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Isoenzyme variation and genetic affinities among four species of the genus *Festuca* L. (Poaceae)

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Abstract: *Festuca* L. is one of the most complicated genera in Poaceae. Polyacrylamide gel electrophoresis was used to study the isoenzyme variation of glutamate-oxaloacetate transaminase, malate dehydrogenase, glutamate dehydrogenase, isocitrate dehydrogenase and 6-phosphogluconate dehydrogenase in natural populations of *F. valesiaca* Schleich. ex Gaud., *F. rupicola* Heuff., *F. dalmatica* (Hack.) K. Richt. and *F. stojanovii* (Acht.) Kozuharov ex Foggi & Petrova. The aim of the present study was to assess isoenzyme variation and genetic affinities among the four species of the genus *Festuca*. Genetic identities (I) and distances (D) were calculated to evaluate qualitative genetic affinities and systematic relationships among the species. Considering the patterns of isoenzyme variation in the studied group, it is evident that *F. dalmatica* and *F. stojanovii* are closely related species. The species *F. valesiaca* and *F. rupicola* are isoenzymatically well characterized as distinct genetic entities. The obtained results generally support recent narrow species concept in the genus *Festuca*.

Key words: *Festuca*, species, isoenzyme variation, genetic affinities, systematic relationships

1. Introduction

Festuca L. (fescue) 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 the genus *Festuca* has undergone drastic changes over the time. More than a century ago, relatively few, broadly defined taxa were recognized (Hackel 1882). Lately, species concepts have become narrower and a large number of finely split taxa are recognized today (Markgraf-Dannenberg 1976, 1978, 1980). *Festuca ovina* L. is an extreme example of this changing species concept. Originally described by Hackel (1882) as a single polymorphic species, lately it was divided into several dozens of species (Markgraf-Dannenberg 1980; Wilkinson & Stace 1981).

The four examined *Festuca* species (*F. valesiaca* Schleich. ex Gaud., *F. rupicola* Heuff., *F. dalmatica* (Hack.) K. Richt. and *F. stojanovii* (Acht.) Kozuharov ex Foggi & Petrova) have a long and varied taxonomic history. They are closely related and belong to the subsection Saxatiles which reflect one of xeromorphic evolutionary lines in the genus *Festuca* (Kozuharov 1982). Formerly they have been referred to as *F. ovina*. The species *F. valesiaca* Schleich. ex Gaud. has been considered a variety (Stojanov & Stefanov 1924) or a subspecies (Stojanov & Stefanov 1948) of *Festuca ovina*. Stojanov & Stefanov (1933) treated *F. dalmatica* (Hack.) K. Richt. as a subspecies of *Festuca ovina*. Achtarov (1953) described *F. stojanovii* as a subspecies of *F. dalmatica*, mainly on the basis of the number of its vascular bundles. Besides, the species *F. stojanovii* has a specific leave cross-section pattern quite different from the *F. dalmatica* pattern. Lately, Kozuharov (1982) made a revision of the genus *Festuca* and proposed the new species *F. stojanovii* (Acht.) Koz., but did not validate (comb. invalid.) this taxonomic decision (Angelov & Kozuharov 1997). Foggi *et al.* (2005) made a new combination *F. stojanovii* (Acht.) Foggi & Petrova within the genus *Festuca*. Besides their morphological and anatomical differences, both taxa have specific ecological preferences. The species *F. dalmatica* is a facultative calcicole occurring in the majority of mountainous floristic districts of Bulgaria between 800 and 1700 m a.s.l. upon calcisols or cinnamonic soils. On contrary, *F. stojanovii* is an obligate calcicole found exclusively in the westernmost part of the country (Znepole floristic

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district) upon eroded cinnamonic soils, between 500 and 1000 m a.s.l. (Angelov & Kozuharov 1997).

The species *F. valesiaca* is quite widely distributed in Europe – from C. Germany to N.C. Russia and southwards to Pyrenees (Markgraf-Dannenberg 1980). The species *F. dalmatica* and *F. rupicola* have a more restricted geographic distribution – C. Europe (Hungary) and Balkan peninsula (Markgraf-Dannenberg 1980). The species *F. stojanovii* is a Balkan endemic taxon, occupying dry stony and grassy places on limestone (Angelov & Kozuharov 1997).

