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Dedicated to Prof. dr. sc. ZVONIMIR DEVIDÉ on the occasion of his 80th birthday

Karyotypes of *Allium senescens* L. ssp. *montanum* (Fries) Holub populations from the Mt. Biokovo region

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The karyotypes of three *Allium senescens* ssp. *montanum* (Fries) Holub populations from the Mt Biokovo region were investigated. This species has been poorly cytogenetically analysed, and with respect to Croatian populations such investigations have never been carried out. The chromosome number, morphology and distribution of heterochromatin were investigated. The number, position and activity of nucleolar organising regions (NORs) were also analysed. In all three populations, only plants with a tetraploid ($2n = 4x = 32$) chromosome complement were found. The chromosome complement consisted of seven groups of four metacentric and one group of four submetacentric chromosomes bearing the satellite on the short arm. Chromosomes exhibit four types of Giemsa C-bands: telomeric, intercalary, centromeric bands and those located on satellites. Eight chromosomes with the characteristic banding pattern were distinguished. Silver staining revealed a maximum of four nucleoli that corresponded to the maximum number of active NORs (Ag-NORs) located terminally on the short arm of four submetacentric chromosomes. The results obtained suggest that *A. senescens* ssp. *montanum* from the Mt Biokovo region is an autotetraploid species.

Key words: *Allium senescens* ssp. *montanum*, karyotype, Giemsa C-banding, nucleolar organising regions, silver staining

Introduction

Allium senescens L. ssp. *montanum* (Fries) Holub is a member of the large subgenus *Rhizirideum*, section *Rhizirideum*, distributed in the very large area of Central and Southern Europe. The species in this ecologically very variable subgenus, including *Allium senescens* ssp. *montanum*, have not been thoroughly cytogenetically analysed. JOACHIMIAK et al. (1987) have described karyotypes of some Polish *Allium montanum* populations and KIM et al. (1989) have given some information about Korean populations. These cytological studies

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have shown that this species possesses 2n = 4x = 32 chromosomes. However, STEARN (1978) stated that the chromosome number could vary from 2n = 16, 24, 32, and in some karyotypes the B-chromosomes (1–4) were described. The present investigation was additionally initiated by the work of RADIĆ (1989) who described the populations from the Mt Biokovo region investigated herein under the name *A. incensiodorum*. The plants in these populations differ in certain morphological characteristics and in flowering time from the original species, *A. senescens* ssp. *montanum*. In *A. incensiodorum* the pedicels are ribbed and scabrid with papillae of very different sizes, the leaves are slightly ribbed and angular above, and perianth is stellate, having segments with large pepillae especially on the keel (RADIĆ 1989). This onion flowers in the summer. In our work we used the name *A. senescens* ssp. *montanum* despite the fact that GREGORY et al. (1998) in Alliorum Nomencaltor accepted the name *A. incensiodorum* proposed by RADIĆ (1989) as a synonym.

The aim of the present study was to analyse the karyotypes of three *A. sensecens* ssp. *montanum* populations from the Mt Biokovo region. Therefore, investigations were carried out to establish the chromosome number and morphology using conventional Feulgen staining technique. Giemsa C-banding technique, a very efficient method in resolving systematic and phylogenetic problems, was used to investigate the distribution of heterochromatin and to identify chromosome markers characteristic of this species. The number, position and activity of the nucleolar organising regions (NORs) were analysed using the silver staining method.

Material and methods

Cytogenetical analysis was carried out with three populations of *Allium sensescens* L. ssp. *montanum* (Fries.) Holub from the Mt Biokovo region: Gornji Tučepi, Saranč-Kozica and Baško polje. Plants collected during 1999 were rooted in tap water for 7–10 days. Karyotypes were prepared from root-tips pretreated with α-bromonaphthalene for 21 h at 4 °C, fixed in 1 : 3 (v/v) absolute ethanol and glacial acetic acid and stained by the Feulgen method. Roots were hydrolysed in 1N HCl at 60 °C for 10 min, rinsed in distilled water and stained in freshly prepared Feulgen stain for 2–4 h. The root-tips were squashed in 45% acetic acid, coverslips were removed by the dry ice method and preparations were air-dried overnight.

