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Genetic Variability of Callus Cultures of Some *Iris* Species

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Abstract–Six species of *Iris* L. genus *(I. setosa, I. ensata, I. oxypetala, I. pseudocorus, I. pumila,* and *I. laevigata)* were comparatively studied using the RAPD and cytogenetic analyses of genomes of their callus cultures and intact donor plants. The closest genetic similarity was observed in callus cultures derived from the same donor plant and the least similarity, in calli from the plants of different genera. During culturing, genetic changes occur on interspecies and intraspecies levels, and the extent of these changes seems to depend on species. A cytogenetic analysis of callus cultures and donor plants of the six *Iris* species showed that the modal class of cell population is constituted by the cells with the diploid chromosome set of parent donor plant. Hence, the *in vitro* cell populations retain the basic genome features of the species despite their significant genetic heterogeneity.

Key words: callus culture, donor plant, genetic variability, Iris genus

INTRODUCTION

Iris genus comprises more than 300 species distributed all over the temperate and partially subtropical climate zones of the Northern Hemisphere.[2] Many of them are common ornamental plants [1, 2]. Some species of this genus, such as *I. sibirica* L., *I. pallida* Lam., and *I. germanica* L., accumulate in their roots essential oils, whose terpenoid ketone constituent irone has a strong and lasting violet smell widely used in perfumery and cosmetics [1, 3–8]. Several plants of *Iris* genus (more exactly the overground parts of *I. setosa* Pall. ex Link, *I. ensata* Thunb., *I. laevigata* Fish. et Mey., *I. pseudocorus* L. [9], and *I. lactea* Pall.) serve as raw material for producing biologically active substances, e.g., mangiferin, on the basis of which the Alpisarin preparation with antiviral activity is produced [10, 11]. Several *Iris* species are endemic (*I. pumila* L. [12], and *I. nigricans* Dinsm. [13]) or relict (*I. oxypetala* Bunge [14]) and need environmental protection.

Ten species of *Iris* genus inhabit the Russian Far East, with four of them (*I. ventricosa* Pall., *I. pseudacorus* L. (*I. maackii* auct., non Maxim.), *I. insata* Thunb. (*I. kempferi* Sieb. ex Lem.), and *I. oxypetala* Bunge (*I. lactea* Pall.)) being rare [15].

In general, irises are vegetatively propagated using rootstocks and bulbs, which limits the number of reproduced plants. For example, *I. hollandica* usually yields on an average 5 daughter bulbs a year. This reproduction rate requires ten years to accumulate the material in amounts sufficient for commercial use. The cross-pollination and extremely long seed germination of many species (from 2 to 5 years) makes the seed reproduction inefficient; moreover, it can additionally lead to splitting the desired characteristics of plant [7]. Currently, a more efficient reproduction of irises uses the *in vitro* tissue culturing along with the traditional vegetative propagation. The plants of several species are regenerated from callus tissues [13, 16–21], protoplasts [13, 22–25], or suspension cultures [19, 26–28].

The goal of this work was the study of genetic variability of callus cultures of the *Iris* genus plants. To this end, the chromosome numbers were determined and the genomic DNA of these cultures and donor plants were analyzed using RAPD. Previously, we successfully used this method to differentiate *Iris* species [29], reveal the intraspecies genetic variability of *I. setosa* from various regions of Russian Far East [30, 31], and estimate the genetic polymorphism in the intact plants and callus lines of *Panax ginseng* C.A. Meyer [32, 33].

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[2]Abbreviations: CTAB, cetyltrimethyl ammonium bromide; PCR, polymerase chain reaction; and RAPD, randomly amplified polymorphic DNA.

EXPERIMENTAL

Subjects of investigation were eight callus lines obtained in our laboratory and donor plants of six iris species: *I. setosa* (sample no. 1) and its callus culture (sample no. 2) [34]; *I ensata* (sample no. 3) and two morphologically different callus lines obtained from its different explants (sample nos. 4 and 5); *I. oxypetala* (sample no. 6) and its callus line (sample no. 7) [35]; *I. pseudacorus* (sample no. 8) and two morphologically different cell lines of it (sample nos. 9 and 10); *I. pumila* (sample no. 11) and its callus culture (sample no. 12); *I. laevigata* (sample no. 13) and its callus culture (sample no. 14). The samples of the intact plants were gathered in the Botanic Gardens of the Far East Division of Russian Academy of Sciences (Vladivostok), except for *I. oxypetala* that was gathered in its natural habitat (Putsilovka village, Primorskii Krai).