Isoenzymes are more reliable genetic markers than those previously used in plant biosystematics. The most significant advantage of isoenzymes is the simple genetic basis of their polymorphism. Being proteins, they can directly reflect alterations in the genome. Hence, changes in the electrophoretic mobility of enzymes provide an extremely useful method of evaluating genetic differences and systematic relationships within taxonomically complicated plant groups.

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

The aim of the present study was to assess isoenzyme variation and genetic affinities among the abovementioned four species of the genus *Festuca*.

2. Materials and methods

In total, 261 individual plants belonging to 8 natural Bulgarian populations of the four *Festuca* species were examined (Table 1). Vouchers are deposited at the Herbarium of Institute of Biodiversity and Ecosystem Research in Sofia (SOM).

The isoforms of enzymes glutamate-oxaloacetate transaminase (GOT, EC 2.6.1.1), malate dehydrogenase (MDH, EC 1.1.1.37), glutamate dehydrogenase (GDH, EC 1.4.1.2), isocitrate dehydrogenase (IDH, EC 1.1.1.6) and 6-phosphogluconate dehydrogenase (6PGDH, EC 1.1.1.44) were resolved by polyacrylamide gel electrophoresis.

Leaf samples (0.1g) were ground in 0.3 ml extraction buffer (0.01M Tris, 0.08 M glycine, 0.005M cysteine and 20% sucrose) at pH 8.3. Ion-exchange resin Dowex 1 x 8 (0.4g / 1g fresh tissue) was added to the extraction buffer to eliminate polyphenols. Homogenates were centrifuged at 10000 rpm for 10 min. The supernatant was used as a source of enzymes. The enzymes were resolved on 7.5% separating gel (3% stacking gel) polyacrylamide slabs using the electrophoretic system of Davis (1964). The length of the separating gel was 7 cm and stacking gels were 2 cm long. Electrophoresis was conducted at 200V until the indicator dye bromophenol blue reached the gel end. Staining of gels followed procedures described by Shaw & Prasad (1970) for MDH and GDH, Przybylska *et al.* (1982) for GOT, Henderson (1965) for 6PGDH and Yeh & O´Malley (1980) for IDH.

Zones of enzyme activity that varied independently of other such zones were considered to be coded by single gene loci. Following Crawford & Smith (1984), different genes (loci) coding the same enzymes (isoenzymes) were designated according to the relative mobility of the enzymes they specify. That is, the gene coding the most anodal isoforms was designated by (1), the next most anodal one, (2), etc. In each locus the allele coding the fastest isoform was designated by (a), the next fastest (b), and so on. Based on the mean allelic frequencies/locus/taxon, genetic identities (I) and distances (D) were calculated (Nei 1972, 1978).

Genetic affinities among the studied *Festuca* species were presented graphically as a dendrogram produced from Nei's distance matrix using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA; Sneath & Sokal 1973).

3. Results

Genetic interpretation of enzyme banding patterns was based on two lines of evidence – the known subunit structure of enzymes and their segregation patterns within species. Three gene loci and dimeric subunit structure are supposed for GOT in *Secale* (Figueiras *et al.* 1984; Perez de la Vega & Allard 1984) and *Triticum* (Jaaska 1976; Salinas *et al.* 1982). Schematic genotypes

Table 1. Species, localities and the number of individuals examined

Species	Locality	Number of individuals	Voucher number
F. valesiaca	Vitosha Mt., around village of Bosnek	29	Co-170
	Vitosha Mt., Boyansko blato	34	Co-172
F. rupicola	Znepole region, around Belidie han	30	Co-106
	Stara planina Mt., around village of Chelopech	35	Co-164
F. dalmatica	Vitosha Mt., around village of Kladnitza	37	Co-111
	Vitosha Mt., around Selimitza	28	Co-112
F. stojanovii	Lozenska Mt., Polovrak	33	Co-115
	Chepan Mt.	35	Co-117

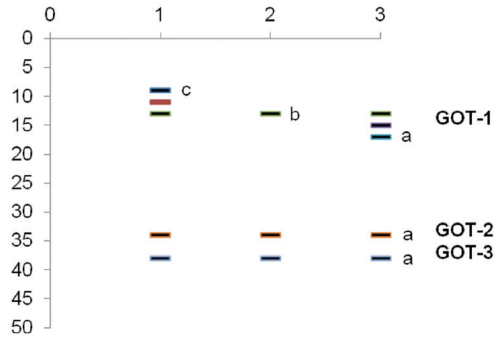

Fig.1. Schematic genotypes of GOT in the studied species of genus *Festuca.* Origin is at top, anode at bottom

