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DNA PLOIDY LEVELS AND INTRASPECIFIC DNA CONTENT VARIABILITY IN ROMANIAN FESCUES (*FESTUCA*, *POACEAE*) MEASURED IN FRESH AND HERBARIUM MATERIAL

Petr Šmarda

Department of Botany, Faculty of Science, Masaryk University in Brno, Kotlářská 2, CZ-611 37 Brno, Czech Republic; e-mail smardap@sci.muni.cz

Abstract: DNA ploidy level estimates are presented for 11 species and two natural hybrids of *Festuca* sampled from 39 locations in Romania. Altogether 48 living samples (22 of them also as one-year-old herbarium specimens) and additional 65 one-year-old herbarium specimens were analyzed using flow cytometry with DAPI staining. The following DNA ploidy levels were assessed: *F. callieri* (4x), *F. ovina* (2x), *F. pallens* (2x), *F. polesica* (2x), *F. pseudodalmatica* (2x, 4x, 5x), *F. pseudovaginata* (2x), *F. pseudovina* (2x), *F. pseudovina* × *F. rupicola* (4x), *F. rupicola* (6x), *F. vaginata* (2x), *F. vaginata* × *F. valesiaca* (2x), *F. valesiaca* (2x) and *F. xanthina* (2x). *Festuca pseudovaginata* is reported for the flora of Romania for the first time.

Measurements of one-year-old herbarium specimens produced significantly smaller sample/standard ratios ($P < 0.001$) and higher coefficients of variance ($P < 0.001$) when compared with fresh plant samples. Several species exhibited marked intraspecific nuclear DNA content variability, documented by non-overlapping double-peaks or bimodal peaks in simultaneous measurements. A maximum difference of about 9.2% in relative DNA contents was observed in *F. pallens*, 5.5% in *F. polesica*, 4.2% in *F. vaginata*, and 3.8% in *F. rupicola*. Diploid *F. xanhina* (*Festuca* sect. *Eskia*) had 1.39–1.58 times higher monoploid relative DNA content in relation to the species of section *Festuca*.

Keywords: Balkan Peninsula, *Festuca ovina* group, *Festuca valesiaca* group, *Festuca* sect. *Eskia*, Flow cytometry, Intraspecific genome size variability, Polyploidy

INTRODUCTION

Many intricate taxonomical groups of *Festuca* L. are composed of polyploid series with only minute morphological and anatomical differences. Ploidy level is one of the most important species characteristics and detailed karyological knowledge has become essential in *Festuca* taxonomy. Studies in species-rich areas in France and Spain, resulting in the description of many new taxa, are good examples showing an effective combination of detailed karyological surveys and classical taxonomical methods (AUQUIER & KERGUÉLEN 1978, FUENTE GARCIA & ORTÚÑEZ RUBIO 1998). Intraspecific variability in the ploidy level is the first indicator for the taxonomic heterogenity of the material studied. Karyological data help clarify the distribution of many taxa, and in a wider population context they may indicate hybrids or rare polyploidization events (ŠMARDA et al. 2005). In contrast to well-documented species-rich areas in the Iberian Peninsula and Central Europe (AUQUIER & RAMELOO 1973, KERGUÉLEN & PLONKA 1989, FUENTE et al. 2001, ŠMARDA & KOČÍ 2003, ŠMARDA et al. 2005), karyological data on *Festuca* species from the Balkan Peninsula are still scarce with

the only detailed survey made by KOŽUHAROV & PETROVA (1991) from Bulgaria. Out of 32 species occurring in Romania (BELDIE 1972, MARKGRAF-DANNENBERG 1980), ploidy levels were documented so far only for *F. arundinacea* SCHREB. (RAICU et al. 1974), *F. airoides* LAM. (EHRENBERGEROVÁ 2001; sub *F. supina* SCHUR), *F. xanthina* DC. (STARLINGER et al. 1994), and *F. rupicola* HEUFF. (ŠMARDA et al. 2005).

The recent increase of karyological data in plants was enabled by the wide application of flow cytometry. It is a fast and cheap method for determining DNA ploidy levels or exact nuclear DNA contents using only a small piece of fresh plant material, and has recently become a popular method in many research applications (SUDA 2004, DOLEŽEL & BARTOŠ 2005). Flow cytometry is also one of the most powerful means to detect intraspecific genome size variability (GREILHUBER 2005, DOLEŽEL & BARTOŠ 2005). Many earlier works dealing with intraspecific genome size variability suffered from methodical errors (GREILHUBER 1998, GREILHUBER 2005), and only a few species have recently been accepted as variable in genome size (GREILHUBER 2005). Simultaneous measurement of two samples yielding a non-overlapping double peak is considered as reliable evidence for a difference in their DNA contents, and thus, for intraspecific variability (GREILHUBER 2005). Differences of around 4% or higher may be detected by this procedure (DOLEŽEL & GÖTHE 1995).

In *Festuca*, flow cytometry has been successfully employed by HUFF & PALAZZO (1998), ARUMUGANATHAN et al. (1999), and by WALLOSEK (1999). More recently, it has been shown that some genera can also be analyzed from dehydrated tissues (SUDA 2004, SUDA & TRÁVNÍČEK 2006), as is the case also for herbarium specimens of *Festuca* (ŠMARDA et al. 2005, ŠMARDA & STANČÍK 2006). The measurement constraints of flow cytometry using herbarium specimens, however, are still poorly known, and should be explored in future studies.

The main aim of the presented study was to complete karyological data for critical taxa of some xerophilous Romanian fescues. Comparisons of flow cytometric measurements in fresh plants and herbarium specimens, and investigation of intraspecific relative genome size variability were of particular interest.

MATERIAL AND METHODS

Plant samples collected in Romania in 2003 originated from rocky steppes, loess steppes and sands. Both living plants and herbarium specimens were studied. Plants were cultivated in the experimental garden of the Faculty of Education, Masaryk University in Brno. Voucher specimens of all samples studied are deposited at BRNU. Localities of individual taxa are listed in the Results, and arranged as follows: administrative region ("Judeţul"), the nearest town/village: locality description, habitat, abundance of the species, geographical coordinates, altitude, collection date, and sample numbers in parentheses. The sample numbers are preceded by either "F" indicating that fresh (cultivated) material was used for flow cytometric analyses, or by "H" indicating that only herbarium specimens were analyzed. The denotation "#F" is used for samples where DNA ploidy was determined from both fresh (cultivated) and herbarium material. Geographical coordinates and altitudes were obtained in the WGS84 coordinate system using a GPS instrument (Garmin-eTrex).