For Giemsa C-banding the method described by KIM et al. (1989) was used with a slight modification. Briefly, the roots were softened in 45% acetic acid for 2–3 h, briefly hydrolised in 1 N HCl and preparations were made by the squash technique. The slides were first incubated in 5% barium hydroxide at room temperature for 8–10 min, washed in running tap water for 30 min and then incubated in 2 × SSC (0.3 M NaCl + 0.03 M Na(III)-citrate) buffer, pH 6.9 at 55–60 °C for 30 min, rinsed in distilled water and stained in 3% Giemsa (Sigma) in 0.067 M Sörensen buffer, pH 6.8–6.9 for 15–20 min.

Silver staining of NORs was performed according to HIZUME at al. (1980). Fixed roots were enzymaticaly softened in 20% of pectinase (Sigma) and 2% cellulase (Calbiochem) in 0.1 M citrate buffer, pH 4.5–4.8 at 37 °C for 40 min, macerated and squashed in 45% acetic acid. The chromosome preparations were impregnated with 50% solution of $AgNO_3$, covered by nylon mesh and incubated at 50–55 °C for 30 min.

Chromosomes were identified and arranged on the basis of decreasing length from microphotographs of 1–3 metaphase plate per population. The nomenclature used for describing karyotype composition followed LEVAN et al. (1964). The short arm length (S), long arm length (L) absolute chromosome length (S+L) and relative chromosome length (%) were measured. The chromosome arm ratio (L/S), standard deviation (s.d.) of absolute and relative chromosome length were also calculated.

Results

Chromosome number and morphology

The karyotypes of *Allium senescens* ssp. *montatum* in each population studied consist of 2n = 4x = 32 metacentric and submetacentric chromosomes (Fig. 1A). Submetacentric chromosomes possess satellites on the short chromosome arm (Fig. 1B). Chromosomes arranged in order of decreasing length are organised in 7 groups of metacentric and 1 group of submetacentric chromosomes. The morphometric measurements showed a high level of homology among the chromosomes in each group (Tabs. 1–3). In the Gornji Tučepi population the chromosomes of the metaphase complement showed a gradual decrease in length from the longest (9.03 μm) to the shortest (4.82 μm) chromosomes (Tab. 1). In the Saranč-Kozica population the chromosome length varied from 8.50 μm for the longest to 4.53 μm for the shortest chromosomes (Tab. 2). The values of chromosome length measurement in the third population, Baško polje, were higher, ranging from 14.03 μm for the longest chromosomes to 7.81 μm for the shortest (Tab. 3). Such discrepancies in the chromosome length could be explained by the fact that in this population only one metaphase plate with extended chromosomes was analysed. The values of relative chromosome length confirmed that there are no differences in chromosome length among populations. The relative length varied from 16.09–16.46 μm for the longest to 8.75–8.96 μm for the shortest chromosomes (Tabs. 1–3).

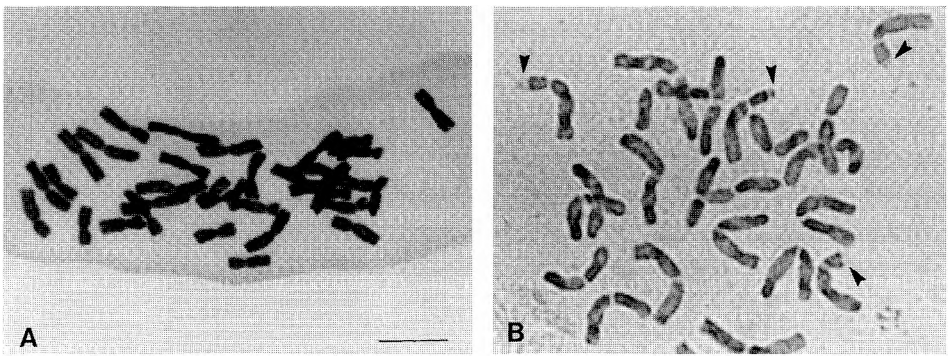

Fig. 1. Karyotype (A) and partial metaphase plate (B) of *Allium senescens* ssp. *montanum* (2n = 4x = 32). Arrows indicate submetacentric chromosomes with satellites. (Bar = 10 μm)

Tab. 1. The length of chromosome arms, arm ratios (L/S), absolute (L+S) and relative chromosome length, and centromere position in *A. senescens ssp. montanum*, Gornji Tučepi population.