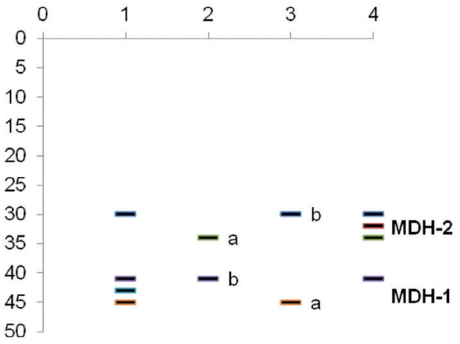

Fig. 2. Schematic genotypes of MDH in the studied species of genus *Festuca*. Origin is at top, anode at bottom

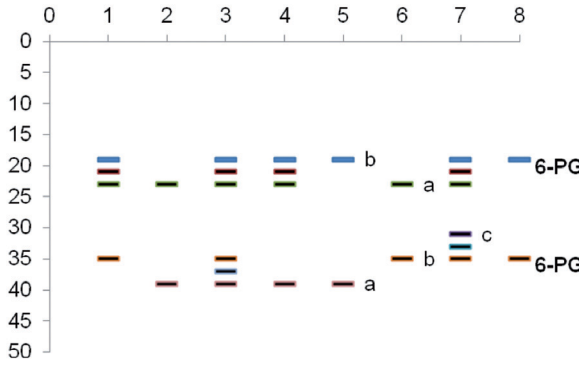

Fig. 3. Schematic genotypes of 6-PGDH in the studied species of genus *Festuca*. Origin is at top, anode at bottom

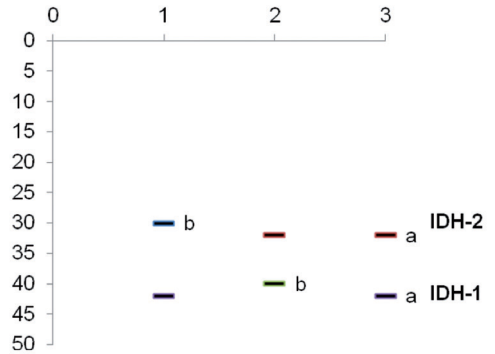

Fig. 4. Schematic genotypes of IDH in the studied species of genus *Festuca*. Origin is at top, anode at bottom

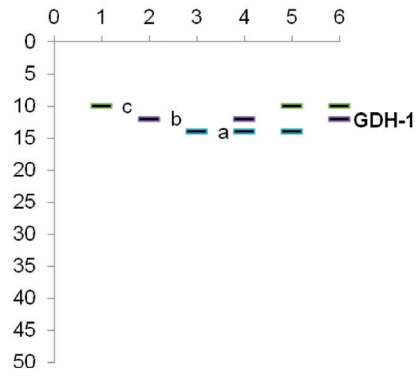

Fig. 5. Schematic genotypes of GDH in the studied species of genus *Festuca*. Origin is at top, anode at bottom

of GOT in the studied species of genus *Festuca* are presented in Fig. 1. Gene loci GOT 1, 2 are monomorhically fixed throughout the whole species group. Locus GOT-3 is variable with three alleles – a, b and c. The enzymes MDH and 6-PGDH are dimers coded by three genes in maize (Goodman *et al.* 1980) and *Secale* (Figueiras *et al.* 1984; Perez de la Vega & Allard 1984). Electrophoretic patterns of MDH and 6-PGDH in the studies species of genus *Festuca* are interpreted as shown in Fig. 2 and 3, respectively. Two gene loci and dimeric subunit structure was proposed for IDH in maize (Stuber & Goodman 1980) and barley (Brown & Munday 1982). Schematic genotypes of IDH and their genetic interpretation in the studies species of genus *Festuca* are presented in Fig. 4. One gene locus has been reported for GDH in *Secale*, *Triticum*, Hordeum, *Arabidopsis thaliana* (Gottlieb 1982; Cammerts & Jacobs 1983). Data about the quaternary structure of GDH are rather controversial. The enzyme is considered to have monomeric (McLeod *et al.* 1983), tetrameric (Bayer 1988) and hexameric (Cammerts & Jacobs 1983) subunit composition. The patterns of variation observed in the studies species of genus *Festuca* (Fig. 5) conform to the first genetic model.

