

Dedicated to Prof. dr. MERCEDES WRISCHER  
on the occasion of her 70<sup>th</sup> birthday.

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## CLASSICAL AND MOLECULAR CYTOGENETIC STUDIES OF TOP ONION, *ALLIUM X PROLIFERUM* (MOENCH) SCHRADER

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Top, tree or Egyptian onion, *Allium x proliferum* (Moench) Schrader,  $2n=2x=16$ , is a minor, vegetatively propagated garden crop in Europe, North America and North East Asia. Several clones of the top onion were found to be locally cultivated in the region of South Croatia under the name “Ljutika-talijanka”. One of these, clone Biorina, was studied by means of both classical cytogenetic techniques (karyotyping and meiosis) as well as molecular techniques (genomic *in situ* hybridization, GISH and flow cytometry). Analysis of a Feulgen stained karyotype revealed its pseudodiploid structure, since among 16 chromosomes no homologous pairs could be detected. A high frequency of heteromorphic bivalents, followed by univalents and a rare occurrence of multivalents, was recorded in meiosis in the pollen mother cells. Although bivalents with random chiasmata prevailed, we observed occasional occurrence of the bivalents with localized chiasmata. All pollen degenerates at the one-nucleate stage, resulting in complete pollen sterility. Using GISH, we identified the parental origin of all 16 chromosomes and confirmed the hybrid status of the analyzed clone: 8 longer chromosomes originated from the *Allium cepa* L. parent, whereas 8 shorter chromosomes originated from the *Allium fistulosum* L. parent. The investigated clone had an intermediate amount of DNA (26.98 pg DNA/2C) as compared to its parent species, *A. cepa* (31.95 pg DNA/2C) and *A. fistulosum* (21.62 pg DNA/2C).

**Key words:** top onion, *Allium x proliferum* (Moench) Schrader, *Allium cepa*, *Allium fistulosum*, genomic *in situ* hybridization, meiosis

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## Introduction

The viviparous garden onion known as top onion, tree onion or Egyptian Onion and propagated vegetatively from inflorescence bulbils or top sets, was previously classified as a variety of the common onion, *Allium cepa* var. *viviparum* (Metzger) Alefeld (HELM 1956, JONES and MANN 1963, MCCOLLUM 1974). SCUBERT et al. (1983) pointed out that for nomenclatural reasons, top onion must be named *Allium x proliferum* (Moench) Schrader.

One of the main characteristics of the top onion is that their inflorescence usually contains a mixture of bulbils and sterile flowers (Fig. 1) or bulbils only. Bulbils often sprout within the transformed umbel, sometimes in two or more tiers.

Several authors suggested that the top onion originated by hybridization between *A. cepa* and some other species, probably *A. fistulosum*. The first to do so reported a diploid chromosome number,  $2n=2x=16$ , as well as evidence that its karyotype contained a heteromorphic pair of satellite chromosomes, one without the large satellite atypically of *A. cepa* (KURITA 1953 cited by MCCOLLUM 1974, BOZZINI 1964). The morphology of the top onion is intermediate between that of *A. cepa* and that of *A. fistulosum*, resembling the  $F_1$  hybrid of these two species (BOZZINI 1964, SINGH et al. 1967, KOUL and GOHIL 1971, MCCOLLUM 1974). By means of Giemsa C-banding, several independent studies proposed its hybrid status (FISKESJÖ 1975, VOSA 1976, SCHUBERT et al. 1983, PUZINA and PAPEŠ 1996). This view was confirmed also by Restriction Fragment Length Polymorphism (RFLP) (HAVEY 1991) and isozyme analysis (MAASS 1997 b).