DNA ploidy level was assessed by flow cytometric measurements on a PA-I Partec ploidy level analyzer at the Department of Botany, Masaryk University in Brno. Young, basal parts of green leaves were used from both fresh plants and herbarium specimens. A modification of a two-step procedure (OTTO 1990) for plant material (DOLEŽEL & GÖTHE 1995) was used to prepare the samples. A piece of plant material was chopped using a sharp razor blade together with a standard in a glass Petri dish containing 0.5 ml Otto I buffer (0.1M citric acid, 0.5% Tween 20). An additional 0.5 ml Otto I buffer was added. The crude nuclei suspension was filtered through a 50 µm nylon mesh. 1 ml of Otto II buffer (0.4M Na_2HPO_4 .$12H_2O$) supplemented with 4',6-diamidino-2-phenylindole (DAPI) at final concentration 4 µg/ml was then added to the nuclei suspension. The youngest leaves of *Lycopersicon esculentum* MILL. "Stupické polní tyčkové rané" (2C = 1.96 pg; DOLEŽEL et al. 1992) were used as an internal standard for measurements of fresh and herbarium samples. Samples were measured once or twice on different days, with the final standard/sample ratio calculated as the mean value. Peak positions and coefficients of variance (CV) were calculated using Partec software incorporated in the flow cytometer used. Data on the number of investigated samples, standard/sample ratios and CVs are given (separately for fresh and herbarium material) in Table 1. To determine the DNA ploidy level of the measured samples, comparative analyses of individuals with known chromosome numbers were performed (ŠMARDA & KOČÍ 2003). The resulting sample/standard peak ratios were then taken as a reference and compared with the measured peak values in the samples of unknown chromosome numbers. Representatives from the same species or related taxon were used: *F. alpestris* F1122 (2n=14) for *F. xanthina*; *F. pallens* F1229 (2n=14) for *F. ovina*, *F. pallens*, *F. polesica*, *F. pseudovaginata*, *F. vaginata*; *F. rupicola* F4 (2n=42) for *F. callieri*, *F. pseudodalmatica*, *F. pseudovina*, *F. rupicola* and *F. valesiaca*. Herbarium material was pressed in newspapers and filter paper under field conditions.

During this study, differences between the ratios obtained for fresh and herbarium specimens were observed. To clarify these differences, measurements with a set of 19 samples (including diploids, tetraploids and hexaploids) represented by both fresh plants and their one-year-old herbarium specimens were carried out. For each sample, a fresh plant and its herbarium specimen were measured consecutively within a few minutes (the fresh plant first). Both fresh and herbarium material was prepared in the same way using the same fresh leaf of the standard. The obtained results were tested for all 19 available pairs of samples using a nonparametric paired Wilcoxon Signed Rank test and nonparametric Spearman correlation using SPSS 8.0 program (SPSS INC. 1998).

Morphological measurements mentioned in the discussion follow the general principles for this genus (MARKGRAF-DANNENBERG 1980, WILKINSON & STACE 1991).

RESULTS AND DISCUSSION

DNA ploidy levels of the analyzed species

Festuca callieri (HACK.) MARKGR. – 2n≈4x

Festuca callieri is a karyologically unclear species, with the distribution ranging from the Balkan Peninsula to Asia Minor. The species was described from the Crimean Peninsula (ALEXEEV 1975), documented there as a tetraploid (TVERETINOVA 1977). The same DNA

Table 1. Summary of flow cytometric characteristics and the DNA ploidy levels observed in the studied *Festuca* species and hybrids. *N* – number of investigated samples. 2C ratio ± s.d. – mean somatic relative nuclear DNA content (sample/standard ratio) of samples ± standard deviation. 1x ratio – mean monoploid relative nuclear DNA content (sample/standard ratio divided by the ploidy level). CV – median values of coefficient of variance of sample peaks in measurements. max. diff. (%) – maximum difference in relative nuclear DNA content found between two samples expressed in the percentage as 100×(larger/smaller content - 1). * – 65 herbarium specimens and 22 herbarium vouchers of cultivated samples.

Species	DNA ploidy level	Fresh (cultivated) samples					Herbarium specimens		
		N	2C ratio ± s.d.	1x ratio	max. diff. (%)	CV	*N*	2C ratio ± s.d.	CV
F. callieri	4x	5	3.035 ± 0.019	0.759	1.4	1.76	11	2.962 ± 0.122	3.92
F. ovina	2x	–	–	–	–	–	1	1.434	4.52
F. pallens	2x	22	1.604 ± 0.034	0.802	9.2	2.18	17	1.549 ± 0.043	3.94
F. polesica	2x	2	1.693 ± 0.065	0.847	5.5	1.86	–	–	–
F. pseudodalmatica	2x	–	–	–	–	–	3	1.405 ± 0.060	3.25
	4x	2	3.065 ± 0.030	0.766	1.4	2.10	10	2.925 ± 0.083	3.18
	5x	1	3.739	0.748	–	1.79	1	3.476	3.81
F. pseudovaginata	2x	1	1.496	0.748	–	1.65	1	1.482	2.45
F. pseudovina	2x	–	–	–	–	–	2	1.380 ± 0.011	3.92
F. pseudovina × *F. rupicola*	4x	–	–	–	–	–	1	3.090	4.52
F. rupicola	6x	4	4.652 ± 0.076	0.775	3.8	1.73	17	4.415 ± 0.173	3.10
F. vaginata	2x	9	1.633 ± 0.024	0.817	4.2	1.62	9	1.543 ± 0.040	2.19
F. vaginata × *F. valesiaca*	2x	1	1.563	0.782	–	1.87	1	1.433	3.12
F. valesiaca	2x	–	–	–	–	–	11	1.415 ± 0.039	3.63
F. xanthina	2x	1	2.368	1.184	–	2.75	2	2.285 ± 0.016	2.50
Total		46	–	–	–	1.91	87*	–	3.22

ploidy level is reported here for plants from the Dobruga Mts. (E Romania). Later, also hexaploids were reported from Crimea (ALEXEEV et al. 1988). Diploid chromosome numbers were also recorded from the localities on the Black Sea coast in Bulgaria (KOŽUHAROV & PETROVA 1991).

