Evaluation of systematic relationships among four taxa of the *Festuca ovina* group (*Poaceae*) by means of isoenzyme markers

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Abstract. Polyacrylamide gel electrophoresis was applied to examine the isoenzyme variation of superoxide dismutase, glucose-6-phosphate dehydrogenase, malate dehydrogenase, NADP-dependent malate dehydrogenase, and α-amylase in natural populations of *Festuca valesiaca*, *F. rupicola*. *F. dalmatica,* and *F. stojanovii*. The purpose was to evaluate the isoenzyme variation and systematic relationships among the above-mentioned species of genus *Festuca.* Two similarity indices (Coefficient of Divergence D_{CD} and phenotypic identity I_h) were calculated in an attempt to evaluate qualitatively the affinities and systematic relationships among the species. The presented isoenzyme data suggest that *F. dalmatica* and *F. stojanovii* are closely related, but different and well defined taxa within genus *Festuca*. The species *F. valesiaca* and *F. rupicola* are also well characterized isoenzymatically as distinct genetic entities. The results support the recent narrow species concept in genus *Festuca*.

Key words: *Festuca*, isoenzyme markers, isoenzyme variation, systematic relationships

Introduction

Festuca L. is one of the most complex genera in *Poaceae*. The high level of morphological variability within *Festuca* makes difficult its systematic interpretation and taxonomic treatment. The species concept in genus *Festuca* has undergone drastic changes. More than a century ago, relatively few broadly defined taxa were recognized (Hackel 1882). Lately, the species definitions became narrower and a large number of finely split taxa have been recognized today (Markgraf-Dannenberg 1976, 1978, 1980). The species *Festuca ovina* L. is an extreme example of this concept of changing species. Originally described by Hackel (1882) as a single variable species, it was recognized in the 1980s as several dozens of species (Markgraf-Dannenberg 1980; Wilkinson & Stace 1981).

The present study includes four species, namely, *Festuca valesiaca* Schleich. ex Gaudin, *F. rupicola* Heuff., *F. dalmatica* (Hack.) K. Richter, and *F. stojanovii* (Acht.) Foggi & Petrova (Foggi & al. 2005). These fescues have been formerly referred to as *F. ovina*. The species *F. valesiaca* was considered a variety (Stoyanov & Stefanov 1924), or a subspecies (Stoyanov & Stefanov 1948) of *Festuca ovina.* Stoyanov & Stefanov (1933) treated *F. dalmatica* as a subspecies of *F. ovina.* Ahtarov (1953) described *F. stojanovii* as a subspecies of *F. dalmatica.* Lately, Kozhuharov (1982) suggested the new species *F. stojanovii* of genus *Festuca*. The examined fescues are dense tuft plants growing in dry grassy habitats.

In the last two decades, several isoenzyme studies of subarctic/arctic (Aiken & al. 1993, 1994; Aiken & Lefkovitch 1995; Guldahl & al. 2001) and temperate zone fescues (Livesey & Norrington-Davis 1991) have been conducted in an attempt to investigate the species delimitation by means of isoenzyme markers.

The purpose of the present study was to reveal isoenzyme variation in an attempt to shed light on systematic relationships among the above-mentioned four species of genus *Festuca.*

Material and methods

The isoforms of enzymes superoxide dismutase (SOD), glucose-6-phosphate dehydrogenase (G-6-PDH), malate dehydrogenase (MDH), NADP-dependent malate dehydrogenase (NADP-MDH), and α-amylase (AMY) were resolved by polyacrylamide gel electrophoresis. Altogether, 261 individual plants belonging to eight natural populations (on the average, 33 plants/population) of the species were examined (Table 1).Vouchers are deposited at the Herbarium of the Institute of Biodiversity and Ecosystem Research (SOM).

Leaves were ground in 0.01 M Tris, 0.08 M glycine, 0.005 M cysteine, and 20 % sucrose with pH 8.3. Ion-exchange resin Dowex 1 × 8 (0.4 g/1 g fresh tissue) was added to the extraction buffer to eliminate polyphenols. Homogenates were centrifuged at 10 000 rpm for 10 min. The supernatant was used as a source of enzymes. The enzymes superoxide dismutase, glucose-6-phosphate dehydrogenase, malate dehydrogenase, and NADP-dependent malate dehydrogenase were resolved on 7.5 % separating gel (3 % stacking gel) polyacrylamide slabs, using the electrophoretic system of Davis (1964). The length of the separating and stacking gel was 8 cm and 2 cm, respectively. Electrophoresis was conducted at 200 V, until the indicator dye Bromphenol Blue reached the gel end. The enzyme α-amylase was run on 6 % polyacrylamide slabs containing 0.5 % soluble starch, for 10 hours at 200 V. Staining of gels followed the procedures of Soltis & al. (1983) for NADP-MDH, Shaw & Prasad (1970) for MDH and G-6-PDH, Baur & Schorr (1966) for SOD, and Reisfeld & al. (1962) for AMY. Each isoform was assigned a number reflecting its gel migration (in mm) from the origin (Perez de la Vega & Allard 1984).