DNA was isolated from lyophilized cell biomass or intact plant leaves using CTAB buffer according to procedure [36] with the following modifications: after CTAB buffer was added for extraction, the samples were incubated at 56°C for 2.5–3 h and precipitated with the CTAB buffer for 2–3 h at room temperature. The obtained DNA was analyzed using 1% agarose gel with ethidium bromide in TBE buffer. The amounts of DNAs were measured by comparison of the band intensities from plant cells and from phage λ of a known concentration.

PCR was carried out in an UNO II 48 (Biometra, Germany) thermocycler using the arbitrarily sequenced tennucleotide primers from Operon (United States). The reaction mixture and the temperature regime were as we previously described [33]. The control sample contained the full amplification mixture except for DNA added. Every measurement was repeated 2–4 times. PCR products were analyzed by electrophoresis in 1.4% agarose gels and photographed in UV light.

The DNA fragment (amplicone) sizes were measured using *Eco*RI + *Hind*III restricts of phage λ DNA (Fermentas, Lithuania) as molecular mass markers.

The amplicones were designated according to the names of primers used for their obtaining and also the sizes in base pairs (bp) they contain.

Data of the RAPD analysis were statistically processed using a comparative computer analysis of the RAPD spectra bands of the studied samples. Electrophoregram negatives were scanned by a Umax Astra 3450 scanner, and then digitalized using a RFLPscanPlus 3.12 program. Only replicable fragment bands were taken into account, and the polymorphism of band intensities was left out of consideration. Dendrograms of the genetic similarity of the studied iris callus cultures and donor plants (UPGMA, NTSYS-pc version 1.7) [37] were constructed on the basis of matrices of D values using the bootstrap procedure (1000 replicas).

Cytogenetic studies were carried out by the conventional procedure of crushed preparations [38]. Metaphase cells from various callus sites were analyzed after 30-day culturing at the stages of various passages (repeated thrice), whereas root apical meristem was investigated for the whole plants. The material was fixed in a 3 : 1 acetic acid–ethanol mixture, then kept in 0.1 N HCl for 15 min, and stained with acetocarmine dye.

RESULTS AND DISCUSSION

The somaclonal variation is well known to arise and cause a genetic variability during the *in vitro* culturing of plant cells and tissues. We chose 7 primers efficient for the iris DNA for studying the genetic variability of iris callus cultures (Table 1). We previously used the OPB-12, OPD-08, OPD-11, and OPD-13 primers to reveal the interspecies polymorphism in the *Iris* genus plants [29], and Shimitzu *et al.* used OPA-01 and OPA-15 for the RAPD analysis of the *I. ensata* and *I. germanica* regenerants derived from protoplasts [25].

All the primers used initiate the syntheses of specific sets of RAPD fragments and permit the differentiation of DNAs for the iris species (Fig. 1). Note that some amplicones in the RAPD spectra of iris DNA—e.g., OPA-15-870 (Fig. 1*a*) and OPA-01-1120 (data not shown)—are common for all the six iris species. Some amplicones are characteristic of several species; e.g., OPB-12-1020 is present in all species, except for *I. ensata* and *I. pumila* (Fig. 1*c*). These common amplicones differ only in the intensity of their bands in the RAPD spectra, which is also characteristic of the RAPD fragments of callus lines and intact

plants of *Panax ginseng* [32, 33]. One of the possible explanations of this polymorphism could be a different copy number of the corresponding DNA loci in the samples under study [39, 40].