Genomic in situ hybridization (GISH) (SCHWARZACHER et al. 1989) has recently become a powerful tool for discrimination between the genomes in a species of hybrid or allopolyploid origin. Labelled total genomic DNA from one of the parental species can be used as a probe, and has often found to be specific enough to mark the chromosomes and chromosome segments from that parent. HIZUME (1994) applied GISH in the study of the genome organization of *Allium wakegi* Araki, the old cultivated crop in Japan, China and Southeast Asia, an onion form analogous to the European top onions (HANELT 1985). He concluded that *A. wakegi* could originate from cross hybridization between close relatives of a form of *A. cepa* and *A. fistulosum*. Using GISH, FRIESEN and KLAAS (1998) verified the hybrid (*A. fistulosum* x *A. cepa*) status of several clones of *A. x proliferum* from the collection of the Institute for Plant Genetics and Crop Plant Research, Gatersleben, Germany.

Several clones of the top onion were found to be locally cultivated in the region of South Croatia under the name "Ljutika-talijanka". In this paper we report the results of GISH applied to one of these clones (clone Biorina), and compare it with the results of the above-mentioned Japanese and German authors. Data obtained by the genome size measurements of the top onion and their parental species (*A. cepa* and *A. fistulosum*) as well as meiosis analyses provide an additional insight into the genome organization of this natural hybrid onion form.

## Material and methods

### Plant material

Several adult plants of top onion in the flowering stage were obtained from the village of Biorina, near Trilj, in South Croatia. Bulbs of common onion (*Allium cepa* cv. "Holland Yellow") were purchased from commercial sources. Seeds of the Japanese bunching onion *Allium fistulosum* L. (CGN16378) were kindly provided by the Centre for Plant Breeding and Reproduction Research, Wageningen, The Netherlands.

### Karyotype analysis

Metaphases were prepared from the root-tip meristems of bulbils from the top onion inflorescence, germinated in tap water for a few days. Root-tips were pretreated with a saturated solution of 1-bromonaphtalene for 24 h in a refrigerator and then fixed in 3:1 absolute ethanol:glacial acetic acid. For microscopic inspection of the karyotype, the root-tips were Feulgen-stained before being crushed in a drop of 2 % acetic orcein.

### Meiosis analysis

Chromosome behaviour at meiosis in pollen mother cells (PMCs) of the top onion was examined. Inflorescences were collected before anthesis. Floral buds were fixed in a Carnoy fixative (6:3:1 solution of absolute ethanol: chloroform: glacial acetic acid) and stained in 2 % acetic orcein.

### Flow cytometry

The protocol used was modified from OTTO (1990). To release the nuclei, leaves were chopped by a razor blade in 1 mL of 0.1 M citric acid containing 0.5 % Tween 20, and the suspension was filtered through a 30  $\mu$ m nylon gauze filter. A 3-fold volume of dye solution containing 5.25  $\mu$ g/mL 4', 6-diamidino-2-phenylindole (DAPI) in 0.4-M di-sodium hydrogen phosphate was added to the filtered suspension. Flow cytometric measurements were performed with a Partec PAS-IIIi flow cytometer equipped with a 100 W high pressure mercury lamp (Osram), UV excitation filter UG1 (Schott), dichroic beamsplitters TK420 longpass and TK500 longpass (Partec), and a longpass filter GG435 (Schott). The diploid *Allium schoenoprasum* (2C genome size 13.4 pg) was prepared in the same way and was used as internal standard.

### Genomic in situ hybridization

#### Chromosome preparation

Enzymatic softening of the root-tip tissue of the top onion clone Biorina, was performed in a mixture of 2.5 % Pectolyase (Sigma R-22) and 2.5 % Cellulase (Onozuka) in 75 mM KCl and 7.5 mM EDTA (pH = 4.0). Root-tips spread preparations essentially followed the method of BUSCH et al. 1994. The chromosomes on the slide were air dried over night and stored at -20 °C until used.

### ***Isolation and labelling of genomic DNA***

Total DNA was isolated using CTAB- buffer according to SHAGHAI-MAROOFF et al. (1984). The genomic DNAs (2µg/reaction) were labelled either with biotin 14 – dATP using BioNick Labelling System (Life Technologies) or with digoxigenin 11– dUTP according to the supplier's instructions.