Enzyme banding patterns observed in *Festuca* are in concordance with the above mentioned studies. Due to weak banding intensity and unsatisfactory resolution, the slowest anodally migrating zones of MDH and 6-PGDH were omitted. Thus, five enzymes, putatively coded by ten gene loci, namely, GOT 1, 2, 3; MDH 1, 2; GDH-1, IDH 1, 2 and 6-PGDH 1, 2 were scored. The studied populations within a taxon were electrophoretically very similar. For this reason, data for all populations in a taxon were pooled and mean frequencies were calculated. Mean allelic frequencies in the studied species are presented in Table 2. Most of alleles were shared by all studied species. Two gene loci, GOT 1 and GOT 2 were monomorphically fixed throughout the whole group. The species *F. valesiaca* and *F. rupicola* were invariant for different alleles at

Table 2. Mean allele frequencies in the studied species of genus *Festuca*

Gene locus	Allele	*F. valesiaca*	*F. rupicola*	*F. dalmatica*	*F. stojanovii*
GOT-1	a	1.00	1.00	1.00	1.00
GOT-2	a	1.00	1.00	1.00	1.00
GOT-3	a	0.00	0.00	0.05	0.22
	b	0.89	0.78	0.92	0.78
	c	0.11	0.22	0.03	0.00
MDH-1	a	0.00	1.00	0.98	0.86
	b	1.00	0.00	0.02	0.14
MDH-2	a	0.50	0.00	0.17	0.00
	b	0.50	1.00	0.83	1.00
GDH-1	a	0.23	0.25	0.10	0.00
	b	0.64	0.34	0.38	0.19
	c	0.13	0.41	0.52	0.81
IDH-1	a	0.00	0.00	1.00	1.00
	b	1.00	1.00	0.00	0.00
IDH-2	a	1.00	1.00	1.00	0.00
	b	0.00	0.00	0.00	1.00
6PGDH-1	a	0.13	0.38	0.63	0.75
	b	0.73	0.62	0.37	0.25
	c	0.14	0.00	0.00	0.00
6PGDH-2	a	0.50	0.62	0.50	0.72
	b	0.50	0.38	0.50	0.28

MDH - MDH 1a and MDH 1b, respectively. Similarly, *F. dalmatica* and *F. stojanovii* were fixed for allele IDH 1a, while allele IDH 1b was invariant in *F. valesiaca* and *F. rupicola*. Four alleles, GOT 3c, MDH 2a, GDH a and IDH 1b, were not detected in *F. stojanovii*. The allele 6-PGDH 1c was not found in *F. rupicola*, *F. dalmatica* and *F. stojanovii*.

Table 3. Genetic identities I (below diagonal) and distances D (above diagonal) for all pair wise comparisons among the studied *Festuca* species

	Species	1	2	3	4
1	*F. valesiaca*	×	0.17	0.34	0.41
2	*F. rupicola*	0.84	×	0,29	0.30
3	*F. dalmatica*	0.71	0.75	×	0.14
4	*F. stojanovii*	0.66	0.74	0.87	×

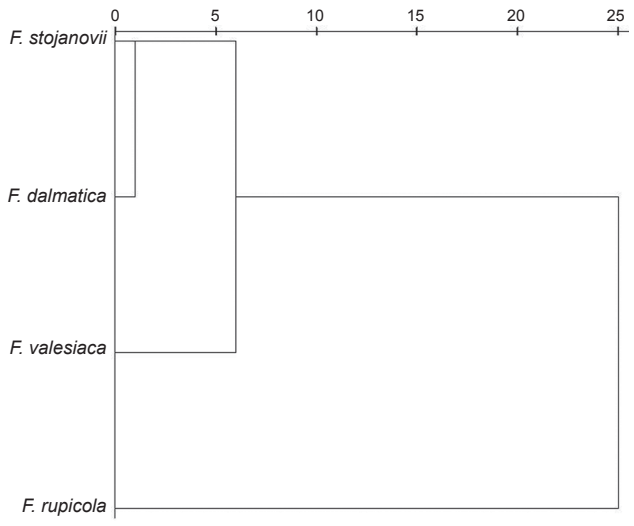

Fig. 6. A dendrogram of genetic distance values for the studied *Festuca* species, generated by the UPGMA clustering method. Scale relative, 0=minimal distance

Genetic identities and distance values for all pair-wise comparisons among the studied species are presented in Table 3. The values of coefficient I varied from 0.67 (*F. valesiaca* vs. *F. stojanovii*) to 0.87, when the latter species was contrasted with *F. dalmatica*. The *species F. dalmatica* and *F. stojanovii* were almost equidistantly positioned from *F. rupicola*. Values of coefficient D were within the range from 0.14 (*F. dalmatica* vs. *F. stojanovii*) to 0.41 when *F. valesiaca* was contrasted with *F. stojanovii*.