Județul Tulcea, Măcin: 3 km ENE of the village, chalk scree isolated on otherwise siliceous rocky ridge, small population, 45°15′50.3″ N, 28°10′04.6″ E, 73 m a.s.l., 16.V.2003 (F398, #F399, #F400, H724, H725, H726). – **Tulcea, Măcin:** 4 km E of the village, siliceous rocks with species-poor vegetation with *Scleranthus perennis* L., small population, 45°15′23.6″ N, 28°11′15.3″ E, 331 m a.s.l., 16.V.2003 (#F382, #F384, H729, H730, H731, H732).

***Festuca ovina* L. – 2n≈2x**

Within this species, both diploids and tetraploids have been frequently documented (cf. ŠMARDA & KOČÍ 2003). In Europe, diploid plants correspond to *F. ovina* subsp. *ovina*. The nearest karyologically investigated population to those studied here is in Ukraine (TVERETINOVA 1977), where also a diploid level was documented.

Județul Alba, Vălişoara: 3.4 km SSE of the village, Cheile Aiudului glen, NW exp. limestone rock cliff with *Helictotrichon decorum* (JANKA) HENRARD, common, 46°22′29.1″ N, 23°35′18.3″ E, 618 m a.s.l., 13.V.2003 (H750).

***Festuca pallens* HOST – 2n≈2x**

In Romania, populations of *F. pallens* occur in the relict vegetation of calcareous canyons and promontories, often composed of relict species such as *Helictotrichon decorum*, *Sesleria rigida* SCHUR or *Seseli devenyense* SIMONK. (CSŰRÖS & POP 1965). Previous studies in *F. pallens* have documented both diploid and tetraploid levels and the existence of several geographically separated but morphologically only partly distinct types (cf. ŠMARDA & KOČÍ 2003). Plants from Romania are similar to the diploids growing on analogous localities in the Western Carpathians (so called Oberösterreich-Niederösterreich type; TRACEY 1980, PILS 1981), which have been documented from most of the natural range of *F. pallens* (ŠMARDA & KOČÍ 2003).

Județul Alba, Ampoița: 1 km E of the village, S-SE exp. rocky slopes of limestone cliff above the village, common, 46°07′04.0″ N, 23°28′54.7″ E, 320 m a.s.l., 12.V.2003 (#F388, H751, H752, H754). – **Alba, Ciuruleasa:** 4.8 km WSW of the village, NW exp. limestone massif, abundant, 46°14′08.3″ N, 22°57′59.5″ E, 959 m a.s.l., 12.V.2003 (F422, F423). – **Alba, Ocoliş:** 4.6 km S of the village, conglomerate rock near the road, common, 16°25′42.1″ N, 23°27′46.5″ E, 410 m a.s.l., 12.V.2003 (F420). – **Alba, Vălişoara:** 3.4 km SSE of the village, Cheile Aiudului glen, NW exp. limestone rock cliff with *Helictotrichon decorum*, abundant, 46°22′29.1″ N, 23°35′18.3″ E, 618 m a.s.l., 13.V.2003 (#F438, #F439, #F440, H747, H748, H749). – **Bihor, Vadu Crişului:** 1 km S of the village on right river bank, limestone promontory above the river, common, 46°58′18.1″ N, 22°30′47.7″ E, 373 m a.s.l., 11.V.2003 (#F385, F386, F387, H761, H762). – **Cluj, Buru:** 3.7 km ESE of the village, gneiss rocky slope and calcareous gravel of the railway yard near the road, common, 46°30′25.2″ N, 23°38′58.2″ E, 370 m a.s.l., 11.V.2003 (#F402, #F403, H759, H760). – **Cluj, Petreşti de Jos:** Cheile Turzi glen and reservation, about 3 km SE of the town centre, steep vertical limestone cliffs of the glen, relict vegetation dominated by *Helictotrichon decorum* and *Sesleria rigida*, common, 46°33′34″ N, 23°41′04″ E, 440 m a.s.l., 11.V.2003 (F406). – **Cluj, Someşu Rece:** S village periphery, above the road, 2.6 km SW of the Gilău village centre, pine forest on S exp. serpentine rocky slope, scattered, 46°44′02.6″ N, 23°21′14.8″ E, 458 m a.s.l., 11.V.2003 (F442, F443). – **Alba, Feneş:** 5 km N of the village, Cheile Feneşului glen, limestone rocky cliffs with *Helictotrichon decorum*, *Sesleria rigida* and *Saxifraga paniculata* MILL., common, 46°09′04.9″ N, 23°17′11.6″ E, 743 m a.s.l., 12.V.2003 (F380, F381). – **Neamț, Bicaz Chei:** W village periphery, along the road, limestone rocks in young pine forest, common, 46°49′29.0″ N, 25°52′02.8″ E, 642 m a.s.l., 14.V.2003 (F417, F418, F419). – **Neamț, Lacu Roşu:** N settlement periphery, bottom of large SE exp. limestone cliff above the cottages, very relict vegetation, scattered, 46°47′56.0″ N, 25°47′35.0″ E, 1170 m a.s.l., 14.V.2003 (F425, F426).

***Festuca polesica* ZAPAŁ. – 2n≈2x**

Populations from Hanu Conachi were previously identified either as *F. vaginata* subsp. *buiae* (PRODAN) BELDIE (BELDIE 1972) or as *F. pallens* var. *arenicola* (PRODAN) NYÁR. et

A. NYÁR. (NYÁRÁDY & NYÁRÁDY 1964). DIHORU (1987), however, demonstrated that they should be assigned to *F. polesica*, a species occurring on sands in neighbouring Ukraine (TVERETINOVA, 1977). The diploid level of *F. polesica* from Romania is in accordance with the results of MIZIANTY & PAWLUS (1984) from Poland and those of TVERETINOVA (1977) from Ukraine.