### ***Genomic in situ hybridization***

Slides were treated with RNase (100 µg/mL in 2x SSC (0.3 M sodium chloride and 0.03 M sodium citrate, pH = 7.0) at 37 °C for 1 h. The slides were then washed three times 5 min in 2X SSC at room temperature (RT), once 5 min at RT in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub> × 7 H<sub>2</sub>O, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>), and once 5 min in PBS + 50 mM MgCl<sub>2</sub>. For the purpose of preserving chromosome morphology, we treated the slides with 4 % paraformaldehyde (FLUKA) in PBS for 15–20 min at RT followed by two times 5 min of washing in PBS. Finally, we dehydrated chromosomes through an ethanol series again. Chromosomes were denatured in 70 % (v/v) deionized formamide in 2x SSC, pH = 7.0 for 2 min, at 68–72 °C, dehydrated through a cold ethanol series and air-dried.

Probe hybridization mixture (20 µL per slide) included the labelled DNA probe (150 ng per slide), 20–50 times that amount of blocking DNA, salmon sperm DNA, 50 % formamide, 2x SSC, 10 % dextran sulphate and 0.1 % SDS (sodium dodecyl sulphate). Blocking DNAs were autoclaved to give DNA fragments between 200 and 500 bp long. We denatured the probe hybridization mixture at 75 °C for 10 min, and quickly cooled it on ice (5 min). The denatured probe mix was located onto the denatured slide preparation (prewarmed at 40–50 °C on a slide warmer) and covered with a coverslip. Hybridization was carried out in a humid chamber, at 37 °C overnight.

Next morning the slides were immersed in 2x SSC (pH = 7.0) at 42 °C to float the coverslips. The slides were then washed 1 × 5 min 2x SSC at 42 °C, 2 × 5 min with 50 % formamide, 2x SSC at 42 °C followed by 5 min 2x SSC at 42 °C. The Coplin jar was taken out from the water bath and cooled at room temperature. The slides were then washed 2 × 5min in 4x SSC at room temperature and incubated with 200 µL of 3 % bovine serum albumin (BSA), 4x SSC, 0.1 % Tween 20 for 30 min in the moist chamber at 37 °C.

Detection of the *A. cepa* biotinylated DNA probe was achieved using fluorescein-isotiocyanate (FITC) conjugated ExtrAvidin (SIGMA) (10 µg/mL) which fluoresced yellow-green under the blue excitation light. Detection of *A. fistulosum* digoxigenin labelled probe was obtained by rhodamine-conjugated anti-digoxigenin (20 µg/mL) that fluoresced red under the green excitation. All detection reagents were made up in 4x SSC, 1 % Tween 20, 3 % BSA. Slides were incubated 30 min in the moist chamber at 37 °C. Washing of the detection solution was carried out in 4x SSC, 1 % Tween 20 (3 × 5 min, 42 °C). The slides were mounted in 20–30 µL antifade solution containing 0.5 µg/mL of DAPI.

The preparations were examined with a Zeiss-Axioplan epifluorescent microscope with appropriate filters. The images of DAPI, FITC and rhodamine fluo-

rescence were acquired separately with a CCD camera. The camera was interfaced to a PC running the ISIS software (Metasystem, Belmont, USA). The images corresponding to DAPI, FITC and rhodamine were superimposed after contrast and background optimisation.

## Results

### Karyotype analysis

Routine karyotype analysis of the clone Biorina revealed its pseudodiploid structure (Fig. 2). Among 16 chromosomes no homologous pairs could be detected (Fig. 5a). Two of the 16 chromosomes resembled satellite chromosomes of *Allium cepa* and *Allium fistulosum*. The top onion chromosome resembling the *A. cepa* satellite chromosome lacked a visible satellite. The chromosome resembling the *A. fistulosum* satellite chromosome possessed a satellite characteristic of *A. fistulosum* (Figs. 2, 5A).