A dendrogram generated from the UPGMA clustering of genetic distances values for all pair-wise comparisons among the studied *Festuca* species is presented in Fig. 6. The dendrogram demonstrates that the species *F. dalmatica* and *F. stojanovii* are the least divergent couple – an indication for their close relatedness. The species *F. rupicola* proved to be the most distant taxon within the studied group of the genus *Festuca*.

4. Discussion

Analysis of isoenzyme data demonstrated that the examined taxa could be clearly discriminated by the used molecular markers. Several monomorphically-fixed allele differences in the isoenzyme structure of studied species were detected. On the other hand, a portion of alleles were not found in some of the examined *Festuca* species. These specific allele 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 in other studies of fescues. Isoenzyme markers were used to assess species boundaries in North American

representatives of the *F. ovina* complex (Aiken *et al.* 1993). Distinct isoenzyme profiles delimited 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 taxa examined (Guldahl *et al.* 2001). Aiken *et al.* (1994) and Aiken & Lefkovitch (1995) also reported unique combinations of bands pertaining to different taxa within the same complex. 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 genetic identity data, it is evident that *F. stojanovii* was the most distant from *F. valesiaca*. The species *F. rupicola* was almost equidistantly positioned from both *F. dalmatica* and. *F. stojanovii* The species *F. dalmatica* and *F. stojanovii* proved to be most closely related taxa within the studied group. Gottlieb (1977) summarized data for numerous electrophoretic studies of congeneric species and conspecific populations. Mean genetic identity value for conspecific populations was 0.95 whereas the respective value for congeneric species was equal to 0.67. Genetic identity values obtained in this study were in most cases much lower than the mean value for conspecific populations. Hence, it could be concluded that the examined *Festuca* species are closely related but discrete genetic entities.

The examined species are dense tufted, predominantly outcrossed wind-pollinated plants. It could be expected that the number of studied populations (8) is enough to reflect adequately the level and patterns of genetic diversity and systematic relationships among the examined *Festuca* species. According to Hamrick & Godt (1990), selfing species had more than 50%, while outcrossed wind-pollinated species had less than 10% of their total genetic diversity among populations. Moreover, genetic diversity at the population level reflects, fairly accurately, diversity at the species level. Species with limited geographic ranges (like *F. stojanovii*) partition their total genetic diversity in much the same way as more widespread species. The previous study of tufted *Festuca* species based on larger population samples (5-8 populations/species) also revealed that most of genetic diversity resides within the populations and the interpopulation diversity is rather low (Angelov 1986).

F. dalmatica and *F. stojanovii* were genetically most closely related taxa within the studied group. In spite of distinct anatomical differences between them, *F. stojanovii* was firstly recognized by Achtarov (1953) as a subspecies of *F. dalmatica* mainly on the basis of the number of vascular bundles. However, the number of bundles in the leaf sclerenchyma – five in *F. dalmatica* and seven in *F. stojanovii*, as well the number of leaf ribs allow for differentiation of these two taxa. Isoenzyme data presented in this study support the opinion of Kozuharov (1982) that *F. dalmatica* and *F. stojanovii* are closely related but different and well defined taxa within the genus *Festuca*. It should be noted that *F. valesiaca* and *F. rupicola*, separated mainly on the basis of subtle morphological differences, were isoenzymatically well characterized as distinct genetic entities. These species are also closely related and clearly distinguishable from *F. dalmatica* and *F. stojanovii*. In the previous study of polyphenolic compounds (Angelov *et al.* 1988), it was demonstrated that the xerophytes *F. valesiaca* and *F. rupicola* are more closely related to each other than to the group of mesophyte species of genus *Festuca*. These findings conformed to the results of the present study. Generally, the studied species of genus *Festuca* exhibit subtle morphological differences. They differ mainly in anatomical characters observed in cross-sections, but their identification is difficult. Isoenzyme data presented here provided evidence that the four taxa are genetically well defined and in good concordance with those reported in the previous studies of other fescue species. In conclusion, the present study generally supports the narrow species concept in the genus *Festuca*.

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