Chromosome type	S	L	S+L	s.d.	Relative length %	s.d.	L/S	s.d.	Centromere position
1	4.34	4.68	9.03	0.55	16.46	0.28	1.08	0.03	m
2	3.82	4.51	8.33	0.59	15.19	0.37	1.18	0.05	m
3	3.59	4.11	7.70	0.51	14.04	0.19	1.15	0.06	m
4	2.89	4.03	6.92	0.18	12.62	0.40	1.39	0.08	m
5	2.75	3.68	6.43	0.46	11.72	0.25	1.34	0.08	m
6	2.55	3.11	5.66	0.20	10.31	0.35	1.22	0.14	m
7	1.98	2.87	4.82	0.17	8.79	0.18	1.45	0.11	m
8	1.60	4.36	5.96	0.48	10.87	0.27	2.73	0.30	sm[s]

L = long arm, S = short arm, s.d. = standard deviation

Tab. 2. The length of chromosome arms, arm ratios (L/S) absolute (L+S) and relative chromosome length, and centromere position in *A. senescens ssp. montanum*, Saranč-Kozica population.

Chromosome type	S	L	S+L	s.d.	Relative length %	s.d.	L/S	s.d.	Centromere position
1	3.98	4.53	8.50	0.78	16.42	0.29	1.13	0.05	m
2	3.68	4.12	7.80	0.60	15.07	0.36	1.12	0.05	m
3	3.43	3.90	7.33	0.79	14.16	0.24	1.16	0.10	m
4	2.68	3.98	6.65	0.63	12.85	0.20	1.49	0.03	m
5	2.65	3.45	6.10	0.73	11.79	0.32	1.31	0.09	m
6	2.43	2.88	5.30	0.49	10.24	0.17	1.20	0.07	m
7	1.90	2.63	4.53	0.62	8.75	0.31	1.40	0.12	m
8	1.45	4.10	5.55	0.66	10.72	0.26	2.66	0.20	sm[s]

L = long arm, S = short arm, s. d. = standard deviation

Tab. 3. The length of chromosome arms, arm ratios (L/S), absolute (L+S) and relative chromosome length, and centromere position in *A. senescens ssp. montanum*, Baško polje population.

Chromosome type	S	L	S+L	s.d.	Relative length %	s.d.	L/S	s.d.	Centromere position
1	6.38	7.65	14.03	0.93	16.09	1.07	1.20	0.08	m
2	5.93	7.05	12.98	0.25	14.88	0.29	1.19	0.10	m
3	4.95	7.05	12.00	0.42	13.76	0.52	1.42	0.25	m
4	5.33	6.08	11.41	0.30	13.09	0.34	1.14	0.11	m
5	4.28	6.30	10.58	0.25	12.13	0.29	1.47	0.22	m
6	4.20	5.40	9.60	0.42	11.01	0.49	1.29	0.15	m
7	3.53	4.28	7.81	0.30	8.96	0.34	1.21	0.08	m
8	2.48	6.30	8.78	0.39	10.07	0.45	2.54	0.92	sm[s]

L = long arm, S = short arm, s.d. = standard deviation

Giemsa C-banding

Giemsa C-banding revealed four types of C-bands: telomeric, intercalary, centromeric bands and satellites (Figs. 2A-C). Telomeric C-bands were present on all chromosomes on one chromosome arm, or both. On some chromosomes telomeric C-bands and those on sat-

ellites were the largest and the most thoroughly stained. Intercalary C-bands were of different intensity and weaker than telomeric C-bands. The weakest C-bands were those in the centromeres. Because of differences in staining intensity of C-bands and different degree of chromosome condensation, chromosome assembling was difficult. However, on the basis of the C-bands position eight marker chromosomes could be distinguished (Figs. 2A-C).