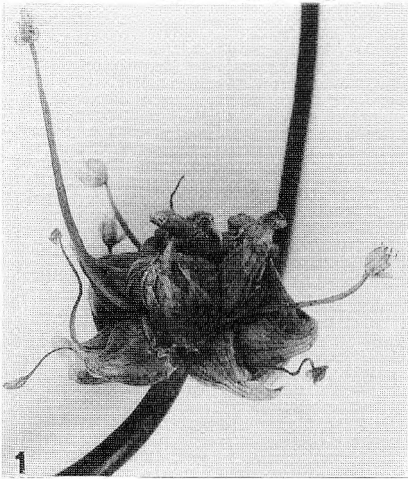


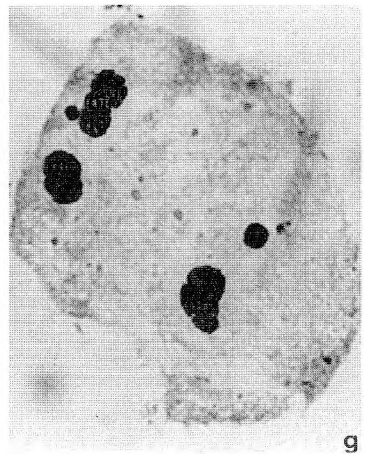
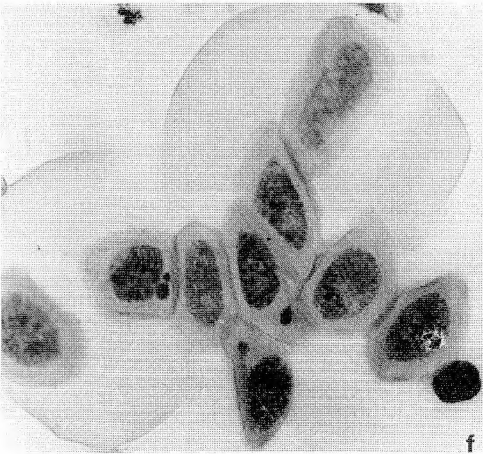
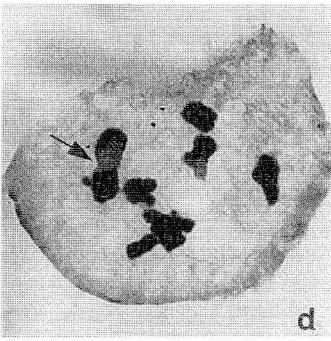
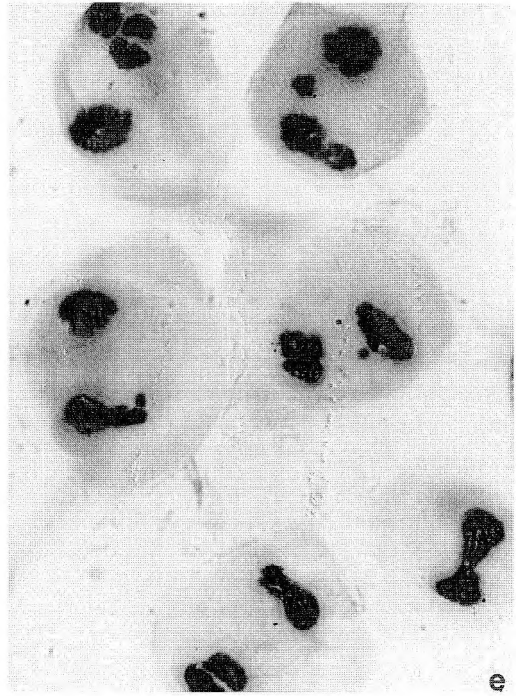
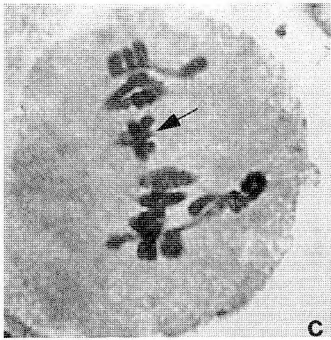
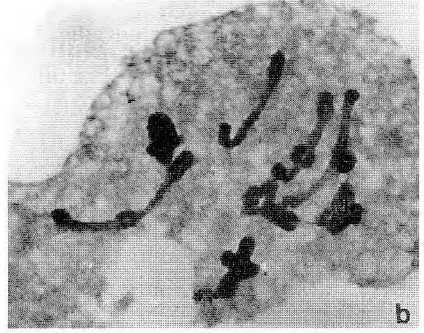
Fig. 1. An inflorescence of *Allium x proliferum* (Moench) Schrader, contains a mixture of bulbils and sterile flowers.