Județul Galați, Hanu Conachi: reservation on the village periphery, about 6.5 km NW of Tudor Vladimirescu town, sands with *Anchusa gmelinii* LEDEB. ex SPRENG. in larger clearing in *Robinia* forest, small population, 45°34′58.0″ N, 27°34′26.5″ E, 16 m a.s.l., 15.V.2003 (F389, F391).

***Festuca pseudodalmatica* DOMIN – 2n≈2x, 4x, 5x**

Especially in the Balkan Peninsula, *F. pseudodalmatica* is still a taxonomically and karyologically problematic species. From Central Europe and Ukraine, tetraploids have been reported (SIMON 1964, ČINČURA 1967, TVERETINOVA 1977, TRACEY 1980, ŠMARDA et al. 2005), while hexaploids were found in Bulgaria (KOŽUHAROV & PETROVA 1991). In addition to these ploidy levels, three diploid plants from two localities in the Mureş region were observed in the present study. Although misidentification and confusion with the sympatrically occurring diploid *F. valesiaca*, could have occurred, the morphological characters (median leaf diameter: 0.4–0.7 mm, spikelet: 6.7–7.9 mm long, second lemma: 4.8–5.9 mm long bearing a 1.2–2.2 mm long awn) fully correspond to the values used to delimit *F. pseudodalmatica* in most floras and determination keys (KRAJINA 1930, BELDIE 1972, ALEXEEV 1975, TVERETINOVA 1977, MARKGRAF-DANNENBERG 1980). In addition, the studied plants grow abundantly on andesite, which is the preferred bedrock of *F. pseudodalmatica* in Slovakia and Hungary (SOÓ 1973a, DOSTÁL 1989). A pentaploid plant was also found together with two other tetraploids in the population near Dubova. In comparison with the two tetraploids, this plant was more robust with larger spikelets (median 8.25 mm versus 7.65 and 7.6 mm in tetraploids), lemmas (median 6.0 mm versus 5.5 and 5.1 mm), longer awns (median 3.4 mm versus 2.35 and 3.0 mm), and stouter tiller leaves (median 0.65 mm versus 0.5 mm in both tetraploids). The pentaploid did not differ in hairiness (having fully glatt lemmas and paleas and densely hairy tiller sheaths), in the sclerenchyma pattern or in the number of veins on tiller leaf cross-sections.

2x – Județul Mureș, Răstolița: 4.3 km W of the village, andesite promontory above the road, dominant, 46°58′26.1″ N, 24°56′14.4″ E, 550 m a.s.l., 13.V.2003 (H744, H745). – **Mureș, Stânceni:** W village periphery near the road, SW exp. andesite rock, abundant, 46°57′38.4″ N, 25°13′04.8″ E, 615 m a.s.l., 14.V.2003 (H746).

4x – Județul Bihor, Vadu Crișului: 1 km S of the village on right river bank, limestone promontory above the river, common, 46°58′18.1″ N, 22°30′47.7″ E, 373 m a.s.l., 11.V.2003 (H763). – **Caraş-Severin, Lăpuşnicel:** 2.5 km NNE of the village, burned grassland on siliceous rocks above the river, abundant, 44°59′36.9″ N, 22°14′43.7″ E, 355 m a.s.l., 20.V.2003 (H775, H776). – **Caraş-Severin, Svinița:** 14 km NNW of the village, limestone cliff above the road and Danube river, abundant, 44°36′01.4″ N, 22°01′22.4″ E, 159 m a.s.l., 19.V.2003 (#F428, H736, H737, H738). – **Caraş-Severin, Dubova:** 12.5 km SSW of the village, *Pinus nigra* ARN. forest on micaeous slope near the road, scattered, 44°31′02.9″ N, 22°11′39.4″ E, 136 m a.s.l., 19.V.2003 (H739, H740). – **Cluj, Someşu Rece:** S village

periphery, above the road, 2.6 km SW of the Gilău village, pine forest on S. exp. serpentine rocky slope, common, 46°44′02.6″ N, 23°21′14.8″ E, 458 m a.s.l., 11.V.2003 (#F444).

5x – Județul Caraş-Severin, Dubova: 12.5 km SSW of the village, *Pinus nigra* forest on micaeous slope near the road, rare, 44°31′02.9″ N, 22°11′39.4″ E, 136 m a.s.l., 19.V.2003 (#F424).

***Festuca pseudovaginata* PENKSZA – 2n≈2x**

This species was described only recently, growing on sands in the Pannonian lowland in Hungary (PENKSZA 2003), and its distribution is not yet completely known. It has not been reported from Romania so far, and this is the first record of its occurrence in this country. Although the diploid ploidy level differs from the tetraploid one found in the type material (ŠMARDA et al., submitted), the studied plant and the type specimens do not differ morphologically (BP!, PENKSZA, pers. comm.). *Festuca pseudovaginata* co-occurs frequently with *F. vaginata* but it flowers much earlier, in early May (PENKSZA 2003), which is also in agreement with the observations on the reported Romanian locality. Further study is needed to clarify the existence of the two ploidy levels in this taxon, taking into account especially its probable recent hybrid origin (ŠMARDA et al., unpubl.data).

Județul Satu Mare, Stănislau: 5 km N of the village, sandpit on sand dune, rare, 47°40′36.5″ N, 22°19′05.2″ E, 140 m a.s.l., 10.V.2003 (#F395).

***Festuca pseudovina* WIESB. – 2n≈2x**

The diploid level reported here is in accordance with records from Russia (ALEXEEV et al. 1988), Austria (TRACEY 1980), Ukraine (TVERETINOVA 1977) and the Czech and Slovak Republics (ŠMARDA et al. 2005). Tetraploid and hexaploid reports for *F. pseudovina* (FELFÖLDY 1947, TRACEY 1980, RYBNICKÁ 1987) probably refer to other taxa or potential hybrids (see below).

