNA polymerase kit according to manufacturer's instructions (Pharmacia) using the deaza-nucleotide kit to avoid band compressions during electrophoresis due to the inherent secondary structures of the ITS regions.

PCR-RFLP of cpDNA. To determine the maternal parent of *A. altyncolicum*, sequences of the rbcL – atpB non-coding region were obtained. PCR-RFLPs were analyzed. Two non-coding regions of the chloroplast genomes were amplified and restricted (trnC-trnD digested with *Acc*I, *Alu*I, *Hinf*I, *San*3AI, and *Cla*I, and trnT-trnD and digested with *Hind*III, *Dra*I, *Eco*RV, *Ban*II, *Hpa*II; DEMESURE & al. 1995, TSUMURA & al. 1995). The resulting restriction sites were compared to restriction sites of a sample of

approximately 130 other species of the genus *Allium* which covers many of the infrageneric taxa in the genus. For outgroup polarization of this sample of species, representatives of *Dichelostemma*, *Agapanthus*, *Tulbaghia*, *Triteleia* and *Nothoscordum* were used.

Analysis of rDNA repeats. Restriction rDNA mapping was carried out as described previously (BORISJUK & al. 1994). Total DNA was digested using restriction enzymes *Eco*RI, *Bam*HI, *Eco*RV, *Dra*I, *Hind*III, *Xba*I and its combinations. DNA fragments were separated on 0.8% agarose gels, transferred to Hybond N^+ filters (Amersham), and hybridized to ^{32}P-labeled inserts of the clones pLoX12 containing 18S rDNA of potato (N. BORISJUK, unpubl.), clone pRZ52 containing the 5.8S rDNA sequence, internal transcribed spacer 2 and 25S rDNA sequences of zucchini (KING & al. 1993) or to the insert of the clone pS-9 coding for the 3′-end of potato 25S rDNA (BORISJUK & al. 1994). Hybridization, washing procedure and rehybridization of the filters were performed according to "Amersham Protocols".

RAPD analysis. Amplification was carried out using six arbitrary 10 bp primers (C02, G13, G19, AB04, AB18, and AC02), obtained from Operon Technologies, Alameda, Calif. The amplification conditions were optimized according to MAAß & 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. Presence and absence of RAPDs was scored only when samples were loaded on the same gel. Only those bands which were unequivocally scorable for their presence/absence in all templates were considered as markers. Similarity of RAPD-patterns was determined by calculation of F-values (twice the number of shared bands in two plants, divided by the total number of bands in the two plants.

Results

C-banding. A total of 35 distinct bands were observed on all 16 chromosomes for 12 analyzed plants of *A. schoenoprasum*, but they were not visible in all metaphases. The two accessions from Altai (Tax 3176 and 3174) were similar. Exact data can be given only for the chromosomal pairs 3, 4, 6 and 8 with a total of 21 C-bands. (Figs. 2a, 3a) These pairs can be used as the marker chromosomes of *A. schoenoprasum*. Chromosome pairs 4, 6 and 8 seem to be identical to chromosome pairs 6, 7 and 8 reported previously by TARDIF & MORISSET (1991).

Two accessions of *A. ledebourianum* (Tax 3170 and 3173) gave a total of 36 distinct bands. Both accessions are similar. In *A. ledebourianum* only the pairs 1, 3, 4, 6 and 8 with 22 bands are distinguishable (Figs. 2b, 3b). The C-banded chromosomes of *A. altyncolicum* could only be arranged in pairs, not in groups of four. This is especially so for the chromosomes with intercalary bands (including two satellite pairs). The two parental karyotypes can be distinguished by six marker chromosomes (Figs. 2c, 3c). C-banding showed that each parent contributed at least three such marker chromosome pairs with exactly identical banding pattern to the *A. altyncolicum* genome. *A. altyncolicum* pairs 9, 11 and 15 are identical to *A. schoenoprasum* pairs 4, 6 and 8, and *A. altyncolicum* pairs 1, 13 and 16, corresponded to *A. ledebourianum* pairs 1, 3 and 8 (Figs. 2 and 3).

