Karyomorphological study of *Leucojum aestivum* (*Amaryllidaceae*) in Bulgaria

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Abstract. Twenty-seven populations of *Leucojum aestivum* in Bulgaria were investigated karyologically as part of a broad biotechnological study. Karyotype analysis is a reliable background for subsequent studies of the experimentally obtained explants. All studied populations had chromosome number $2n = 22$. The morphometric and statistical analyses revealed that the karyotype morphology of all populations was constant, with the exception of one telocentric pair which often showed minor length polymorphism in the long arm.

Key words: *Leucoujm aestivum*, idiograms, interpopulation variation, karyotypes

Introduction

Leucojum aestivum L. is an Euro-Asian element distributed throughout Europe, Caucasus, North and Northwest Iran (Mill 1984). In Bulgaria it occurs in seven of all twenty floristic regions, forming relatively old populations with predominating vegetative propagation and different types of alkaloid biosynthesis, i.e. it forms chemoraces (Stefanov 1990).

Leucojum aestivum is an important source of pharmacologically active alkaloids that, so far, have been obtained only from natural populations. At the same time the species is endangered in the Bulgarian flora and its exploitation is restricted. Therefore it has been subjected to a profound biotechnological study for alternative approaches of bioproduction of alkaloids (NATO Project no. SfP 974453). Part of this study is a karyological analysis of plants from the wild populations of *L. aestivum* that are subsequently used for *ex situ* cultivation and in *in vitro* experiments.

So far the karyotype of *L. aestivum* has been the subject of many karyological studies, most of which provide only the chromosome number of the species (see Fedorov 1969; Goldblatt 1981, 1988; Goldblatt & Johnson 1990, 1991, 1998, 2000, 2004). Detailed karyological analysis has been conducted by Stefanov (1990), Bareka & al. (1998), Bareka & al. (1999), D'Amato & Bianchi (1999), and Marcucci & Tornadore (1999). All studies show that the karyotype of *L. aestivum* consists of 11 mostly asymmetrical chromosome pairs.

Karyological data about Bulgarian populations of *L. aestivum* have been published by Stefanov (1990). However, that study does not give information about all known localities of the species, while the current study covers 27 populations of *L. aestivum* in Bulgaria (Table 1) and performs a comparative analysis of their karyotype characteristics.

The main goal was to detrmine the level of karyotypic similarity in the material of wild origin. The results present an opportunity to compare the karyotype characteristics of plants from different chemoraces and can serve as a reliable reference for the changes that occur in the karyotypes of the experimentally obtained explants.

Table 1. List of populations studied.

No	Locality	Geographical coordinates	No	Locality	Geographical coordinates
1.	Gradina	42°08' N, 25°12' E	15	Antimovo 2	44°00' N, 22°56' E
2.	Blatets	42°38' N, 26°32' E	16	Antimovo 3	44°00' N, 22°56' E
3.	Ormana	42°32' N, 26°31' E	17	Archar 1	43°47' N, 23°01' E
4.	Palaouzovo	42°32' N, 26°44' E	18	Archar 2	43°47' N, 23°01' E
5.	Dolnata Ova	41°49' N, 26°08' E	19	Balabana	42°16' N, 26°34' E
6.	Blatoto	42°24' N, 27°40' E	20	Biser	41°52' N, 25°60' E
7.	Vesselie	42°18' N, 27°40' E	21	Vinitsa	42°08' N, 25°08' E
8.	Petkaki	42°15' N, 27°42' E	22	Dolna Topchiya	42°15' N, 26°24' E
9.	Karaagach	42°13' N, 27°45' E	23	Debelata Koriya	42°22' N, 24°47' E
10.	Arkoutino	42°19' N, 27°45' E	24	Lozenski Put II	41°46' N, 26°10' E
11.	Kalinata	42°42' N, 27°40' E	25	Osmar	43°13' N, 26°51' E
12.	Chairi	42°48' N, 27°32' E	26	Kochovo	43°14' N, 26°48' E
13.	Baltata	43°22' N, 28°03' E	27	Zidarovo	42°20' N, 27°24' E
14.	Antimovo 1	44°00' N, 22°56' E			

Material and methods

Plant material was collected from 27 populations (Table 1). Between 10 and 30 plants per population were collected, depending on the population size.