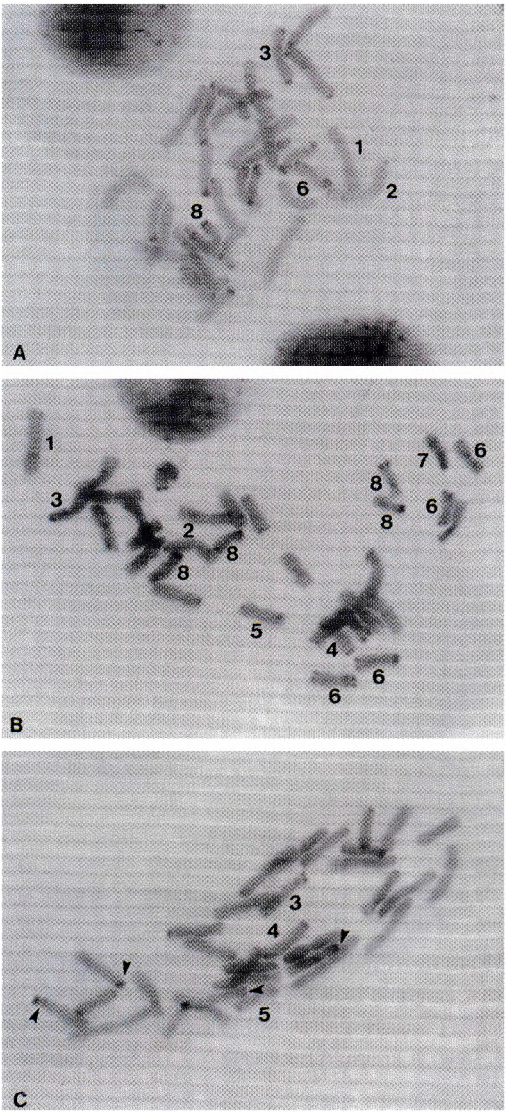

Fig. 2. C-banding pattern in metaphase chromosomes of *Allium sensescens* ssp. *montanum* revealed by Giemsa C-banding: A) – C) On the basis of C-band position, eight marker chromosomes (1–8) can be distinguished. The arrowheads indicated C-bands at satellites on the short arm of submetacentric chromosomes. The different size of these C-bands corresponds to the extension of secondary constrictions.

The longest chromosome 1 exhibits medially located weak intercalary C-bands on both chromosome arms and a weak telomeric C-band on one arm (Figs. 2A, B). Chromosome 2 possesses telomeric C-bands on both arms and three intercalary C-bands could be observed. Two intercalary C-bands are more prominent while the third one is visible on some chromosomes (Figs. 2A, B). Chromosome 3 is characterised by prominent telomeric C-bands and intercalary C-bands proximal to telomeres on both arms. Chromosomes 4 and 5 possess only intensively stained telomeric C-bands almost of the same size (Figs. 2B, C). Chromosome 6 is distinguished by two of the most prominent intercalary C-bands on both arms and two of the largest telomeric C-bands (Figs. 2A, B). Chromosome 7 possesses a telomeric C-band on the short arm and a weak intercalary C-band located medially on the short arm (Fig. 2B). Submetacentric chromosome 8 is characterised by the most intensively stained C-band located terminally on the short arm that corresponds to the satellite. In some metaphase plates the secondary constrictions (SCs) are highly extended and together with satellites they produced the largest C-bands in the karyotype (Fig. 2C). On the long arm of this chromosome a faint telomeric C-band is also observed.

Activity of NORs

Silver staining confirmed that NORs were located in SCs and satellites on the short chromosome arm of the four submetacentric chromosomes (Fig. 3A). The maximum of four active NORs (Ag-NORs) was concordant with the maximum number of nucleoli in the interphase cells (Tab. 4, Fig. 3G). Cells with fewer than 4 nucleoli were also observed (Figs. 3D-F). Table 4 showed that in all populations and individuals studied cells with 2 and 3 nucleoli appeared more frequently than did those with 1 or 4. The number of Ag-NORs at metaphase chromosomes also varied from 1–4 (Fig. 3B). In some cells chromosomes with highly extended Ag-NORs (Fig. 3C) and partially active NORs were observed.

Tab. 4. Contribution of interphase nuclei with different numbers of nucleoli in three populations of *Allium sensescens* ssp. *montanum* as revealed by silver staining.

Analysed plants	Number of cells	Percentage of cells with 1–4 nucleoli			
		1	2	3	4
		Population Gornji Tučepi			
1	660	11.06	24.70	42.12	22.12
2	679	19.73	32.99	43.15	18.85
3	488	11.68	32.78	37.50	18.03
Total/Average	1827	14.16	30.16	40.92	19.67
		Population Saranč-Kozica			
1	1020	11.47	37.00	39.70	13.82
2	796	10.30	30.90	41.08	17.70
3	464	19.82	34.70	36.64	8.62
Total/Average	2280	13.86	34.20	39.14	13.38
		Population Baško Polje			
1	660	11.06	24.70	42.12	22.12
2	679	19.73	32.99	43.15	18.85
3	488	11.68	32.78	37.50	18.03
Total/Average	1827	14.16	30.16	40.92	19.67