GISH. Different conditions of in situ hybridization and probe mixing which were used revealed some visible differences in chromosomes of *A. altyncolicum*. After standard in situ hybridization without blocking DNA in the probe, all chromosomes give a very strong hybridization signal. Different concentrations of the probe and blocking DNA as well as different denaturation procedures and

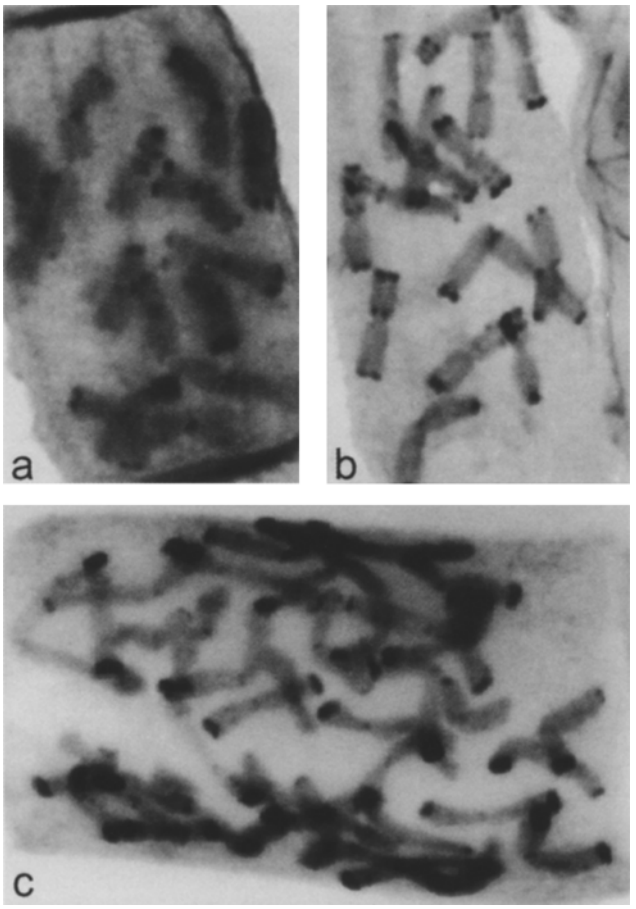

Fig. 2. C-banded metaphases. *a Allium schoenoprasum* (Tax 3176), *b A. ledebourianum* (Tax 3170), *c A. altyncolicum* (Tax 3157)

washing conditions resulted in distinct differences among the experiments. The best result was obtained after GISH using biotin-labeled total genomic DNA from *A. schoenoprasum* (Tax 9/8) as a probe and DNA of *A. ledebourianum* (Tax 3173) as blocking DNA in the relative concentrations 1:40. In this case GISH distinguished four parental chromosomes of *A. ledebourianum* without signal and two satellite chromosomes with very faint signal (pink fluorescence) while the other chromosomes gave a very strong hybridization signal (green-yellow fluorescenc, Fig. 1i). Obviously, these chromosomes are highly related to the chromosomes of the *A. schoenoprasum* genome. Probe mix ratios of biotinylated DNA to blocking DNA of 1 : 10–1 : 20 gave only small differences in intensity signals. Biotinylated DNA of *A. schoenoprasum* and blocking DNA of *A. ledebourianum* gave a much stronger hybridization signal than biotinylated DNA of *A. ledebourianum* and blocking DNA of *A. schoenoprasum*. GISH using biotin-labeled genomic DNA from *A. schoenoprasum* as a probe and DNA of *A. ledebourianum* as blocking DNA in concentration 1 : 100, followed by a stringent wash clearly distinguished only the telomere sequences of 26 chromosomes, probably due to the highly repetitive motive present in the *A. schoenoprasum* genome.