Județul Cluj, Moldonoveşti: 0.9 km SE of the village, siliceous rocks along the road, common, 46°29′33.8″ N, 23°39′37.1″ E, 421 m a.s.l., 11.V.2003 (H758). – **Satu Mare, Stănislau:** 5 km N of the village, sandpit on sand dune, scattered, 47°40′51.4″ N, 22°19′37.9″ E, 114 m a.s.l., 10.V.2003 (H765).

***Festuca pseudovina* × *F. rupicola* – 2n≈4x**

The tetraploid level is intermediate between the putative parental species, diploid *F. pseudovina* and hexaploid *F. rupicola*. Both assumed parents occur sympatrically with the reported hybrid and are also documented in this study (see samples H765, H766). Misidentification with *F. wagneri* (DEGEN, THAISZ et FLATT) DEGEN, THAISZ et FLATT, a tetraploid taxon from similar sandy habitats in neighbouring Hungary (BAKSAY 1961; sub *F. conflicta* BAKSAY, ŠMARDA et. al. 2005) can undoubtedly be ruled out. The hybrid has three clearly separated sclerenchyma bands in the leaf cross-section, and tiller leaves without macrohairs, whereas for *F. wagneri* an interrupted to closed sclerenchyma ring in the leaf cross-section and tiller leaves with conspicuous macrohairs have been observed (PENKSZA & ENGLONER 2000).

Morphological characters of the putative hybrid show intermediate values. Small stems up to 13 cm with short panicles 3–4 cm long, and 5.6–6.2 mm long spikelets with short lemmas

3.8–4.1 mm long resemble *F. pseudovina*, whereas lemmas densely covered with long hairs on the surface, 1.8–2.2 mm long awns, and stouter tiller leaves are typical of *F. rupicola*.

Județul Satu Mare, Stănislau: 5 km N of the village, sandpit on sand dune, scattered, 47°40′36.5″ N, 22°19′05.2″ E, 140 m a.s.l., 10.V.2003 (H764).

***Festuca rupicola* HEUFF. – 2n≈6x**

Presented results from Romania confirm the hexaploid level in this species, as it was documented in previous analyses on material from Romania (ŠMARDA et al. 2005), neighbouring Moldova and Ukraine (TVERETINOVA 1977, ALEXEEV et al. 1988), and in the studies from Central Europe (TRACEY 1980, MIZIANTY & PAWLUS 1984, PILS 1984).

Județul Alba, Ampoița: 1 km E of the village, S-SE exp. rocky slopes of limestone cliff above the village, common, 46°07′04.0″ N, 23°28′54.7″ E, 320 m a.s.l., 12.V.2003 (H753). – **Bacău, Răcăciuni:** 4.5 km NNW of the village, E exp. degraded pasture on loamy substrate, dominated by *Teucrium chamaedrys* L., *Poa pratensis* L. and *Artemisia pontica* L., sparse, 46°22′08.6″ N, 26°58′13.0″ E, 155 m a.s.l., 15.V.2003 (H734). – **Caraş-Severin, Moldova Veche:** 1.5 km S of the town centre, near factory on Danube river bank, small spoil heap on stabilized sands, rare, 44°42′46.2″ N, 21°38′29.5″ E, 78 m a.s.l., 19.V.2003 (H777). – **Caraş-Severin, Prigor:** 12.5 km NNW of the village, overgrowing clearing with a house near the forest road, oak-hornbeam forest fringe, the only large tuft, 45°01′51.1″ N, 22°02′15.7″ E, 605 m a.s.l., 20.V.2003 (#F429). – **Cluj, Petreşti de Jos:** 3.2 km E of the village, ridge above the country road to Cheile Turzii glen, pasture on limestone, common, 46°34′15.2″ N, 23°41′43.3″ E, 616 m a.s.l., 11.V.2003 (H757). – **Galați, Draganești:** 3 km E of the village, forest track margin, *Robinia* forest on sand, several plants, 45°47′06.0″ N, 27°30′12.8″ E, 23 m a.s.l., 15.V.2003 (#F379, H735). – **Galați, Hanu Conachi:** reservation on the village periphery, about 6 km NW of Tudor Vladimirescu town, stabilized sands on margin of *Robinia* forest, small population, 45°34′54.6″ N, 27°34′33.7″ E, 10 m a.s.l., 15.V.2003 (#F430). – **Gorj, Bumbeşti-Jiu:** 12 km N of the village, siliceous rock above the river near the road, larger population, 45°16′46.6″ N, 23°23′21.4″ E, 468 m a.s.l., 21.V.2003 (#F412, H770, H771, H772). – **Hunedoara, Călan:** 3 km N of the village, on right bank of Strei river, burned pasture on siliceous rocks, sparse, 45°45′44.1″ N, 23°00′38.0″ E, 235 m a.s.l., 22.V.2003 (H722). – **Ialomița, Țăndărei:** 6 km ENE of the village, fringe of forest and scrubs dominated by *Acer tataricum* L. with *Poa pratensis*, isolated tufts, 44°39′48.5″ N, 27°44′20.3″ E, 9 m a.s.l., 17.V.2003 (H789). – **Mehedinți, Vârciorova:** about 200 m SE of the bridge and the turn-off to the monastery, above the road, scree below the stabilized calcareous rock, scattered, 44°42′51.3″ N, 22°28′52.6″ E, 95 m a.s.l., 18.V.2003 (H788). – **Neamț, Lacu Roşu:** N settlement periphery, grassland near tourist path below the SE exp. large limestone cliff, small population, 46°47′53.0″ N, 25°47′35.9″ E, 1142 m a.s.l., 14.V.2003 (F427). – **Satu Mare, Stănislau:** 5 km N of the village, sandpit on sand dune, scattered, 47°40′51.4″ N, 22°19′37.9″ E, 114 m a.s.l., 10.V.2003 (H766). – **Tulcea, Horia:** 3.5 km S of the village, near the road, open acidophilous termophilous oak forest with *Quercus pubescens* WILLD. and *Q. frainetto* TEN., common, 44°59′50.9″ N, 28°26′55.8″ E, 220 m a.s.l., 16.V.2003 (H728).