Table 1. Characteristics of primers used

Primer	Nucleotide sequence (5'→3')	Number of polymorphic/allowed loci					
		I. setosa	*I. ensata*	*I. oxypetala*	*I. pseudacorus*	*I. pumila*	*I. laevigata*
OPB-12	CCTTGACGCA	0/11	2/10	3/14	4/15	10/13	14/19
OPD-08	GTGTGCCCCA	4/13	3/13	7/12	3/15	10/17	15/19
OPD-11	AGCGCCATTG	6/19	5/12	4/16	5/13	14/22	17/22
OPD-13	GGGGTGACGA	2/6	6/15	2/15	5/13	11/16	9/16
OPA-01	CAGGCCCTTC	3/13	12/17	0/12	2/12	9/17	6/15
OPA-15	TTCCGAACCC	1/10	2/15	6/19	6/13	7/13	8/14
OPA-17	GACCGCTTGT	8/19	6/16	3/13	4/7	10/14	6/10
Total		24/91	36/98	36/98	29/88	71/112	75/115

A comparison of DNAs from callus cultures and donor plants showed that they display similar but not identical RAPD spectra. The number of loci detected by each primer in DNA of various species (allowed loci) and the loci whose callus lines differ from those of donor plants (polymorphic loci) are given in Table 1. The proportion of polymorphic loci varies in different species: it is 26% for *I. setosa*, 37% for *I. ensata*, 25% for *I. oxypetala*, 33% for *I. pseudacorus*, 63% for , *I. pumila*, and 65 % for *I. laevigata*. Some polymorphic amplicones are present only in the callus RAPD spectra, for example OPA-17-1294 in *I. ensata*, OPB-12-860 in *I. laevigata*, and OPB-12-1135 in *I. pseudacorus*, whereas the others (e.g., the major fragments of OPA-17-1080 in *I. setosa* and OPA-17-900, OPB-12-580, OPB-12-490, and OPB-12-380 in *I. pumila)* can be detected only in the amplified fragments of donor plants (Fig. 1). Significant differences were found between the callus and donor plant RAPD-spectra for *I. pumila* and *I. laevigata* species (Fig. 1). A similar picture was observed when iris DNA was amplified with the other used primers.

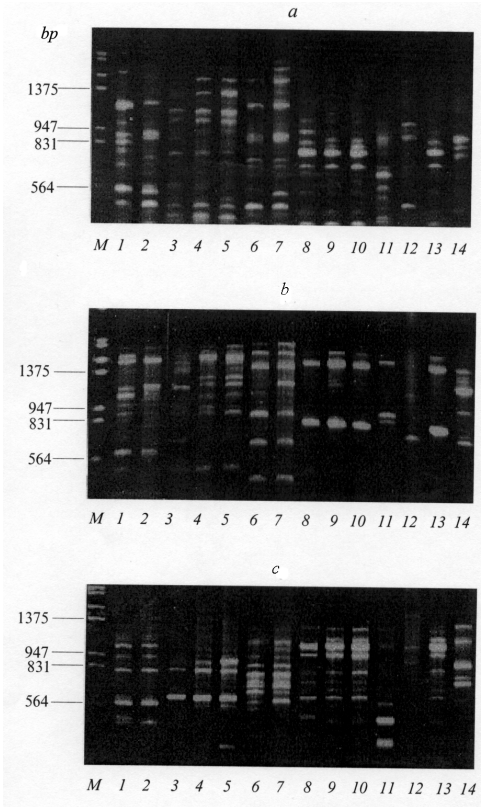

Fig. 1. Amplification products for DNAs of the callus cultures and intact plants of six iris species obtained with the help of (*a*) OPA-15 primer, (*b*) OPA-17 primer, and (*c*) OPB-12 primer; bands: *1*, *I. setosa* plant and *2*, callus culture; *3*, *I. ensata* plant and *4*, *5*, callus cultures; *6*, *I. oxypetala* plant and *7*, callus culture; *8*, *I. pseudacorus* plant and *9*, *10*, callus cultures; *11*, *I. pumila* plant and *12*, callus culture; *13*, *I. laevigata* plant and *14*, callus culture; *M*, *Eco*RI + *Hind*III restricts of phage λ DNA.

These results indicate some genetic changes that occur during the introduction into an *in vitro* culture and continuous culturing of the *Iris* spp. These changes involve, on the one hand, the structures of loci revealed by the used primers in the donor plant DNA and, on the other hand, the sequences outside these loci, which results in new amplicones appeared in addition to the majority of fragments characteristic of donor plant DNA. Note also that plant DNA is often subjected to variations when analyzed by RAPD.