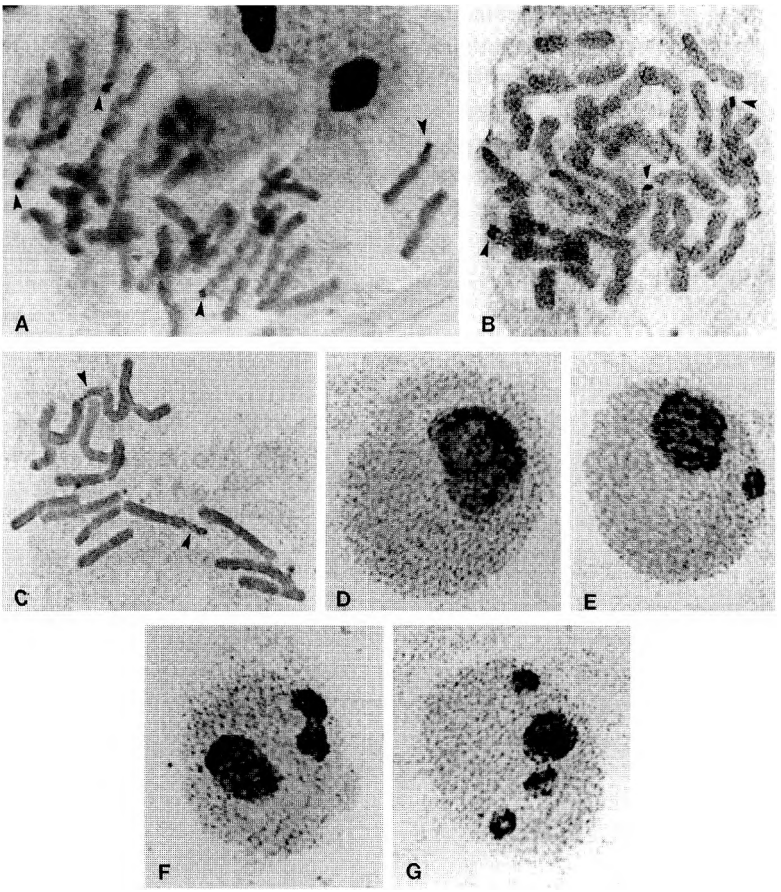

Fig. 3. Silver stained metaphase chromosomes and interphase cells of *Allium senescens* ssp. *montanum*: A) four Ag-NORs at the telomeric position on the short arm of submetacentric chromosomes (arrowheads), B) metaphase plate with three Ag-NORs (arrowheads), C) metaphase chromosomes with two extended Ag-NORs (arrowheads), D) –G) interphase nuclei with one to four nucleoli.

Discussion

The populations of *Allium senescens* ssp. m*ontanum* from the Mt Biokovo region investigated in this paper are interesting for cytogenetical analysis with respect to the different morphological characteristics of this onion, which RADIĆ (1989) described under the name *A. incensiodorum* (the original and accepted name is *A. senescens* ssp. *montanum*).

Present investigation showed that *A. senescens* ssp. *montanum* in all three populations from Biokovo mounatain region has a tetraploid 2n = 4x = 32 chromosome complement. Morphometric analysis showed that this species has a symmetrical karyotype consisting of seven groups of four metacentric and one group of four submetacentric chromosomes on which the satellites are terminally located on the short chromosome arm. The data for absolute chromosome length in all three populations showed a high level of homology among

the chromosomes within each chromosome group, and the measurement of relative chromosome length showed no difference in morphology and chromosome length among the populations. Morphometric data presented for populations from the Mt. Biokovo region are in agreement with those given by JOACHIMIAK et al. (1987) for Polish populations of *A. montanum*. Some differences perceived in the length of the smallest chromosomes probably appeared as a result of different chromosome condensation due to the pretreatment used.