***Festuca vaginata* WILLD. – 2n≈2x**

The localities in the southwestern part along the Danube river form the southern border of the natural range of this Pannonian species. The diploid level is in accordance with previous reports from Hungary and Slovakia (PÓLYA 1949, BAKSAY 1956, SCHWARZOVÁ 1967, HORÁNSZKY et al. 1972, ŠMARDA et al. 2005).

Județul Satu Mare, Stănislau: 5 km ± N of the village, sandpit on sand dune, large population, 47°40′36.5″ N, 22°19′05.2″ E, 140 m a.s.l., 10.V.2003 (#F394). – **Dolj, Piscuteț:** about 3 km S of the village, disturbed place on sand dune with *Alcanna tinctoria* (L.) TAUSCH near the Danube, small population, 43°49′59.5″ N, 23°08′08.6″ E, 34 m a.s.l., 18.V.2003 (F414, F415). – **Mehedinți, Izvoarele:** SW village margin, about 6.5 km NW of Gruia town, local sandpit on stabilized sand dune near Danube river, larger population, 44°18′07.4″ N, 22°39′06.5″ E, 48 m a.s.l., 18.V.2003 (F432, F433, F434, F435, F436, F437, H779, H780, H781, H782, H783, H784, H785, H786).

***Festuca vaginata* × *F. valesiaca* – 2n≈2x**

The only tuft of this hybrid plant was found in disturbed sandy grassland dominated by *F. valesiaca* with rare occurrence of *F. rupicola* (both documented in this study). The second putative parent, *F. vaginata*, occurs on the nearby river island Moldova Veche (BELDIE 1972). Diploid level has been reported in this hybrid combination also from the sands in Austria (TRACEY 1980) and Hungary (ŠMARDA et al. 2005).

The investigated plant is morphologically intermediate between the parents. Its habitus, tuft formation, and cross section of tiller leaves with seven veins and long inner hairs are similar to *F. vaginata*, while lemmas bearing 0.8–1 mm long awns and partly scabrid leaves are rather typical of *F. valesiaca*. The sclerenchyma pattern is also intermediate. *Festuca valesiaca* forms three separate sclerenchyma strands in tiller leaves, while *F. vaginata* has a continuous sclerenchyma ring; the hybrid shows an interrupted sclerenchyma ring. The sclerenchyma pattern may resemble that of *F. wagneri*, another species inhabiting sandy areas. However, the latter species is tetraploid (BAKSAY 1961; sub *F. conflicta* BAKSAY, ŠMARDA et. al. 2005) and has macrohairs (PENKSZA & ENGLONER 2000), thus, it does not conform to the morphology of the assumed hybrid.

Județul Caraş-Severin, Moldova Veche: 1.5 km S of the town centre, near the factory on Danube river bank, small spoil heap on stabilized sands dominated by *Festuca valesiaca*, the only tuft, 44°42′46.2″ N, 21°38′29.5″ E, 78 m a.s.l., 19.V.2003 (#F409).

***Festuca valesiaca* GAUDIN – 2n≈2x**

The diploid level reported in Romania agrees with the results obtained from most of the natural range of this species (TVERETINOVA 1977, TRACEY 1980, PILS 1984, VÁCHOVÁ 1987, ALEXEEV et al. 1988, JAROLÍMOVÁ 1992, NAZAROVA & GOUKASIAN 1995, ŠMARDA et al. 2005). The hexaploid level recorded in *F. valesiaca* from Kashmir (KOUL & GOHIL 1991) probably refers to another taxon. Based on the author's field experience, the tetraploid reports by FELFÖLDY (1947; sub *F. pseudovina* in the original work or *F. valesiaca* according to SOÓ 1973b) from Hungary probably refer to *F. pseudodalmatica*. A wide distribution of the tetraploid cytotype of *F. valesiaca* in Hungary was presented by HORÁNSZKY et al. (1972),

however, they did not refer to any precise locality or any published chromosome number counts.

Județul Alba, Ampoița: 1 km E of the village, S-SE exp. rocky slopes of limestone cliff above the village, common, 46°07′04.0″ N, 23°28′54.7″ E, 320 m a.s.l., 12.V.2003 (H755). – **Alba, Vălişoara:** 4 km SSE of the village, below the Cheile Aiudului glen, pasture on limestone, scattered, 46°22′08.3″ N, 23°35′24.6″ E, 420 m a.s.l., 13.V.2003 (H742, H743). – **Arad, Vărădia de Mureş:** 4.5 km WNW of the village, S exp. steppe slope above the road, siliceous rocks dominated by *Melica ciliata* L., *Teucrium chamaedrys* and *Sedum* spp., scattered, 46°02′12.9″ N, 22°04′48.7″ E, 181 m a.s.l., 22.V.2003 (H723). – **Caraş-Severin, Dubova:** between Cazanele Mici and Cazanele Mari glens, opposite the Peştera Ponicova caves, limestone rock near the road, common, 44°35′46.4″ N, 22°15′15.3″ E, 189 m a.s.l., 19.V.2003 (H741). – **Caraş-Severin, Moldova Veche:** 1.5 km S of the town centre, near the factory on Danube river bank, small spoil heap on stabilized sands, abundant, 44°42′46.2″ N, 21°38′29.5″ E, 78 m a.s.l., 19.V.2003 (H778). – **Cluj, Petreşti de Jos:** 3.2 km E of the village, ridge above the country road to Cheile Turzii glen, pasture on limestone, abundant, 46°34′15.2″ N, 23°41′43.3″ E, 616 m a.s.l., 11.V.2003 (H756). – **Hunedoara, Călan:** 3 km N of the village, on right bank of Strei river, burned pasture on siliceous rocks, abundant, 45°45′44.1″ N, 23°00′38.0″ E, 235 m a.s.l., 22.V.2003 (H721). – **Hunedoara, Ponor:** E village periphery, intensive pasture on limestone rock above the village, common, 45°30′50.6″ N, 23°09′02.2″ E, 555 m a.s.l., 21.V.2003 (H773). – **Mehedinți, Vârciorova:** 200 m SE of the bridge and the turn off to the monastery, scree below the stabilized calcareous rock above the road, scattered, 44°42′51.3″ N, 22°28′52.6″ E, 95 m a.s.l., 18.V.2003 (H787). – **Tulcea, Horia:** 3.5 km S of the village, near the road, open acidophilous termophilous oak forest with *Quercus pubescens* and *Q. frainetto*, common, 44°59′50.9″ N, 28°26′55.8″ E, 220 m a.s.l., 6.V.2003 (H727).