For the karyological study, root tips were pretreated with 0.5 % colchicine at room temperature and fixed in ethanol:acetic acid (3:1) for about 24 h at 4 °C. After hydrolysis in 1 N HCl for 15 min at 60 °C and cytoplasm clearing in 1N HCl:diethyl ether (1:1) the root meristem was stained in Schiff's reagent for 2 h. After washing in SO_2-water, the root tips were squashed in 45 % acetic acid, and after freezing and dehydration in 96 % ethanol, permanent slides were obtained through mounting in Entellan.

For the comparative analysis of the karyotypes five well spread metaphase plates from each population were measured, using the image analysis software MicroImage 4.0 (Media Cybernetics, L.P., USA). The lengths of the short (S) and long (L) arms, and the satellites (Sat) were measured. The original values were normalized on the basis of 200 % for the total karyotype length. These values were used to calculate the means and the standard deviation of the length of the short and long arms, the satellites and the total chromosome length (T). For calculation of the centromeric index (Ci), the following formula was used: Ci = (L/T) x 100. The chromosomes are arranged in the idiograms in a descending order, strictly following the values of T. The chromosome types were classified according to Levan & al. (1964). The idiograms were drawn with MS Paint for Windows 98.

For karyomorphometric analysis, we explored the variation of the total chromosome length (T). This value was subjected to One-Way ANOVA analysis (Statistica 6.0, StatSoft, USA).

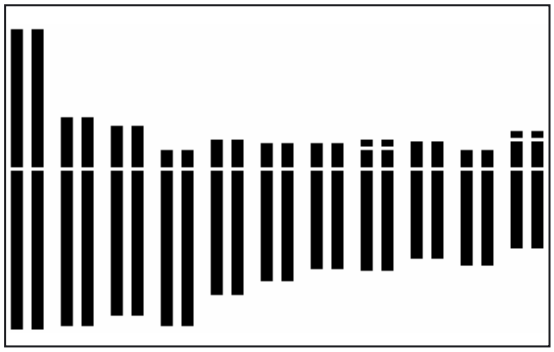
Results and discussion

The karyotypes of all studied populations correspond to the formula $2n = 2m + 1st + 2t + 2sm\text{-}S - AT + 1st\text{-}SAT = 22$. All studied populations had chromosome number $2n = 22$ in contrast to Heitz (1926) and Dobeš & al. (1997), who found the chromosome number $2n = 20 - 24$, and Magulaev (1986), who registered 24 chromosomes. No differences in the number of SAT-pairs were registered, in contrast to Bareka & al. (1999). B-chromosomes were also absent in the Bulgarian populations.

The karyotype is highly asymmetrical in shape (Fig. 1). The longest chromosome pair is metacentric and ranges from 17.52 to 18.42 µm, while the shortest one (5.65–6.37 µm) is submetacentric, with satellites. The majority of the chromosome pairs are subtelocentric and their length varies from 6.77–12.06 µm. Only one pair (number 4 in the idiogram) is telocentric, with Ci = 89.05 – 91.71 varying among populations. This chromosome pair shows very often minor structural heterozygosity with respect to the relative size of the long chromosome arm (Fig. 2). The length varies from 8.85 (Gradina population) to 9.70 (Debelata Koriya population), i.e. approx. 9 % difference. A similar phenomenon was detected by Bareka & al. (1998) and Bareka & al. (1999) in Greek populations. Other variations in the chromosome shape, within and between the studied populations, were not detected (Table 2).

ANOVA of the total chromosome length (T) showed statistically significant differences ($F_{26, 243} = 1.79$; $p<0.05$) only for chromosome pair 11 (Table 3). In all remaining pairs interpopulation variation was not significant.

The high karyotypic homogeneity indicates a high karyotypic stability in the material from natural sources and provides a reliable basis for biotechnological experiments. The genetic variability (somaclonal variation) as observed in the *in vitro* produced tissue cultures is due to several mechanisms: endomitosis,



	1	2	3	4	5	6
Sat						
S	7.98 ± 0.44	2.90 ± 2.34	2.35 ± 0.14	0.99 ± 0.13	1.57 ± 0.30	1.43 ± 0.18
L	9.24 ± 0.46	8.96 ± 0.43	8.40 ± 2.24	9.13 ± 1.06	7.22 ± 0.33	5.14 ± 0.55
T	17.23 ± 0.77	11.87 ± 2.43	10.76 ± 2.25	10.12 ± 1.10	8.80 ± 0.53	7.80 ± 0.60
Ci	53.65 ± 1.43	77.37 ± 10.09	76.26 ± 9.97	90.19 ± 1.40	82.20 ± 2.37	81.56 ± 1.80
	7	8	9	10	11	
Sat		0.42 ± 0.06			0.45 ± 0.14	
S	1.41 ± 0.11	1.06 ± 0.2	1.47 ± 0.18	0.99 ± 0.12	1.47 ± 0.16	
L	5.72 ± 0.46	5.75 ± 0.4	5.14 ± 0.55	5.48 ± 0.25	4.54 ± 0.34	
T	7.14 ± 0.43	6.85 ± 0.59	6.62 ± 0.68	6.47 ± 0.31	6.37 ± 0.42	
Ci	80.12 ± 2.10	84.08 ± 3.13	77.72 ± 1.74	84.72 ± 1.58	71.31 ± 3.60	