Table 2. Total matrix of genetic similarity (D) between the studied samples

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	1.0000													
2	0.8481	1.0000												
3	0.3537	0.3311	1.0000											
4	0.3681	0.3593	0.8077	1.0000										
5	0.3879	0.3905	0.7721	0.9540	1.0000									
6	0.3636	0.3195	0.2658	0.3103	0.3295	1.0000								
7	0.3669	0.3468	0.2716	0.3146	0.3333	0.8778	1.0000							
8	0.3490	0.3268	0.3098	0.3291	0.3375	0.3875	0.3902	1.0000						
9	0.3006	0.3057	0.2603	0.3086	0.3171	0.3658	0.3809	0.3809	1.0000					
10	0.2895	0.2949	0.2483	0.2733	0.2822	0.3681	0.3712	0.8299	0.9536	1.0000				
11	0.3226	0.3648	0.2973	0.3171	0.3253	0.3735	0.3647	0.2667	0.2597	0.2614	1.0000			
12	0.2631	0.3333	0.2483	0.3478	0.3681	0.2945	0.2874	0.2585	0.3046	0.2933	0.5359	1.0000		
13	0.3087	0.3660	0.2535	0.2911	0.3125	0.3250	0.3049	0.6111	0.5811	0.5850	0.2400	0.2721	1.0000	
14	0.3291	0.3580	0.2252	0.2515	0.2722	0.3077	0.3006	0.3529	0.3312	0.3461	0.2264	0.2564	0.5098	1.0000

Note: 1–14 are the DNA samples (cf. the Experimental section).

The matrices for the genetic distances (D) for all six *Iris* species and their callus cultures were calculated on the basis of RAPD analysis with 7 primers (273 loci in total) (Table 2). The results of the cluster analysis performed by the UPGMA method with the use of bootstrap procedure are shown in Fig. 2. The bootstrap indices that indicate the data conformity and serve as an internal criterion of their reliability varied from 24 to 100%. The branches in the sites with less than 50% values cannot be regarded as reliable.

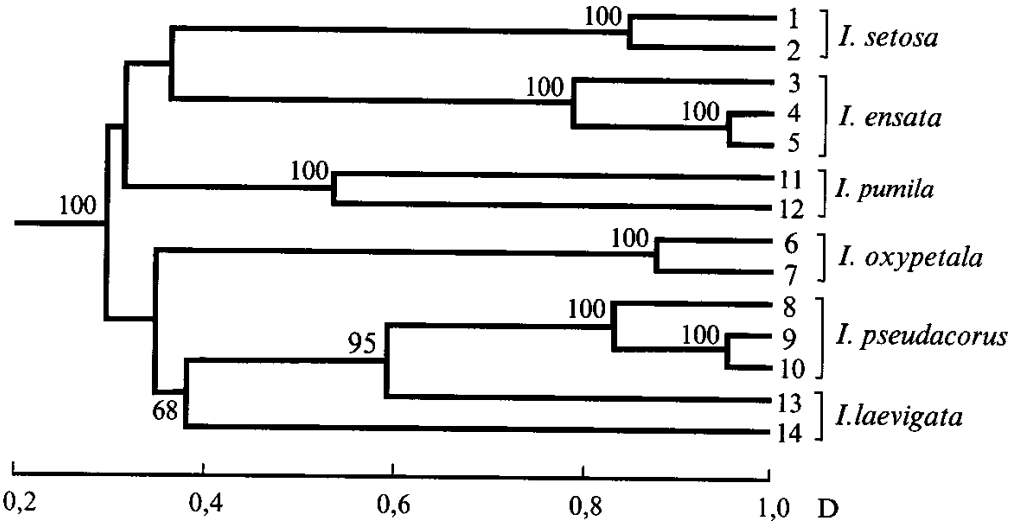

Fig. 2. Dendrogram of the genetic similarity of callus cultures and the corresponding donor plants.

A dendrogram of the genetic similarity indicates that all the tested samples are reliably separated according to their species (Fig. 2). The average interspecies distances are 0.240–0.6111 (Table 2). The callus lines obtained from the same donor plant have the closest similarity: the corresponding D values for the samples of *I. ensata* (nos. 4 and 5), and *I. pseudacorus* (nos. 9 and 10) are 0.9540 and 0.9536, respectively. The similarity indices for the callus cultures of different species correlate with those for the corresponding donor plants (Table 2).