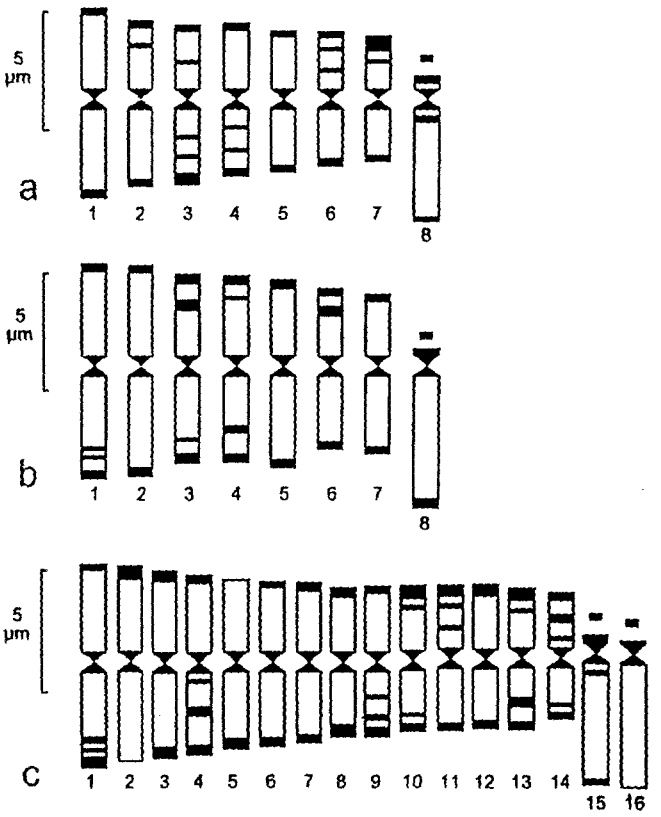

Fig. 3. C-banded karyograms of three *Allium* species. *a Allium schoenoprasum* (mean of 13 metaphases), *b A. ledebourianum* (mean of 18 metaphases); *c A. altyncolicum* (mean of 21 metaphases)

The GISH assay of the triploid artificial hybrid *A. altyncolicum* × *A. schoenoprasum* gave a result similar to the *A. altyncolicum* experiment. Figure 1j shows metaphase chromosome preparations of the hybrid (21b) after GISH with *A. schoenoprasum* DNA labelled as a probe and blocking DNA of *A. ledebourianum* (ratio 1 : 40). GISH distinguished one satellite parental chromosome of *A. ledebourianum* (pink fluorescence) and three other chromosomes with a more faint signal. The other chromosomes probably have a high sequence similarity to the *A. schoenoprasum* genome, indicated by a very strong hybridization signal (green-yellow fluorescence).

Sequences of ITS. The entire 632 bp DNA sequence of the ITS1, ITS2, and the intervening 5.8s RNA region were determined for *A. schoenoprasum*, *A. altyncolicum* and *A. ledebourianum*. All three species were identical in this region.

PCR-RFLP of cpDNA. The *rbc*L–*atp*B sequences of *A. schoenoprasum*, *A. altyncolicum*, and *A. ledebourianum* were identical. The chloroplast genomes of all but one species of sect. *Schoenoprasum* (*A. schmitzii* was not available) however share a synapomorphic *Cla*I mutation in the trnT–trnD region which has not been found in any other taxon of *Allium* outside of sect. *Schoenoprasum* (Mes & al. 1997). Consequently, the chloroplast genomes of these species are clearly monophyletic. One *Dra*I restriction site mutation in the trnCD region distinguished *A. ledebourianum* from the other two species. However, whether or not this is an autapomorphic mutation of *A. ledebourianum* or a synapomorphy for *A. schoeno-*

prasum and *A. altyncolicum* is unclear since a large amount of length variation is present in this chloroplast region in *Allium*. One additional length mutation of about 10–20 bp in the trnT–trnD was detected for *A. ledebourianum* when compared to *A. altyncolicum and A. schoenoprasum*. Unfortunately, polarisation is equally problematic as in the trnC–trnD region. Thus, although the mutations found are by no means unambiguous, a somewhat higher degree of divergence of their chloroplast genome has been found in *A. ledebourianum*.