The examined enzymes were not subjected to genetic analysis. With the exception of *Festuca rupicola*, the studied species were polyploids. Hence, it was difficult to discriminate between isozymes and allozymes and to interpret the electrophoretic patterns in genetic terms. For this reason, two phenetic parameters were employed: 1) isoform presence/absence, and 2) isoform frequency. Mean frequencies of isoforms were calculated for each species. Using isoform frequency, mean values of D_{CD} (Coefficient of Divergence after Clark 1952, see Stuessy 1990) and the measure of phenotypic identity (I_h) of Hedrick (1971) were calculated according to the equations:

$$D_{CD} = \left[\frac{1}{N} \sum_{i=1}^{N} \left(x_{ij} - x_{ik} \right)^2 \right]^{\frac{1}{2}}$$

where ***N*** is the total number of isoforms for each enzyme, ***xij*** and ***xik*** – the frequency of ***i*-th** isoform in taxa ***j*** and **k,**

and

$$I_h = 2 \sum_{j=1}^{n} P_{jx} P_{jy} \; / \; \sum_{j=1}^{n} P^2_{jx} + \sum_{j=1}^{n} P^2_{jy,}$$

where $\mathbf{P_{jx}}$ and $\mathbf{P_{jy}}$ are the frequencies of **j-th** isoform in species **x** and **y,** and **n** is the number of isoforms of each enzyme.

Results

The mean isoform frequencies of superoxide dismutase are shown in Table 2. Eleven isoforms were detected electrophoretically. Isoforms 38, 43, 49, and 54 were monomorphically fixed across the entire group, while isoform 34 was invariant in *Festuca valesiaca* and *F. rupicola* only. Isoform 67 was monomorphic and species – specific for *F. valesiaca*. Isoform 30 was common for *F. valesiaca* and *F. rupicola,* but it was absent in *F. stojanovii* and *F. dalmatica*. Isoform 34 was not found in the studied populations of *F. stojanovii*. The Coefficient of Divergence D_{CD} varied within the range from 0.22 in the comparison between *F. stojanovii* and *F. dalmatica* to 0.50 when the former was compared with *F. valesiaca*. The pair-wise comparisons among the species resulted in a phenotypic identity coefficient I_h equal to 0.79, when *F. valesiaca* and *F. stojanovii* were contrasted. The highest values (I_h=0.95) were obtained in the comparison of the species *F. dalmatica* and *F. stojanovii.*

Table1. Species, populations and number of examined individuals.

Species	Locality	Number of individuals	Voucher number
F. valesiaca	Sofia region, MtVitosha, around Bosnek village	29	Co-170
	Sofia region, Mt Vitosha, Boyansko Swamp	34	Co-172
F. rupicola	Znepole region, around Belidie Han	29	Co-106
	Stara Planina Mts, around Chelopech village	35	Co-164
F. dalmatica	Sofia region, Mt Vitosha, Kladnitza village	37	Co-111
	Sofia region, Mt Vitosha, around Selimitza chalet	28	Co-112
F. stojanovii	Sofia region, Mt Lozenska, peak Polovrak	32	Co-115
	Znepole region, Mt Chepan,	35	Co-117

Table 2. Mean isoform frequencies of superoxide dismutase in the studied species of genus *Festuca*.

Species	Isoform										
	23	26	30	34	38	43	47	49	54	58	67
F. valesiaca	0.83	1.00	0.11	1.00	1.00	1.00	0.27	1.00	1.00	0.86	1.00
F. rupicola	0.12	1.00	0.28	1.00	1.00	1.00	0.74	1.00	1.00	0.34	0.00
F. dalmatica	0.48	0.84	0.00	0.14	1.00	1.00	0.28	1.00	1.00	0.48	0.00
F. stojanovii	0.15	0.38	0.00	0.00	1.00	1.00	0.28	1.00	1.00	0.14	0.00

Totally, five isoforms of glucose6-phosphate dehydrogenase were detected in the studied species (Table 3). Most isoforms were shared by all examined species. Isoform 4 was not found in the studied populations of *Festuca dalmatica.* Isoform 28 was monomorphically fixed across the entire group. In respect to D_{CD}, the range of its variation was from 0.17 (*F. valesiaca* vs. *F. dalmatica*) to 0.28, when the latter was contrasted to *F. rupicola*. Coefficient I_h varied within a narrow range (0.91–0.98) for most pair-wise comparisons among the examined species.