Results obtained to date for the distribution and amount of constitutive heterochromatin visible as C-banding pattern on the chromosomes confirm that the Giemsa C-banding technique is a very efficient method to settle systematic and phylogenetic problems between taxa. As reported by many authors, C-bands are evolutionarily conserved in the genus *Allium*, and could be helpful in the detection of taxonomic relationships among different *Allium* species as well as in the identification of progenitors in interspecific hybrids (EL-GADI and ELKINGTON 1975, VOSA 1976, JAMILENA et al. 1990, TARDIF and MORISSET 1991, PUIZINA and PAPEŠ 1996, BESENDORFER et al. 1997). In the present study, Giemsa C-banding pattern has shown that the chromosomes of *Allium sensescens* ssp. *montanum* have a high amount of heterochromatin, located mostly on telomeres and satellites with an appropriate number of intercalary C-bands. With some exceptions our results are in agreement with those of JOACHIMIAK et al. (1987). The differences mostly concerned the heterochromatin at centromeres that were hardly visible in karyotypes of populations from Biokovo. On the other hand, JOACHIMIAK et al. (1987) obtained centromeric C-bands in Poland populations and KIM et al. (1989) described C-bands near the centromeres as the most widely stained bands in the karyotypes of tetraploid *A. sensecens* (2n = 32) and diploid *A. senescens* var. *minor* (2n = 16) from Korea. Such differences could be explained by a lower amount of constitutive heterochromatin at the centromeric region in the populations from Biokovo. The other explanation could be that alkaline treatment was too destructive for the heterochromatin in this region even when the same Giemsa C-banding technique used by KIM et al. (1989) was applied. The heteromorphism of intercalary and telomeric C-bands visible as different staining intensity between homologue chromosomes was also noticed in present study. This heteromorphism could be connected with recombination events during meiosis, when unequal chromosome exchange may occur. On the other hand, problems with the Giemsa C-banding technique also have to be considered. JOACHIMIAK et al. (1987) also described some variability of C-bands, especially those in the group of the largest chromosomes 1 and 2, where some minor intercalary C-bands were not visible on all chromsomes. Regardless of this, the Giemsa C-banding pattern allowed them to conclude that the Polish populations of *A. montanum* are autotetraploid. On the other hand, it was on the basis of the Giemsa C-banding pattern that KIM et al. (1989) confirmed that the *A. senescens* from Korea was amphidiploid.

The Ag-NORs in *A. senescens ssp. montanum* populations corresponded to C-bands located terminally on submetacentric chromosomes. The maximum number of Ag-NORs at the metaphases was four and corresponded to the maximum number of nucleoli in the interphase cells in all populations studied. However, the number of Ag-NORs in some metaphase plates was lower than the maximum, which could be explained by the fact that silver staining technique visualises NORs functionally active during preceding interphase. In the interphases, the lower number of nucleoli than the maximum (predominant were

cells with 2 and 3 nucleoli) could be a result not only of NOR inactivation but also of the association of active NORs, which then form one nucleolus. The differences in the sizes of Ag-NOR in the chromosomes noticed in the *Allium* species studied here have been described for many plant species. MOSCONE et al. (1995) stated that polymorphism in Ag-NOR size in several species in the genus *Capsicum* could appear, not only between homologue chromosomes within the cell and between the cells of the same individual, but also or even more between individuals. In the present study, no differences in Ag-NOR sizes between individuals within populations or between populations were observed. BESENDORFER et al. (1997) have shown that in *Allium commutatum* the differences in the sizes of Ag-NORs, which appeared between homologue and nonhomologue chromosomes, corresponded with the extension of secondary constrictions. In some metaphase chromosomes of *A. sensescens* ssp. *montanum* with extremely extended Ag-NORs the satellites could not be distinguished from the secondary constrictions. This indicated that ribosomal rRNA genes could be located in both, SCs and satellites. However, fluorescent *in situ* hybridisation (FISH) with the rDNA probe would have to be applied to confirm our assumption. In some chromosomes, silver deposits were located only on a small portion of the extended SC, which implies that only a few clusters of rRNA genes within NORs are active. Different sizes of Ag-NORs, noticed in the *Allium* investigated in this paper, are probably positively correlated with the level of transcription activity due to the requirement of the cells for ribosomes, rather than the domination of some NORs over the other, which is known as nucleolar dominance. Nucleolar dominance was first discovered in plants, and has been described in interspecific and intergeneric hybrids within numerous plant genera (FLAVELL and O'DELL 1979, LACADENA et al. 1984, CHEN and PIKAARD 1997). Therefore, nucleolar dominance could be an indicator of the hybrid origin of certain species.

Taken together, the morphometric data, Giemsa C-banding patterns and the activity of NORs suggest that the *Allium senescens* ssp. *montanum* studied here is autotetraploid with a karyotype very similar to that described by JOACHIMIAK et al. (1987). However, an analysis of meiotic events has to be performed to confirm our assumption. For a better understanding of karyotype evolution mechanisms in *Allium*, we plan comparative analyses of Mediterranean populations and those in continental areas in Croatia with the accent on heterochromatin distribution, detection of NOR position by fluorescent *in situ* hybridisation (FISH) and their activity as well as chromosome pairing during meiosis.

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