RLFP of the rDNA gene. The restriction sites for all enzymes in the rRNA coding regions were identical for all species analyzed (Fig. 4), and match the positions of corresponding sites published previously for other *Allium* species (HAVEY 1992). On the other hand, *A. schoenoprasum, A. altyncolicum* and *A. ledebourianum* differ significantly from each other in length and restriction site within the intergenic spacer (IGS) region. Two different rDNA repeat variants were revealed in each genome: 10.6 and 12.1 kb in *A. schoenoprasum*, 11.9 and 13.9 kb in *A. altyncolicum*, and 11.5 and 12.9 kb long in *A. ledebourianum*. The position of *Dra*I in the IGS of *A. schoenoprasum* and *A. ledebourianum*, and *Eco*RV in *A. altyncolicum* and *A. ledebourianum* appear to be rather species specific. There is only one site (*Eco*RV) which is common to *A. ledebourianum* and *A. altyncolicum* and which was not found in the IGS of *A. schoenoprasum.* Most of the differences between two different rDNA repeats of the same species were due to the length

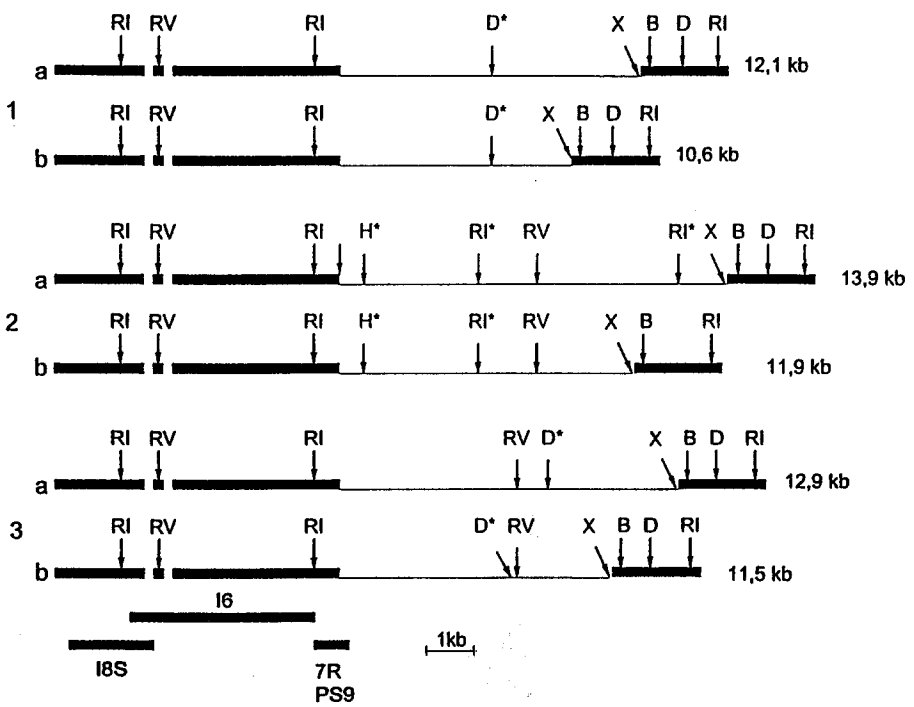

Fig. 4. Relative location of restriction enzyme sites in the nuclear 45s ribosomal DNA of three *Allium* species. *1a,b A. schoenoprasum* (Tax 3176); *2a,b A. altyncolicum* (Tax 3157); *3a,b A. ledebourianum.* R1 *Eco*R1; RV *Eco*RV; H *Hind* III; X *Xba*; B *Bam* H1; D *Dra*1. * site resistant to digestion

variation of the IGS region between *Dra*I/*Eco*RV sites, which are characteristic for the AT-rich IGS region of many plant species (McMullen & al. 1986, Gründler & al. 1991, Borisjuk & Hemleben 1993), and the 5′-end of 18S rRNA gene. The situation is complicated by the enormous variability of the IGS in *Allium* which is evident even among five accessions of *A. schoenoprasum* (Fig. 5).