The *in vitro* genetic variability values for the *I. setosa*, *I. oxypetala*, and *I. pseudacorus* species correlate in scale with the intraspecies variability (D values are from 0.7721 to 0.8778). The callus lines of *I. pumila* and *I. laevigata* significantly differ from the donor plants (see Table 2 and Fig. 2); their genetic variability values correspond to the interspecies variability (D = 0.5359 and 0.5098, respectively). The fact that some plants have the values of genetic variability close to the interspecies variability when cultured *in vitro* was

first observed at a comparative genome study of the intact plants and cell cultures of *Crepis capillaris* L. and *Rauwolfia serpentina* Benth. by the methods of restriction analysis and blot-hybridization [41]. The authors reported that a continuous *in vitro* culturing is accompanied by genomic changes (changes in repetition fractions), whose scale exceeds the level of the interspecies variability. Our genetic analysis of the cell lines and intact ginseng plants [32, 33] revealed only the changes of the intraspecies level. In this study of representatives of the *Iris* genus, we found that the genetic changes of both inter- and intraspecies levels may occur during the culturing and their intensity depends on the species used.

Chromosome number of a species is one of its most important cytological characteristics, which is employed for answering the questions of the species taxonomy, phylogeny, genetics, and some practical selection problems. All the whenever observed chromosome numbers (2n) for each plant are listed in a handbook of chromosome numbers of flowering plants [42] regardless of whether a somatic or generative organ of plant was a source of the analyzed cell. The majority of the representatives of *Iris* genus have a considerable variability in respect of their chromosome numbers. For example, *I. germanica* can have 24, 36, 44, 48, or 60 chromosomes; *I. pseudacorus,* 24, 30, 32, or 34; *I. pumila,* 30 or 32; *I. setosa,* 34, 36, or 38; and *I. versicolor,* 72, 84, 100, 105, 108, or 112 chromosomes. However, several iris species demonstrate no variations in chromosome numbers: *I. kaemferi,* 24; *I. klattii,* 44; *I. laevigata,* 32; *I. nigricans,* 20, and *I. sibirica,* 28 [42].

Table 3. The determined ploidy of intact plant cells and cell cultures, %

	I. setosa		*I. ensata*		*I. oxypetala*		*I. pseudacorus*		*I. pumila*		*I. laevigata*	
	1	2	3	4	6	7	8	9	11	12	13	14
Diploid	60	38	36	38	45	45	42	43	43	36	35	36
Hypodiploid	16	37	52	40	20	24	46	44	30	63	62	53
Hyperdiploid	24	25	12	20	35	31	11	13	27	9	3	11
Aneuploid	40	58	54	50	28	28	38	34	51	66	44	48

Note: 1, 3, 6, 8, 11, 13 are the samples of intact plants; 2, 4, 7, 9, 12, 14 are the callus culture samples.