Fig. 2. *Allium x proliferum*, clone Biorina,  $2n=2x=16$ , Feulgen-stained metaphase chromosomes. Arrows indicate two subtelocentric-satellite chromosomes. The *A. cepa* subtelocentric chromosome lacks a visible satellite. Bar scale: 10  $\mu$ m.

### Meiosis analysis

Divisions in the pollen mother cells (PMCs) of the top onion clone Biorina were reduced as compared to the parent species *A. cepa* and *A. fistulosum*. At the diakinesis and metaphase I stage, heteromorphic bivalents were the most frequent chromosomal configurations (Fig. 3a-c). Additionally we observed univalents and, occasionally, multivalents (Fig. 3d). The type of chromosome configurations with their average frequency is presented in Table 1. Bivalents mainly possessed random chiasmata (*cepa* type), but occasionally we observed bivalents with localized chiasmata (*fistulosum* type) (Fig. 3c). Ring bivalents were most common, but usually 2 or more rods were present in each cell.



Tab. 1. Analysis of chromosome pairing in diakinesis and metaphase I in *Allium x proliferum*, clone Biorina.

Chromosome configuration	Number of PMCs analyzed	Frequency (%)
8 (II)	23	44.23
2 (I) + 7 (II)	18	34.62
4 (I) + 6 (II)	9	17.31
1 (I) + 6 (II) + 1 (III)	1	1.92
2 (I) + 5 (II) + 1 (IV)	1	1.92
Total	52	100.00

Tab. 2. Nuclear DNA content (pg) of *Allium x proliferum*, clone Biorina, and of two parental species, *A. cepa* and *A. fistulosum*.

Species	Chromosome number (2n)	Total DNA content (pg)
<i>Allium cepa</i> cv. "Holland Yellow"	16	31.95 ± 0.23
<i>Allium fistulosum</i> L.	16	21.62 ± 0.09
<i>Allium x proliferum</i> (Moench) Schrad.	16	26.98 ± 0.26

In both the first and the second meiotic division of the top onion, clone Biorina, frequent irregularities occurred at post-metaphase stages and in tetrad formation. Nearly one third (30.7 %) of the analyzed PMCs during anaphase I had some abnormalities. The most common deviation was the presence of chromatic bodies (originating from univalents and fragments) outside of the spindle (21.9 %). Bridges and bridges with fragments were observed in 8.77 % of the analyzed anaphases I. In telophase I the frequency of abnormalities increased to 44 % of PMCs. The lagging chromosomes (univalents) and fragments formed micronuclei in 35.57 % of all analyzed telophases I. Chromatin bridges and lagging univalents were observed in 8.4 % of the analyzed PMCs. In some cases a complete failure of division of chromosomes was observed (Fig. 3e and f). 30 % of the analyzed microspores carried one or more micronuclei (Fig. 3g) All pollen degenerates before pollen mitosis, resulting in a complete pollen sterility.