RAPD analysis. The six selected primers produced 125 bands from which only six bands are present in all 18 accessions of the three species. Thirty-three bands were restricted to the *A. ledebourianum* samples, 32 bands are restricted to the four accessions of *A. altyncolicum* and only eight bands are restricted to the 12 samples of *A. schoenoprasum*. Generally, the RAPD patterns varied substantially even between accessions of one species. The four accessions of *A. altyncolicum* have similaritis of the RAPD patterns between 53.5% and 75.7% (Table 2). The RAPD patterns between accessions of *A. ledebourianum* and *A. altyncolicum* have a similarity between 28.2% and 36.4%, and the similarity of RAPD patterns between accessions of *A. schoenoprasum* and *A. altyncolicum* varied between 42.5% and 26.6%, with the accessions from Altai having a similarity between 36.5% and 42.5%. Only one amplified fragment (c. 730 bp by using primer G13) was common to *A. ledebourianum* and *A. altyncolicum*, but it was not found in any accession of *A. schoenoprasum* (Fig. 6). Testing of this fragments for homology by hybridization (Rieseberg 1996) gave a positive result (Fig. 7). The 0.73-kb fragment from *A. ledebourianum* (Tax 3170) was used as a hybridization probe to determine whether it was homologous to the equivalently sized fragment in *A. altyncolicum*. It hybridized to *A. altyncolicum* fragments, indicating that comigrating 0.73-kb fragments found in the two species were homologous. The presence of other hybridization

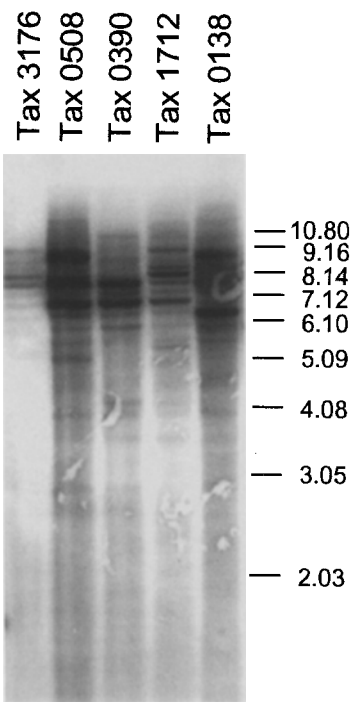

Fig. 5. Intraspecific variation of some accessions of *Allium schoenoprasum* after restriction analysis using *Eco*R1, hybridized with the clone pS-9, wich covers the 3'-end of 25S rDNA

Table 2. Similarity of RAPD-patterns (F-values) in 18 accessions of *Allium altyncolicum* and allies

	3170	3173	3157	2170	0679	0433	3176	3174	3892	0079	0138	3446	3897	0934	0192	0390	1256	0702
3170																		
3173	68.8																	
3157	30.3	34.2																
2170	34.2	36.4	75.7															
0679	35.6	30.8	60.0	55.7														
0433	28.2	30.6	57.7	57.5	53.4													
3176	29.0	29.7	40.3	36.8	38.4	38.8												
3174	27.0	25.8	38.4	40.5	36.5	40.9	54.7											
3892	27.6	30.5	28.4	28.9	33.8	29.9	40.7	43.4										
0079	33.9	28.4	40.5	37.2	42.5	41.2	51.8	44.1	33.9									
0138	28.3	25.0	36.1	32.9	34.2	40.6	38.6	38.6	32.7	39.0								
3446	30.4	26.7	32.4	32.9	32.4	30.3	33.9	33.9	32.7	34.5	53.2							
3897	23.4	24.2	29.9	27.2	26.6	27.8	26.6	24.6	29.3	21.7	23.8	21.3						
0934	26.6	23.5	37.8	31.3	32.5	40.3	36.1	29.7	32.8	32.3	37.9	33.3	34.4					
0192	35.0	35.5	43.7	40.0	50.0	46.9	33.9	38.3	40.0	38.7	40.4	38.2	24.2	40.0				
0390	34.5	35.0	37.5	36.0	39.4	42.2	37.9	33.3	37.0	38.3	40.0	35.2	31.7	39.7	55.8			
1256	30.4	28.8	30.6	29.3	30.6	38.7	31.6	36.4	38.0	41.8	41.2	38.8	25.4	38.2	55.1	62.2		
0702	30.6	31.3	39.7	34.6	43.7	40.3	40.7	36.1	40.0	41.0	31.1	31.0	30.2	40.0	55.6	47.3	46.2	