Table 3. Mean isoform frequencies of glucose-6-phosphate dehydrogenase in the studied species of genus *Festuca*.

Species	Isoform				
	4	10	13	20	28
F. valesiaca	0.22	0.31	0.27	0.24	1.00
F. rupicola	0.48	0.58	0.34	0.42	1.00
F. dalmatica	0.00	0.22	0.44	0.56	0.00
F. stojanovii	0.29	0.42	0.16	0.22	1.00

Six isoforms of malate dehydrogenase were resolved in the studied species (Table 4). Regarding isoforms 24, 26 and 28, the populations of all species were monomorphic and uniform. Isoform 5 was fixed in the species *Festuca valesiaca* and *F. rupicola*. Similarly, isoform 30 was invariant and common for *F. stojanovii* and *F. dalmatica*. The values of D_{CD} varied from 0.41 (*F. valesiaca* vs. *F. rupicola*) to 0.62, when the former species was compared with *F. dalmatica.* The phenotypic identity coefficient I_h ranged from 0.74 (*F. valesiaca* vs. *F. dalmatica*) to 0.90, when the former species was contrasted to *F. rupicola*.

Table 4. Mean isoform frequencies of malate dehydrogenase in the studied species of genus *Festuca*.

Species	Isoform					
	5	6	24	26	28	30
F. valesiaca	1.00	0.86	1.00	1.00	1.00	1.00
F. rupicola	1.00	0.74	1.00	1.00	1.00	0.00
F. dalmatica	0.00	0.32	1.00	1.00	1.00	0.00
F. stojanovii	0.00	0.49	1.00	1.00	1.00	1.00

In total, five isoforms of NADP-malate dehydrogenase were electrophoretically resolved (Table 5). Most isoforms were shared by all examined species. Isoform 23 was monomorphically fixed across the entire group, while isoform 19 was invariant and common for *Festuca rupicola* and *F. valesiaca* only. Isoform 13 did not occur in the studied populations of *F. stojanovii.* In terms of coefficient D_{CD}, the species *F. valesiaca* and *F. stojanovii* demonstrated the highest affinity (D_{CD}=0.22) within the studied group. On the contrary, *F. rupicola* was most distant (D_{CD}=0.35) from *F. stojanovii*. The values of coefficient I_h were high and ranged from 0.90 (*F. dalmatica* vs. *F. stojanovii*) to 0.96 in the pairwise comparisons of *F. valesiaca* with *F. dalmatica* and. *F. stojanovii.*

Table 5. Mean isoform frequencies of NADP-malate dehydrogenase in the studied species of genus *Festuca*.

Species	Isoform				
	13	15	17	19	23
F. valesiaca	0.31	0.31	0.31	1.00	1.00
F. rupicola	0.72	0.48	0.64	1.00	1.00
F. dalmatica	0.53	0.27	0.16	0.86	1.00
F. stojanovii	0.00	0.38	0.52	0.72	1.00

The isoenzyme structure of α-amylase is presented in Table 6. Isoforms 16 and 17 occurred with different frequencies in all examined species. Isoforms 14 and 18 were not found in the studied populations of *Festuca dalmatica* and *F. stojanovii.* Isoform 26 was monomorphically fixed across the entire group. With the exception of the species pair *dalmatica- stojanovii* (D_{CD}=0.05), the values of coefficient D_{CD} fell within the 0.26–0.34 range, when *F. stojanovii* was contrasted to *F. rupicola* and *F. valesiaca,* respectively. The values of coefficient I_h varied from 0.79 (*F. valesiaca* vs. *F. stojanovii*) to 0.99, when the latter species was compared with *F. dalmatica.*

Table 6. Mean isoform frequencies of α-amylase in the studied species of genus *Festuca*.

Species	Isoform				
	14	16	17	18	26
F. valesiaca	0.00	0.87	0.11	0.05	1.00
F. rupicola	0.48	0.58	0.07	0.10	1.00
F. dalmatica	0.00	0.27	0.39	0.00	1.00
F. stojanovii	0.00	0.18	0.47	0.00	1.00

The mean values of coefficients I_h and D_{CD} are presented in Table 7. The lowest value of coefficient D_{CD} was obtained, when *Festuca dalmatica* and *F. sto-*

janovii were contrasted. A comparatively low value of coefficient D_{CD} was calculated in the comparison between *F. valesiaca and F. rupicola.* An analysis of the mean I_h values demonstrated a similar pattern. The highest values of I_h were found in the comparisons between the species pairs *F. dalmatica – F. stojanovii* and *F. valesiaca – F. rupicola*. All other pair-wise comparisons of the species resulted in lower values, which varied within a comparatively narrow range – an indication of nearly equidistant positions of the species within the group.