Fig. 3. Meiosis in pollen mother cells (PMCs) of top onion, clone Biorina. a, b, c and d) Diakinesis and metaphase I, heteromorphic bivalents are the most frequent chromosomal configuration. c) Arrow indicates a bivalent with localized (*fistulosum* type) chiasmata. d) Arrow indicates a quadrivalent formation. e and g) Telophase I and telophase II, severe disturbances in the PMCs division. f) Microspores containing micronuclei. Bar scale: 10 µm.

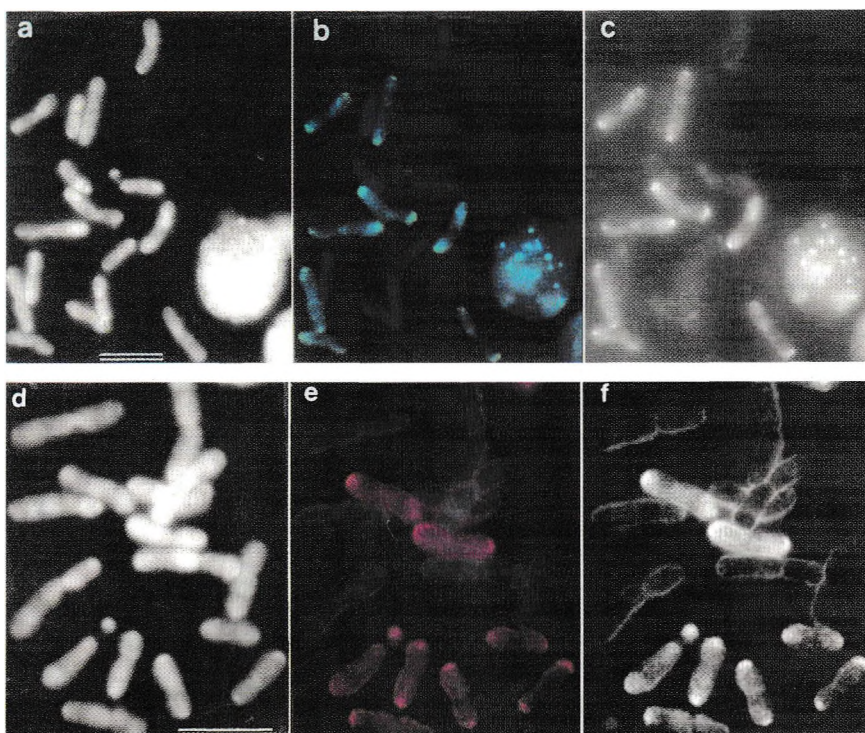


Fig. 4. Fluorescent microphotographs of the metaphase chromosomes of the top onion clone Biorina,  $2n=2x=16$ , after genomic *in situ* hybridization. a and d) A complete DAPI stained metaphases. b-c) GISH using biotin-labelled genomic DNA from *A. cepa* as a probe and unlabelled DNA of *A. fistulosum* as blocking DNA. In colour picture (b) the labelled chromosomes with FITC, corresponding to the C genome, are yellow-green. e-f) GISH using digoxigenin-labelled genomic DNA from *A. fistulosum* as a probe and unlabelled DNA of *A. cepa* as blocking DNA. In colour picture (e) the labelled chromosomes with rhodamine, corresponding to the F genome, are red. Bar scale: 10  $\mu$ m.

#### Genomic *in situ* hybridization (GISH)

*In situ* hybridization of *A. cepa* biotinylated genomic DNA mixed with unlabelled, blocking DNA from *A. fistulosum* to the chromosomes of the clone Biorina resulted in eight, relatively long labelled chromosomes (Fig. 4b and c). The size and morphology of these chromosomes were very similar to those of *A. cepa* and we assigned this genome as the C genome (Fig. 5c). The intensity of hybridization was strongest at the telomeric regions of the labelled chromosomes and the GISH pattern of the labelled chromosomes was therefore reminiscent of their C-banding pattern.