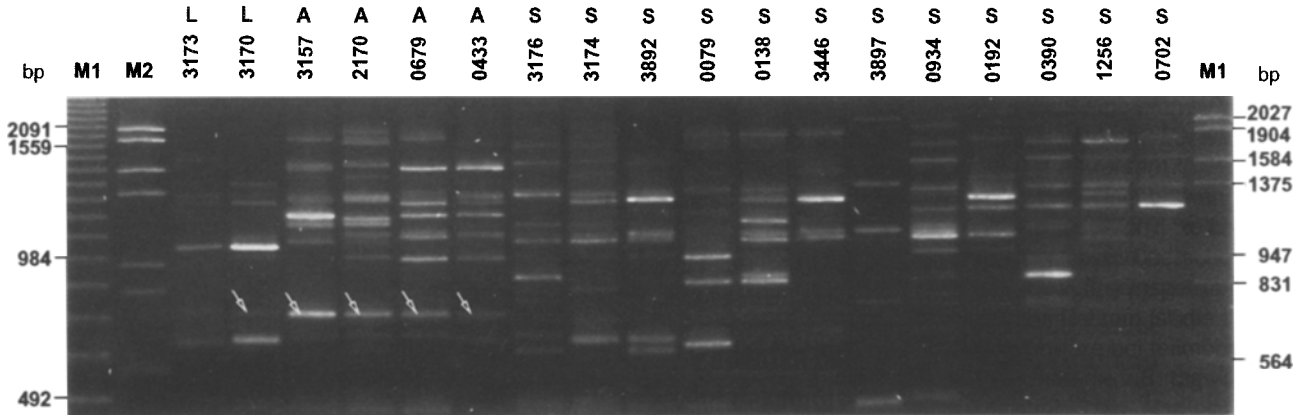

Fig. 6. Amplified bands of the total DNA from 18 specimens of three *Allium* species with 10-mer primer OPG-13. L *A. ledebourianum*, A *A. altyncolicum*, S *A. schoenoprasum*; M1 marker 123, M2 marker Lamda DNA/*Eco*RI+*Hind*III. Origin of the specimens see Table 1

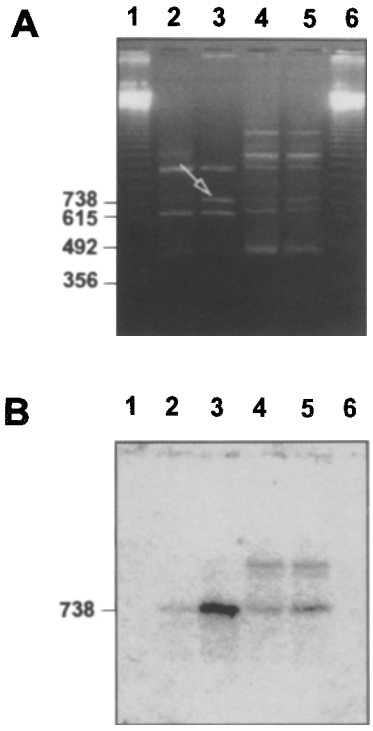

Fig. 7. Homology tests for RAPD fragment G13-730. Lines 1 and 6 are the 123-kb ladder (Gibco BRL). Lanes 2 and 3 were amplified from *Allium ledebourianum* (Tax 3173 and 3170, respectively), lanes 4 and 5 from *A. altyncolicum* (Tax 3157). *A* Amplification products stained with ethidium bromide. *B* The 0.73-kb fragment from *A. ledebourianum* (Tax 3170) was used as a hybridization probe; it hybridizes to *A. altyncolicum* fragments, indicating that comigrating 0.73-kb fragments found on the two species are homologous

signals is considered less important when compared to the signal of the 0.73 kb fragment.

Analysis of artificial hybrids *A. altyncolicum* × *A. schoenoprasum* resulted in a principally different picture (Fig. 8). Thus RAPD patterns of the parental species,