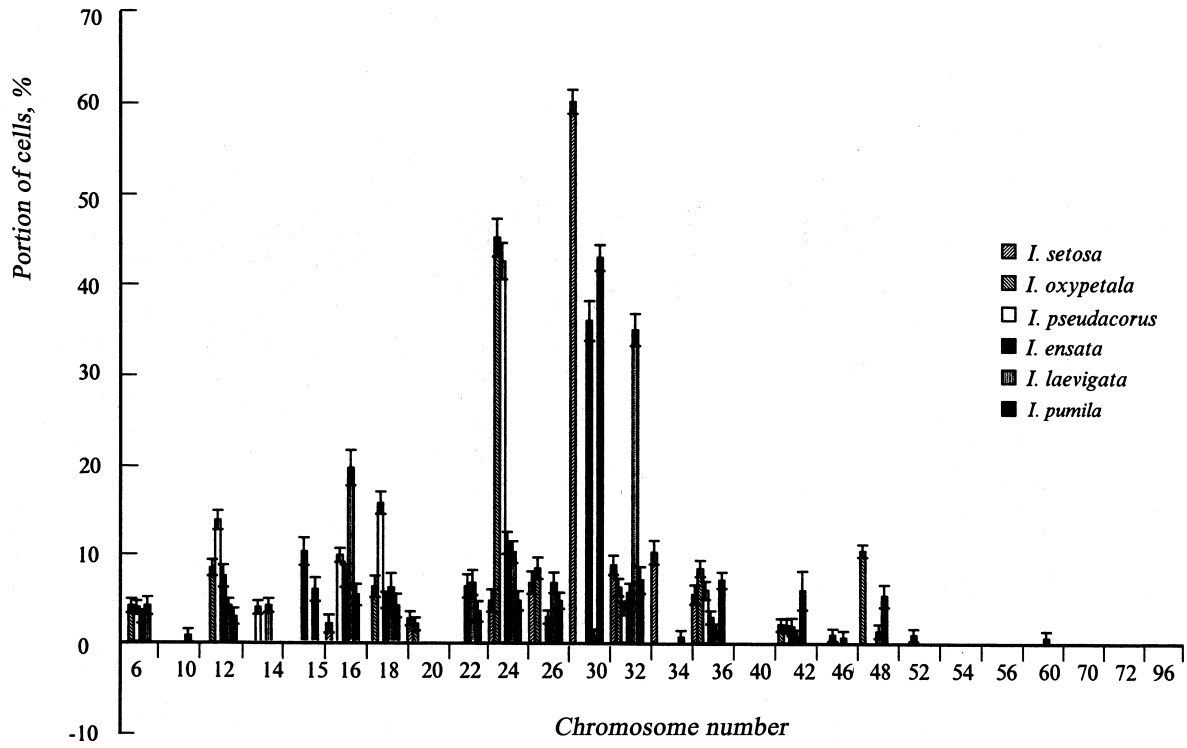

Fig. 3. Distribution of chromosome numbers in the cells of apical meristem of the donor plant roots.

A collection of the *I. setosa* plants gathered in 1995–1998 in various areas of the Russian Far East was established in our Institute. Their chromosome numbers (2n) were as follows: 30, 32, and 34 for the plants

from Primorskii krai; 30, for Sakhalin plants; 38, for plants from Amurskaya oblast, Magadan and Chukotka; 30, for plants from Kuriles Kunashir, Shikotan, Chirpoya, Ketoya, and Rasshua; 30 and 32 for plants from Urup; 32, for plants from Shiashkotan and Anekotan; 30 and 34, for plants from Paramushir and Green island; and 32, 32, and 36, for plants from Simushir. Thus, not only high morphological and ecological variability level [13], but also a strong genetic variability in respect of both DNA structure [30, 31] and chromosome numbers (2n = 30, 32, 34, 36, and 38) are characteristic of *I. setosa*. A wide distribution of the species over the Asian continent (Middle and East Siberia, Russian Far East, and the North Pacific islands) is probably due to this characteristic. High genome pliability serves as one of the protective mechanisms against unfavorable environmental and stress factors in plants with their attached way of life; it is efficient for both individual organisms and the whole population.

Our cytogenetic analysis of the apical meristem cells of the donor plant roots, which were a source of primary callus, resulted in establishing their chromosome numbers (2n): 30 for *I. setosa,* 30 for *I. ensata*, 24 for *I. oxypetala*, 24 for *I. pseudacorus*, 30 for *I. pumila*, and 32 for *I. laevigata*. A mixoploidy (the chromosome numbers from 6 to 60) with the predominance of the diploid chromosome set (35–60%) is characteristic of the investigated iris species. A frequency of the cell occurrence with other than diploid chromosome sets and their ploidy varied (Fig. 3, Table 3). Note that the aneuploid cells are frequent (28–54%) among the studied plant cell populations, which is characteristic of the vegetatively propagated and apomictic species.