When *A. fistulosum* digoxigenin labelled DNA probe mixed with 30 times more *A. cepa* unlabelled blocking DNA was applied to denatured metaphase chromosomes of the top onion, clone Biorina, we obtained eight, relatively short labelled chromosomes (Fig. 4e, f). The size and morphology of these chromo-



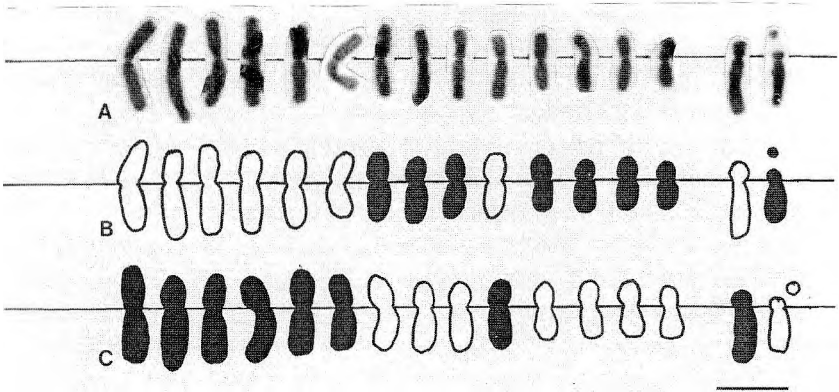


Fig. 5. Metaphase chromosomes of the top onion clone Biorina,  $2n=2x=16$ , arranged on the basis of decreasing chromosome length. A) Cutouts of Feulgen-stained chromosomes from Fig. 2. Only the satellite chromosomes from the two parental species can be identified with certainty. B) Eight black coloured chromosomes represent the F (*A. fistulosum*) genome (reconstructed from Figs. 4d and f). C) Eight black coloured chromosomes represent the C (*A. cepa*) genome (reconstructed from Figs. 4a and c). Using GISH the two parental genomes are unequivocally identified. Bar scale: 10  $\mu\text{m}$ .

somes was very similar to those of *A. fistulosum* and we assigned this genome as the F genome (Fig. 5b). The shorter subtelocentric with a big satellite was the easiest to recognize among this group of labelled chromosomes. The strongest intensity of the hybridization was observed in the terminal regions, whereas centromeric regions were usually more weakly labelled.

The results of GISH clearly confirm the hybrid (*A. cepa*  $\times$  *A. fistulosum*) status of the analyzed clone of the top onion. Of 16 chromosomes, 8 longer chromosomes are derived from *A. cepa* parent (C genome), whereas 8 smaller chromosomes are derived from *A. fistulosum* parent (F genome) (Fig. 5).

#### Genome size measurement

The results of genome size measurements of the top onion clone Biorina, and the two parent species *A. cepa* and *A. fistulosum* are shown in Table 2. The results showed that the analyzed clone possessed an intermediate size of genome (26.98 pg DNA/2C) as compared to *Allium cepa* (31.95 pg DNA/2C) and *Allium fistulosum* (21.62 pg DNA/2C).

#### Discussion

Interspecific hybridization is well known in *Allium* as well as in other angiosperm groups. By the erection of crossing barriers it is a form of speciation in crop plants, often involving the generation of vegetatively propagated forms. To study hybrid crops and their suspected parents, initial karyotype and meiosis analysis followed by GISH and flow cytometry techniques provide a powerful tool for an unequivocal resolution of past hybridization events.

In the conventional (Feulgen) stained karyotype of the top onion only one chromosome pair, the satellited one, can be identified with certainty. Although other chromosomes differ mutually in respect of the chromosome length, their morphology is very similar and it is impossible to differentiate between the two parental genomes. Working on interspecific hybridization between onion and distant *Allium* species, KELLER et al. (1996) suggested that simple karyotyping could be useful for the initial verification of the interspecific hybrids, provided that the chromosomes of the parental complements differ in size, number and/or morphology.