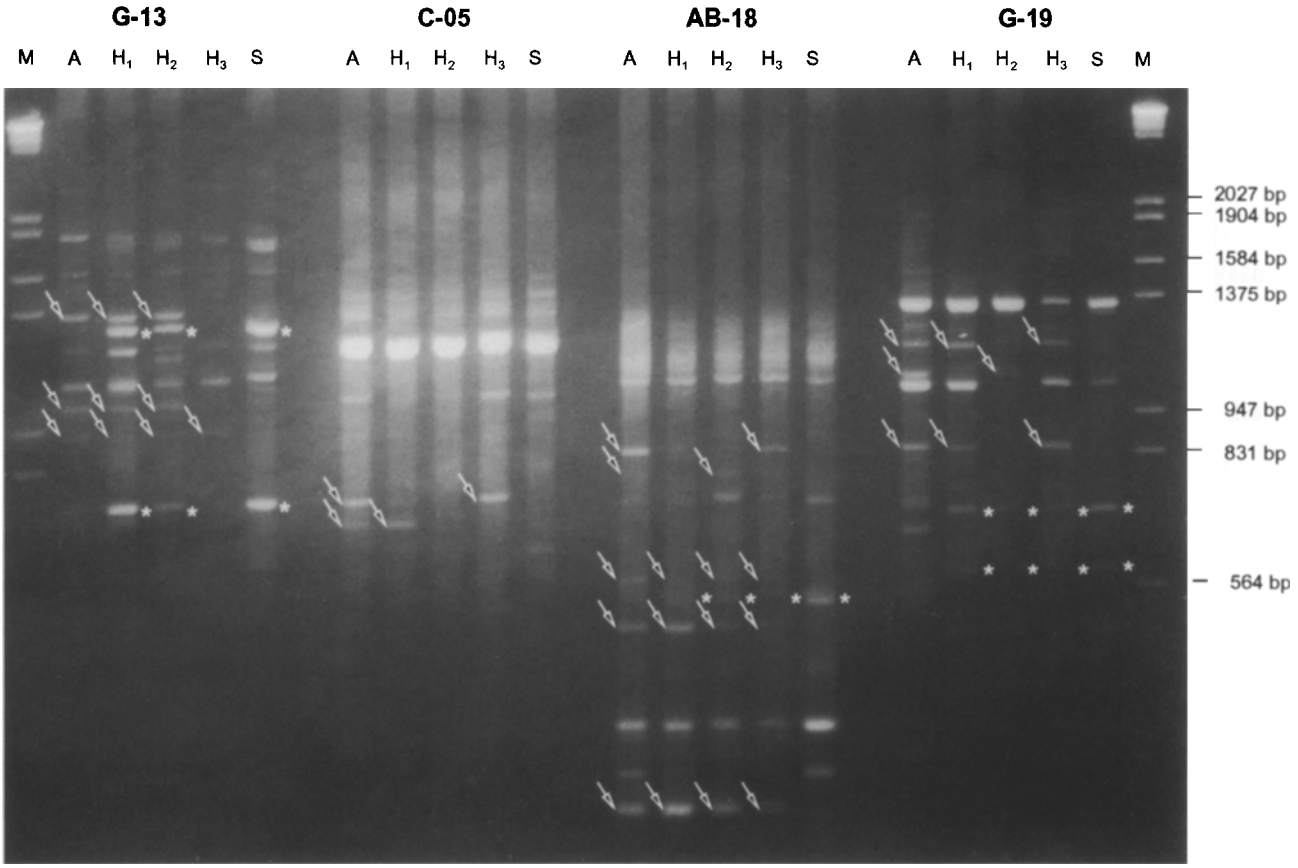

Fig. 8. Amplified bands of the total DNA with four different 10-mer primers. A *Allium altyncolicum* (Tax 2170), S *A. schoenoprasum* (9/8), H1–H3: the artificial triploid hybrids *A. altyncolicum* × *A. schoenoprasum* (7a, 13d and 21b, respectively). Fragments shared by artificial hybrids and *A. altyncolicum* are indicated with points, and fragments shared by artificial hybrids and *A. schoenoprasum* indicated with asterisks

A. altyncolicum and *A. schoenoprasum*, when compared to their artificial triploid hybrids, confirm hybrid origin of the triploids.