Festuca xanthina ROEM. et SCHULT. – 2n≈2x

The investigated samples originated from the area of the locus classicus near Băile Herculane, and were shown to be diploid in accordance with the study of STARLINGER et al. (1994).

Județul Caraş-Severin, Băile Herculane: 5.5 km NNE of the town centre, steep limestone cliffs with *Pinus nigra*, common, 44°55′21″ N, 22°28′06″ E, 550 m a.s.l., 21.V.2003 (#F411, H774).

Relative nuclear DNA content of fresh plant material versus herbarium specimens

A comparison of the sample/peak ratios obtained from fresh samples and their one-year-old herbarium specimens, measured one week later, showed permanent and significant differences between the obtained values. Consecutive measurements (with only a few minutes delay) of fresh and herbarium samples gave similar differences. Sample/standard ratios obtained from the measurements of herbarium specimens were lower for 18 of 19 samples than those obtained from the measurements of fresh plants ($P < 0.001$, Fig. 1). The differences ranged from +0.4% to -9.9% (median -4.1%). Measurements of

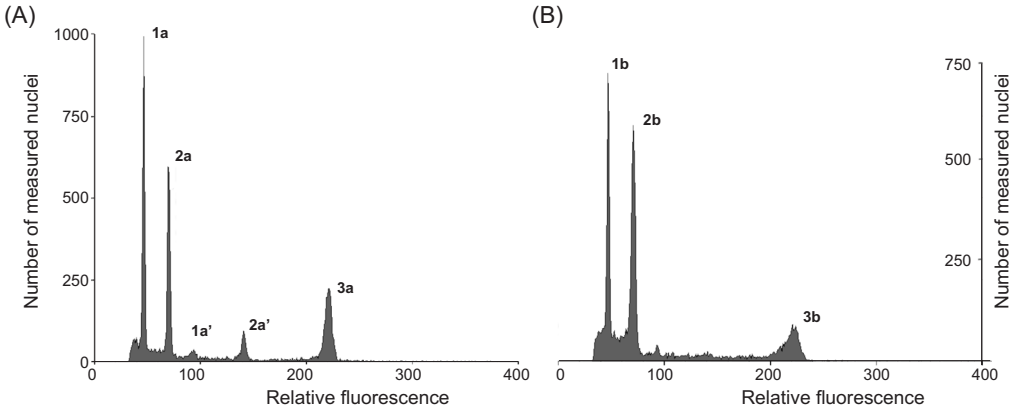

Fig. 1. Comparison of flow cytometric histograms obtained from the consecutive measurements of fresh plants (A) and their one-year-old herbarium specimens (B) showing lower standard/sample ratio and higher CVs for the latter. 1 – standard *Lycopersicon esculentum*; 2 – F395 diploid *F. pseudovaginata*; 3 – F412 hexaploid *F. rupicola*. Denotations with apostrophs indicate corresponding G2/M peaks. Peak characteristics are given in the following order: mean, CV, sample/standard ratio. Peak 1a: 46.75, 1.60, 1.000; Peak 2a: 70.49, 1.24, 1.508; Peak 3a: 220.90, 1.06, 4.725; Peak 1b: 47.07, 1.59, 1.000; Peak 2b: 70.43, 2.48, 1.496; Peak 3b: 218.34, 2.18, 4.702.

herbarium specimens always resulted also in higher coefficients of variance ($P < 0.001$, Fig. 1) when compared with the corresponding fresh plants. This finding is in agreement with the results by SUDA (2004). The observed shifts of peak positions seem not to be critically influenced by the quality of peaks and ploidy level of the samples. No significant correlations were observed ($P > 0.2$) between the rate of the peak shift and either the CV of the specimen, or the difference between CVs of herbarium and fresh material, or the ploidy level. Several processes can be considered to explain the observed pattern, e.g., the loss of DNA during the death of cells, presence of secondary metabolites originating during cell degradation, different concentrations of metabolites in plants sampled in natural conditions and in plants from cultivation. In this respect, measurements of herbarium material require further critical studies.

Although the observed difference is significant, it is not too high to disable parallel determination of the ploidy level in both herbarium and fresh samples. Still, one should be aware of these observations, and results from fresh and herbarium material should be treated separately.

Inter- and intraspecific nuclear DNA content variability

Almost all species with several populations studied here exhibited notable intraspecific relative DNA content variability (Table 1). This variability is beyond instrumental or methodological errors, and in *F. pallens* and *F. polesica* it was also confirmed by simultaneous measurements of the two most distant samples, which showed non-overlapping double peaks (Figs. 2A, B). The differences observed within *F. vaginata* and *F. rupicola* were on the detection limit of the instrument, and in simultaneous measurements only bimodal