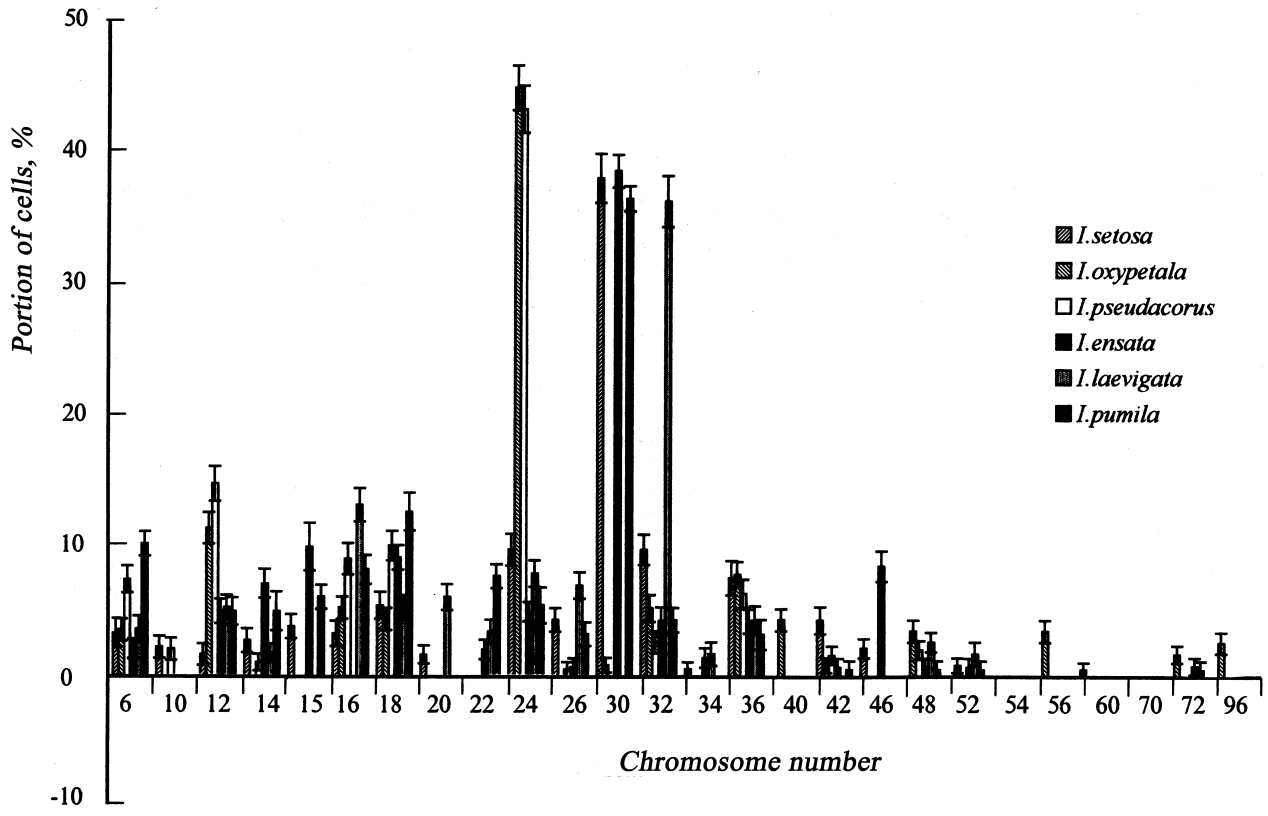

Fig. 4. Distribution of chromosome numbers in the metaphase cells of callus cultures.

It is well known that considerable structural and functional changes, predominantly chromosome aberrations, occur in cells introduced into the *in vitro* culturing [33, 43–46]. The mixoploidy with a broad range (6–96) of chromosome numbers (Fig. 4) is also characteristic of the studied iris populations of cultured cells. At the introduction into the *in vitro* culture, the modal class contains diploid cells with the chromosome number equal to that of the corresponding donor plant. The distribution of the cells according to their chromosome numbers is represented in Table 3. The fraction of hypoploid cells in *I. setosa* and *I. pumila* increases twofold and the portion of hyperploid cells threefold decreases in *I. pumila,* whereas it 3.5 times increases in *I. laevigata* without any significant changes in the portion of aneuploid cells. However, despite some changes, the structure of the cell population in callus cultures mainly remain the same as in the donor plants of the corresponding species.

Thus, we studied the representatives of 6 iris species and established with the help of RAPD analysis that they undergo some genetic changes during the *in vitro* culturing. These changes involve the primary DNA sequence, and their level corresponds to both intra- and interspecies variability levels. The iris tissue cultures predominantly remain diploid and retain the basic features of the species as a whole, and the individual genotype of donor plant, in particular.

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