Discussion

The allotetraploid nature of *A. altyncolicum* with *A. schoenopasum* and *A. ledebourianum* as its parental species has been indicated by C-banding data. However, this examination could not specify the precise contributions of the parental genomes, since only three chromosome pairs can be unequivocally attributed to each parent. Morphologically, *A. schoenoprasum* and *A. ledebourianum* have fully identical karyotypes. The early assumption that *A. altyncolicum* is a segmental allopolyploid could be ascertained by our GISH data. Six

chromosomes in *A. altyncolicum* are derived from *A. ledebourianum* and the other 26 chromosomes are from *A. schoenoprasum.*

All molecular data show an extremely close (phylo)genetic relationship among the three species. Especially GISH and C-banding indicated that both the genome of *A. schoenoprasum* and of *A. ledebourianum* contributed to *A. altyncolicum.* The accessions of *A. schoenoprasum* from the Altai mountains are most closely related to *A. altyncolicum*, according to the RAPD data. However, we could not definitely resolve which species was the maternal parent of *A. altyncolicum* because the chloroplast DNA mutations could not be polarized.

The data on rDNA repeats obtained do not allow us to draw any conclusions concerning the origin of the tetraploid onion *A. altyncolicum* because only one accession for each species was used. The natural tetraploid hybrid possesses different rDNA repeats in terms of IGS length and localisation of restriction sites as compared to its progenitors. Similar results have been obtained by analysis of *Brassica napus*, an allotetraploid hybrid of *B. oleracea* and *B. campestris* (Borisjuk & Miroshnichenko 1989), and *Nicotiana tabacum*, a natural hybrid of *N. plumbaginifolia* and *N. sylvestris* (Borisjuk & al. 1989). Nevertheless, the direct comparison of IGS nucleotide sequences of *N. tabacum* and its natural parents (Volkov & Borisjuk, unpubl.), which show different rDNA restriction maps, revealed a suprisingly high similarity between tobacco and one of the progenitors, *N. plumbaginifolia.* The intergenic spacer of *N. plumbaginifolia* differs from that of *N. tabacum* in the number of subrepeats and some rare nucleotide changes. Intra- and interspecific variation in the number of rDNA repeats are also well documented in other *Allium* species (Maggini & Garbari 1977, Schubert & Wobus 1985).

The relatedness among the species studied probably influences the experimental conditions of the GISH experiments. Since it is very easy to determine the genetic divergence using one of the molecular techniques described, we were able to calibrate GISH conditions fairly rapidly.

In the case of *A. altyncolicum* we are dealing with a naturally segmental allopolyploid species. Its hybrid origin has been significantly masked by many generations of independent evolution during which *A. altyncolicum* "adapted" the two different parental genomes to produce a well-functioning combination. Probably, this is the reason why it was practically impossible to separate the *A. schoenoprasum* and *A. ledebourianum* genomes in *A. altyncolicum* on the basis of rDNA repeats and RAPDs. *Allium altyncolicum* does not represent just a simple sum of two genomes but has formed a third independent genome. It crucially differs from artificial hybrids of the F1 generation, in which the RAPD pattern was almost the sum of the two parental ones.

The fact that only a part of the *A. ledebourianum* genome is identified in *A. altyncolicum*, could be explained by two alternative hypotheses. First there is a possibility that of the 16 chromosomes of *A. ledebourianum*, 10 resemble very much *A. schoenoprasum* chromosomes. The other possibility is that after the hybridization between *A. schoenoprasum* and *A. ledebourianum* but before polyploidization backcrossing with *A. schoenoprasum* has occured. The GISH experiments stained whole chromosomes in *A. altyncolicum.* Since no alternations of genomic regions of the parental species have been found in this species, the second scenario is considered more likely. However, there is a possibility that this

is merely a consequence of the experimental GISH conditions, which are known to be very critical (ANAMTHAWAT-JONSSON & al. 1996). The other molecular markers may facilitate the distinction between the two schemes mentioned above.

We thank P. HAVRANEK, Olomouc, for the living material of the artificial triploid hybrid. This research was supported by A. v. HUMBOLDT Foundation and by DFG project HA 1848/3-1 for N. FRIESEN.

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Address of the authors: N. FRIESEN, N. BORISJUK, T. H. M. MES, M. KLAAS, P. HANELT, Institute for Plant Genetics and Crop Plant Research, D-06466 Gatersleben, Germany, E-mail: friesenn@ipk-gatersleben.de

Accepted December 9, 1996 by M. HESSE and I. KRISAI-GREILHUBER