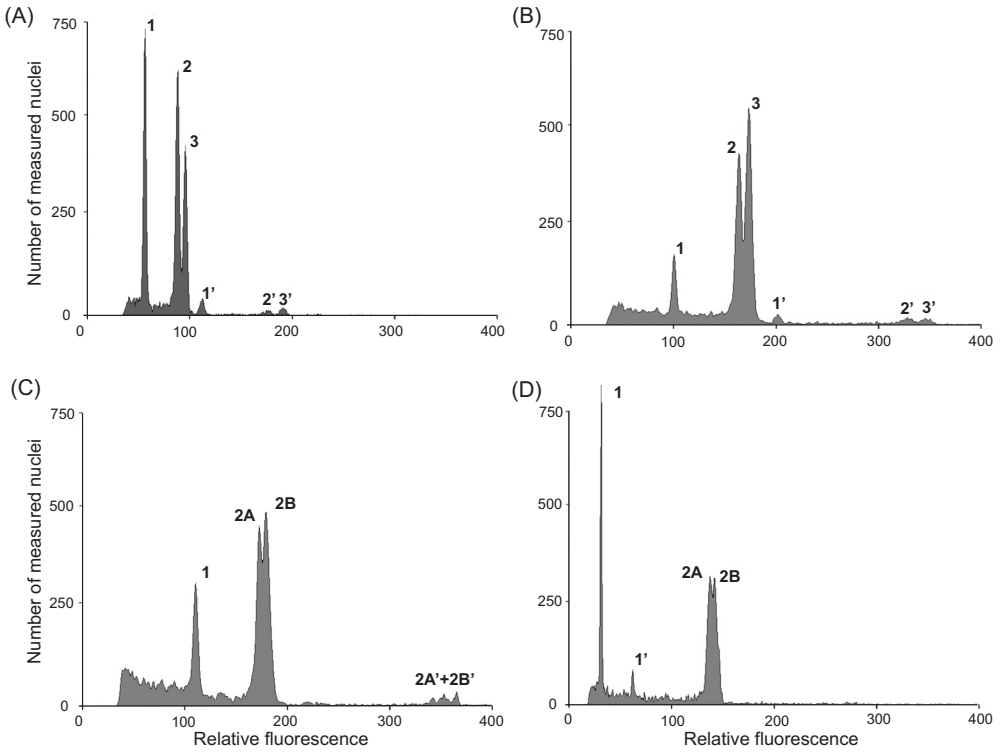

Fig. 2. Intraspecific difference in the relative nuclear DNA content of two simultaneously measured samples proved as a non-overlapping double peak (A, B) or an apparent bimodal peak (C, D). Denotations with apostrophs indicate corresponding G2/M peaks. *Lycopersicon esculentum* was used as standard in all these analyses.
A: 1 – standard; 2 – diploid *F. pallens* (F380, loc. Cheile Feneşului glen); 3 – diploid *F. pallens* (F387, loc. Vadu Crişului). B: 1 – standard; 2 – diploid *F. polesica* (F389); 3 – diploid *F. polesica* (F391); both samples from the same locality in Hanu Conachi. C: 1 – standard; 2A – diploid *F. vaginata* (F394, loc. Stănislau, NW Romania); 2B – diploid *F. vaginata* (F434, loc. Izvoarele, SW Romania). D: 1 – standard; 2A – hexaploid *F. rupicola* (F412, loc. Bumbeşti-Jiu); 2B – hexaploid *F. rupicola* (F427, loc. Lacu Roşu).

peaks were observed (Figs. 2C, D). The maximal differences obtained from separate (Table 1) and simultaneous measurements (Figs. 2A–D) were almost the same. The largest intraspecific difference of about 9% (Table 1, Fig. 2A) was observed in *F. pallens*. This species was sampled from several populations across its wide geographical range, and the differences in the DNA content seem to be geographically correlated. While two plants from Cheile Feneşului glen (samples F380 and F381) showed the sample/standard ratios of 1.56 and 1.58, respectively, all three plants (F385, F386, F387) from Vadu Crişului (110 km NW of the former) had the standard/sample ratios of 1.65, 1.66 and 1.70, i.e., up to 9% higher than in the former population (Fig. 2A). Similarly, the samples of *F. vaginata* from Stănislau and Pisculet (NW Romania) had smaller DNA content than samples from the population near

Izvoarele (SW Romania), and around a 4% maximum difference in DNA content was observed in this case between the two most contrasting samples (Fig. 2C). The most distant samples of *F. rupicola* in respect of the DNA content (F412 and F427, Fig. 2D) also originated from geographically remote localities (about 250 km apart). In the case of *F. polesica*, a 5.5% difference was observed between the two samples originating from the same site (Fig. 2B).

Intraspecific DNA content variability has also been previously documented from other grasses, as in *Zea mays* (RAYBURN et al. 1989), *Dactylis glomerata* (REEVES et al. 1998), and most recently in *Dasypyrum villosum* (GREILHUBER 2005). Although intraspecific genome size variability has been reported in grasses more frequently, this phenomenon seems to be only genus- or species-specific. LYSÁK et al. (2000) and LE THIERRY d´ENNEQUIN et al. (1998) have found that the genome size of *Sesleria albicans* and some *Setaria* species are stable. While DNA content variability has usually been studied within single species, comparative analyses of the presented study allowed exploring this phenomenon in several *Festuca* species. Intraspecific variation was revealed in many of them, and thus, this genus appears to be of particular interest for further studies.

One source of intraspecific genome size variability observed in *Festuca* may be the presence of B chromosomes, commonly documented in this genus (ÖNDER & JONG 1977, PILS 1980, MIZIANTY & PAWLUS 1984, KOŽUHAROV & PETROVA 1991, WILKINSON & STACE 1991, ŠMARDA & KOČÍ 2003). However, the DNA content and the presence of B chromosomes are not always directly correlated (POGGIO et al. 1998, PALESTIS et al. 2004). Another source explaining the intraspecific variation may be the activity of retrotransposons, which is assumed to be the main mechanism of recent genome size variation in plants (BENNETZEN et al. 2005). The activity of retrotransposons may be associated with different microclimatic gradients and may be ecologically determined, as found e.g. in *Hordeum spontaneum* (KALENDAR et al. 2000). Extreme habitats of rocky or sandy steppes with a sharp microclimatic gradient may be one of the reasons for the recent DNA content variability in fescues. Because small differences in DNA content were observed among some of the species studied here, genome size variability in some taxa may also reflect on-going microevolutionary and speciation processes (MURRAY 2005).

The most outlying values of the DNA content among the taxa studied here were observed in *F. xanthina*. *Festuca xanthina* (*Festuca* sect. *Eskia* WILLK.) apparently differs in the monoploid relative nuclear DNA content, which is about 1.39–1.58 times higher than in the remaining species (all from sect. *Festuca*). This was also suggested for the karyologically proven sample of diploid *F. alpestris* ROEM. et SCHULT. (2n=14, ŠMARDA & KOČÍ 2003) and for some other species from the *Eskia* section (ŠMARDA et al., unpubl. data). Since WALLOSEK (1999) observed nearly regular intervals (1-, 2-, 3-fold) in the relative genome size of different ploidy levels (2x, 4x, 6x) in other species of this section from the Alps, it may be assumed that the whole *Eskia* section has a distinct monoploid DNA content.

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