In comparison with the classical techniques, GISH provides to be a much more powerful tool for distinguishing the chromosomes of different origin in the hybrid or allopolyploid species. In this work we have shown that using GISH, each chromosome of the top onion karyotype can be unmistakably identified. The preferential hybridization of the *A. cepa* genomic probe to the C genome of the top onion and that of the *A. fistulosum* probe to the F genome, indicated that both parental genomes are still complete and, after the spontaneous hybridization process, have remained karyotypically unchanged.

The results of GISH obtained in this work for the clone Biorina are in concordance with the results of GISH obtained by HIZUME (1994) in *Allium wakegi* and the results of FRIESEN and KLAAS (1998) obtained for several strains of top onion from the collection of the Institute in Gatersleben. In all three independent studies, where GISH experiments were conducted under more or less different conditions, the parental chromosomes derived from *A. fistulosum* and *A. cepa* were identified, providing the hybrid status of all the analyzed clones of the top onion. These three studies support the opinion of HANELT (1985) that both the European and the Japanese strains of the top onion have the same parentage.

The C-banding pattern in *A. cepa* and its close relatives is characterized by broad telomeric C-bands in all chromosomes, and some chromosomes additionally exhibit weaker interstitial C-bands (FISKESJÖ 1975, SCHUBERT et al. 1983, PUJIZINA and PAPEŠ 1996). As KUIPERS et al. (1997) found in the genus *Astroemeria*, we observed a coincidence of hybridization signals and C-banding pattern in our experiment. In both experiments, the labelled chromosomes showed the strongest intensity of hybridization at the telomeric regions, whereas the centromeric region exhibited weaker intensity of hybridization. Using GISH in *A. wakegi* HIZUME (1994) did not report such a GISH pattern, but FRIESEN and KLAAS (1998) obtained a result similar to ours. The telomeric regions of the chromosomes of *A. cepa* and its relatives consist of highly repetitive satellite and/or rDNA sequences (BARNES et al. 1985, IRIFUNE et al. 1995, PICH et al. 1996). Since these sequences hybridize with a DNA probe faster than other non-repetitive sequences, coincidence between GISH and Giemsa C-banding pattern is a logical consequence.

A high frequency of heteromorphic bivalents in meiosis of the top onion, as found in this work, indicates that the genomes of *A. cepa* and *A. fistulosum* contain sufficient homologous sequences to provide the pairing and recombination between them during the prophase of meiosis. In spite of that, the produced pollen is completely sterile. Similar results were regularly obtained by several

breeders who created numerous artificial hybrids between *A. cepa* and *A. fistulosum* (EMSWELLER and JONES 1935, LEVAN 1941, VAN DER MEER and VAN BENNEKOM 1978, ULLOA-G et al. 1994). ULLOA-G et al. (1995) studied some of these artificial hybrids and backcross progeny and concluded that nucleo-cytoplasmic incompatibility might be the cause underlying the species barrier between *A. cepa* and *A. fistulosum*.

Until recently the Indian triploid onion "Pran" (SINGH et al. 1967) was considered to be another representative of natural hybrids between *A. cepa* and *A. fistulosum* (KOUL and GOHIL 1971, ANIMA and KOUL 1983). Molecular analyses of the Indian triploid onion form "Pran" (HAVEY 1991, MAASS 1997 a) contradicted these reports. HAVEY (1991) concluded that "Pran" arose from the hybridization of *A. cepa* with an unresolved seed parent. PUIZINA et al. (1999) and PUIZINA and PAPEŠ (1996, 1997) identified the Croatian triploid onion "Ljutika" as a European equivalent to "Pran". By means of GISH it was shown that "Ljutika" might possess a complex triparental genome organization. Two of the parental species were identified to be *A. cepa* and *Allium roylei* STEARN, respectively (PUIZINA et al., in press). Spontaneous hybridization between *A. cepa* and other *Allium* species seems, obviously, to be a very interesting and not fully explored field of research.

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