Fig. 1. Idiogram and chromosome measurements in the Chairi population of *L. aestivum*.

Table 2. Range of variation of the centromeric index of the chromosome pairs among populations.

Chromosome type	Centromeric index	
	min	max
m	52.41	55.30
sm	69.64	74.98
st	76.14	81.56
	76.77	85.83
	77.05	79.89
	77.37	82.55
	77.70	80.12
	81.56	85.12
	82.11	84.38
	83.00	87.03
t	89.05	91.71

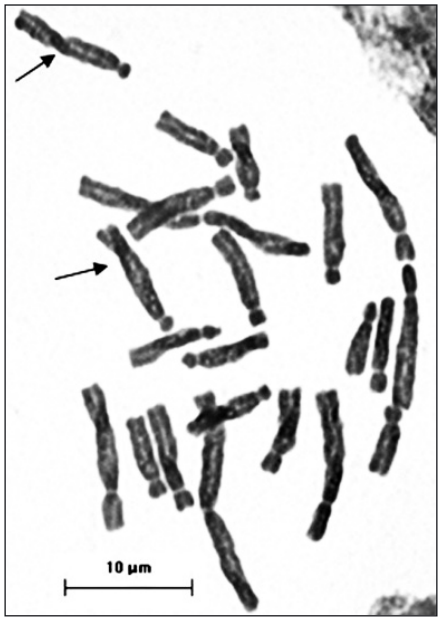


Fig. 2. Root tip metaphase of a plant from the Antimovo population of *L. aestivum*. Heterozygosity in the telocentric (t) chromosome pair is indicated (LA = 9.40 µm and LB = 8.30 µm).

Table 3. Summary for ANOVA based on total chromosome length (T) of an 11 chromosome pairs set; *statistical significance level < 0.05.

Chromosome pair		Sum of squares (SS)	Degrees of freedom	Mean square (MS)	F	P
1	population	17.81	26	0.69	1.3	0.136456
	Error	124.97	243	0.51		
2	population	8.06	26	0.31	1.4	0.086534
	Error	52.62	243	0.22		
3	population	9.29	26	0.36	1.2	0.196819
	Error	69.70	243	0.29		
4	population	9.47	26	0.36	1.5	0.059176
	Error	58.71	243	0.24		
5	population	6.98	26	0.27	1.16	0.276214
	Error	56.25	243	0.23		
6	population	4.30	26	0.17	0.79	0.755450
	Error	50.77	243	0.21		
7	population	5.18	26	0.20	1.22	0.222831
	Error	39.86	243	0.16		
8	population	4.96	26	0.19	1.32	0.142652
	Error	35.04	243	0.14		
9	population	4.97	26	0.19	1.29	0.161284
	Error	35.91	243	0.15		
10	population	3.39	26	0.13	1.05	0.398850
	Error	30.07	243	0.12		
11	population	8.201	26	0.315	1.79	0.012908*
	Error	42.820	243	0.176		

chromosomal aberration, DNA amplification, transposable elements, and point mutations. Polyploidy in the form of endoreduplicated cells is frequently induced in cultures via two essential mechanisms: chromosome endoreduplication and spindle failure leading to restitutional mitosis (D'Amato 1985). Larkin and Scowcroft (1981) suggested that chromosome structural changes could be a major mechanism to generate somaclonal variation. Chromosome type aberrations result from breakage and reunion of broken chromosome ends in the pre-DNA synthesis phase (G_1) of the cell cycle (Kihlman 1966).

Stanilova & al. (1994) have established $2n = 22$ in *in vitro* regenerants of *L. aestivum*. Our initial karyological study of callus cultures obtained from *L. aestivum in vitro* reveals some disturbances in the mitotic division. They result mainly in chromosome breakages and formation of micronuclei. Further comparative morphological studies of the karyotype of the tissue and organ cultures can reveal the frequency of somaclonal variation in *in vitro* regenerated *L. aestivum*.

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