Table 7. Mean values of the Coefficient of Divergence D_{CD} and phenotypic identity I_h for all pair-wise comparisons among the examined species.

Species	Coefficient of Divergence D_{CD}				Phenotypic identity I_h			
	1	2	3	4	1	2	3	4
F. valesiaca	0.00							
F. rupicola	0.29	0.00			0.92	1.00		
F. dalmatica	0.36	0.37	0.00		0.87	0.80	1.00	
F. stojanovii	0.31	0.38	0.25	0.00	0.88	0.86	0.92	1.00

Discussion

An analysis of the isoenzyme data has demonstrated that the examined taxa could be clearly discriminated by the employed molecular markers. Several monomorphically-fixed isoform differences in the isoenzyme structure of the studied species were detected. On the other hand, some isoforms were not found in some of the examined *Festuca* species. These specific combinations form distinct isoenzyme patterns which clearly distinguish the respective species from all other taxa within the group.

Similar patterns of isoenzyme variation have been found during other studies of the fescues. Isoenzymes were used to assess the species boundaries in the North American representatives of the *Festuca ovina* complex (Aiken & al. 1993). Distinct isoenzyme profiles have delimited some discrete entities within the complex. An extensive study of the *F. brachyphylla* complex, which has been formerly referred to *F. ovina*, revealed unique diagnostic bands and distinct banding patterns for all four examined taxa (Guldahl & al. 2001). Unique combinations of the bands pertaining to different taxa within the same complex have been also reported (Aiken & al. 1994; Aiken & Lefkovitch 1995). Other isoenzyme studies have also demonstrated that fescues and other grasses may be separated by extreme allele frequency differences (Warwick & Aiken 1986; Davis & Manos 1991; Davis & Goldman 1993).

Considering the coefficients I_h and D_{CD}, it becomes evident that *Festuca dalmatica* and *F. stojanovii* are closely related, as shown by the surveyed isoenzymes. In spite of the distinct anatomical differences between them, *F. stojanovii* was first recognized by Ahtarov (1953) as a subspecies of *F. dalmatica,* mainly on the basis of the vascular bundles' number. However, the number of leaf sclerenchyma bundles – four in *F. dalmatica* and six-seven in *F. stojanovii* – as well as the number of leaf ribs have allowed to differentiate these two taxa. Isoenzyme data presented in this study support the opinion of Kozhuharov (1982) that *F. dalmatica* and *F. stojanovii* are closely related, but different and well-defined taxa within genus *Festuca*. Mention deserves the fact that the species *F. valesiaca* and *F. rupicola,* separated mainly on the basis of subtle morphological differences, were also well characterized isoenzymatically as distinct genetic entities. These species are more or less equidistantly positioned within the studied group and clearly distinguished from *F. dalmatica* and *F. stojanovii.* In an earlier study of polyphenolic compounds (Angelov & al. 1988) it was shown that xerophytes *F. valesiaca* and *F. rupicola* are more closely related to each other than to a group of mesophyte species of genus *Festuca*. These findings support the results of the present study.

The studied species of genus *Festuca* have exhibited some subtle morphological differences. They differed mainly in anatomical characters observed by cross-sectioning, but their identification was difficult. The isoenzyme data presented here provide evidence that the four taxa have been genetically well-defined entities. The results are in good concordance with those reported in the above-mentioned studies of other fescue species. And finally, the present study supports the recent narrow-species concept in genus *Festuca*.

References

Ahtarov, B. 1953. Genus *Festuca* L. (Vlasatka) in Bulgaria. – Izv. Bot. Inst. (Sofia), **3:** 1-89 (in Bulgarian).

Aiken, S. & Lefkovitch, L. 1995. *Festuca edlundiae* (*Poaceae*), a high arctic, new species compared enzymatically and morphologically with similar *Festuca* species. – Syst. Bot., **20:** 374-392.

Aiken, S., Consaul, l., Davis, j. & manos, P. 1993. Systematic inferences from variation in isoenzyme profiles of arctic and alpine caespitose *Festuca* (*Poaceae*). – Amer. J. Bot., **80:** 76-82.

Aiken, S., Spidle, A. & May, B. 1994. Allozyme and morphological observations of *Festuca hyperborea* compared with *F. baffinensis* and *F. brachyphylla* (*Poaceae*) from Canadian Arctic. – Nordic J. Bot., **14:** 137-143.

Angelov, G., Edreva, A. & Kozhuharov, S. 1988. Biosystematic study on species of genus *Festuca* L. I. Chromatographic analysis of polyphenolic compounds. – Fitologiya, **35:** 3-12 (in Bulgarian).

Baur, B. & Schorr, R. 1969. Genetic polymorphism of tetrazolium oxidase in dogs. – Science, **166:** 1524-1535

Davis, B. 1964. Disc electrophoresis. I. Method and application to human serum proteins. – Ann. New York Acad. Sci., **121:** 404-427.

Davis, J. & Goldman, D. 1993. Isozyme variation and species delimitation among diploid populations of the *Puccinellia nuttalliana* complex (*Poaceae*): character fixation and discovery of phylogenetic species. – Taxon, **42:** 585-599.

Davis, J. & Manos, D. 1991. Isozyme variation and species delimitation in the *Puccinellia nuttalliana* (*Poaceae*) complex: an application of the phylogenetic species concept. – Syst. Bot., **16:** 431-445.

Foggi, B., Scholz, H. & Valdés, B. 2005. The Euro+Med Treatment of *Festuca* (*Gramineae*) – new names and new combinations in *Festuca* and allied genera. – Widenowia **35**(2): 241-244.

Guldahl, A., Borgen, L. & Nordal, I. 2001. Variation in the *Festuca brachyphylla* (*Poaceae*) complex in Svalbard, elucidated by chromosome numbers and isozymes. – Bot. J. Linn. Soc., **137:** 107-126.

Hackel, E. 1882. Monographia Festucarum Europaearum. T. Fisher, Kassel.

Hedrick, P. 1971. A new approach to measuring genetic similarity. – Evolution, **25:** 276-280.

Kozhuharov, S. 1982. Studies on composition, distribution and phytogeographic links of genus *Festuca* in Bulgaria. –God. Sofiisk. Univ. "Kliment Ohridski" Biol.Fak. 2, Bot., **75:** 11-20 (in Bulgarian).

Livesey, V. & Norrington-Davies, J. 1991. Isoenzyme polymorphism in *Festuca rubra* L. – Euphytica, **55:** 52-79.

Markgraf-Dannenberg, I. 1976. Die Gattung *Festuca* in Griechenland. – Veröff. Geobot. Inst. Rübel Zürich, **56:** 92-182.

Markgraf-Dannenberg, I. 1978. New taxa and names in European *Festuca* (*Graminae*). – In: **Heywood V**. (ed.), Flora Europaea, Notulae Systematicae. – Bot. J. Linn. Soc., **76:** 322-328.

Markgraf-Dannenberg, I. 1980. *Festuca*. – In: **Tutin, T.G. & al.** (eds.), Flora Europaea. Vol. **5:** pp. 149-150. Cambridge Univ. Press, Cambridge.

Perez de la Vega, M. & Allard, R. 1984. Mating system and genetic polymorphism in populations of *Secale cereale* and *S. vavilovii*. – Canad. J. Genet. Cytol., **26:** 306-317.

Reisfeld, R., Lewis, U. & Williams, D. 1962. Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. – Nature, **195:** 281-283.

Shaw, C & Prasad, R. 1970. Starch gel electrophoresis of enzymes – a compilation of recipes. – Biochem. J., **4:** 297-310.

Soltis, D., Haufler, C., Darrow, D. & Gastony, G. 1983. Starch gel electrophoresis of ferns: a compilation of grinding buffers, gel and electrode buffers, and staining schedules. – Amer. Fern J., **73:** 9-27.

Stoyanov, N. & Stefanov, B. 1924. Flora of Bulgaria, Vol. **1**. State Printing House, Sofia (in Bulgarian).

Stoyanov, N. & Stefanov, B. 1933. Flora of Bulgaria, Ed. 2. Guttenberg Press, Sofia (in Bulgarian).

Stoyanov, N. & Stefanov, B. 1948. Flora of Bulgaria, Ed. 3. Univ. Press, Sofia, (in Bulgarian).

Stuessy, T. 1990. Plant Taxonomy. The Systematic Evaluation of Comparative Data. Columbia Univ. Press, New York.

Warwick, S. & Aiken, S. 1986. Electrophoretic evidence for the recognition of two species in annual wild rice (*Zizania*, *Poaceae*). – Syst. Bot., **11:** 464-473.

Wilkinson, M. & Stace, C. 1981. A new taxonomic treatment of the *Festuca ovina* aggregate (*Poaceae*) in the British Isles. – Bot. J. Linn. Soc., **106:** 347-397.