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Isoenzyme Variation and Genetic Relationships among four Balkan Endemics of the *Festuca ovina* group (*Poaceae-Poeae*)

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With 3 Figures

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Summary

ANGELOV G. B. 2003. Isoenzyme variation and genetic relationships among four Balkan endemics of the *Festuca ovina* group (*Poaceae-Poeae*). – Phyton, (Horn, Austria) 43 (2): 271–280, 3 figures. – English with German summary.

The isoenzyme systems SOD, ACP, DIA and CAT were studied in four Balkan endemics of the genus *Festuca* L., belonging to the group of *Festuca ovina* [*F. oviniformis* VETTER, *F. thracica* (ACHT.) MARKGR.-DANN., *F. hirtovaginata* ACHT.) MARKGR.-DANN. and *F. hercegovinica* MARKGR.-DANN.]. Most isoforms and isoenzyme phenotypes were shared by all fescues examined. However, each of four species possessed unique isoenzyme phenotypes. One unique isoform each for *F. oviniformis* and *F. hirtovaginata* was also revealed. Despite of their close morphological resemblance, the four fescues proved to be discrete entities as jugded by the set of enzymes surveyed. *F. oviniformis* seemed to be the most distinct within the studied group, while *F. hirtovaginata, F. thracica* and *F. hercegovinica* were nearly equidistant from each other. Isoenzyme data presented are generally in concordance with the narrow species concept in the genus *Festuca*.

Zusammenfassung

ANGELOV G. B. 2003. Isoenzym-Variabilität und genetische Beziehungen von vier Balkan-Endemiten der *Festuca ovina*-Gruppe (*Poaceae-Poeae*). – Phyton (Horn, Austria) 43 (2): 271–280, 3 Abbildungen. – Englisch mit deutscher Zusammenfassung.

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Die Isoenzym-Systeme SOD, ACP, DIA und CAT werden für vier Endemiten der Balkanhalbinsel aus der *Festuca ovina*-Gruppe studiert [*F. oviniformis* VETTER, *F. thracica* ACHT.) MARKGR.-DANN., *F. hirtovaginata* ACHT.) MARKGR.-DANN. and *F. hercegovinica* MARKGR.-DANN.]. Die meisten Isoformen und Isoenzym-Phänotypen sind allen vier *Festuca*-Arten gemeinsam. Aber jede der vier Arten besitzt spezifische Isoenzym-Phänotypen. Je eine spezifische Isoform wurde für *F. oviniformis* und *F. hirtovaginata* gefunden. Trotz der großen morphologischen Ähnlichkeit sind die vier Festucen auf Grund der Enzym-Muster distinkte Einheiten. *F. oviniformis* ist anscheinend innerhalb der studierten Arten die am stärksten abgesetzte, während *F. hirtovaginata, F. thracica* und *F. hercegovinica* zueinander nahezu äquidistant erscheinen. Die Isoenzym-Daten decken sich mit einem engen Art-Konzept innerhalb der Gattung *Festuca*.

Introduction

The fescues *Festuca oviniformis* VETTER, *F. thracica* ACHT.) MARKGR.-DANN., *F. hirtovaginata* (ACHT.) MARKGR.-DANN. and *F. hercegovinica* MARKGR.-DANN. are restricted to the southern parts of Balkan peninsula (Bulgaria, Greece, former Yugoslavia), thus being Balkan endemics. *F. oviniformis* is reported as endemic to the mountains above Comotini in NE Greece (MARKGRAF-DANNENBERG 1980). This taxon has been overlooked in other taxonomic treatments of genus *Festuca* in Greece (MARKGRAF-DANNENBERG 1976, STRID 1991). *F. oviniformis* has been found in SW Bulgaria – Strouma Valley (KOZUHAROV & PETROVA 1991) and SE Bulgaria – the southeastern parts of Rhodopes (KOZUHAROV 1985). The four species are perennial, dense caespitose xerophytes occurring in open grassy and stony places. These species exhibit different edaphic preferences. *F. oviniformis* grows on serpentinites, while *F. hercegovinica* prefers silicates. The species *F. thracica* and F. *hirtovaginata* grow mainly on calcareous substrates. The study of Bulgarian populations (KOZUHAROV 1985, KOZUHAROV & PETROVA 1991) showed that *F. thracica* is diploid (2n=14), *F. hirtovaginata* and *F. hercegovinica* are tetraploids (2n=28), while *F. oviniformis* is hexaploid (2n=42).

The species concept in the genus *Festuca* has undergone drastic changes. More a century ago, relatively few, broadly defined taxa were recognized (HACKEL 1882). Now that species definitions become more narrow and a large number of finely split taxa is recognized today. *F. ovina* is an extreme example of this changing species concept. Originally described by HACKEL 1882 as a single variable species it was recognized in the eighties as several dozens species (MARKGRAF-DANNENBERG 1980, WILKINSON & STACE 1981). *F. thracica* and *F. hirtovaginata* have also a varied and complex taxonomical history. In some older taxonomic treatments they have been considered as forms of *F. duriuscula* (ACHTAROV 1953) or varieties/ subspecies of *F. ovina* (STOJANOV & STEFANOV 1948: 150, STOJANOV & al. 1966: 124). Lately, these taxa were critically revised by MARKGRAF-DAN-

NENBERG (1976, 1978, 1980) who elevated them to species rank. *F. hercegovinica* was only recently described (MARKGRAF-DANNENBERG 1978).

In the last decade several isoenzyme studies of subarctic / arctic (AIKEN & al. 1993, AIKEN & al. 1995; AIKEN & al. 1995, GULDAHL & al. 2001) and temperate zone fescues (LIVESEY & NORRINGTON-DAVIS 1991, WILSON 1999) were conducted in attempt to investigate species delimitation by means of isoenzyme markers. To our knowledge, no isoenzyme studies of Balkan endemic fescues have been performed so far.

The purpose of the present study was to employ electrophoresis to determine isoenzyme variation and genetic affinities among the above-mentioned four Balkan endemics of the genus *Festuca*. Additionally, isoenzyme data might contribute to their more precise delimitation.

Material and Methods

Living plants were collected from natural populations (Table 1) and they were cultivated under greenhouse conditions. Voucher specimens were deposited in the Herbarium of Institute of Botany, the Bulgarian Academy of Sciences (SOM). Fresh leaves were used as source of enzymes. The extracting buffer was 0.01 M tris, 0.08 M glycine, 0.05 M cysteine, pH 8.3. Ion-exchange resin Dowex 1 × 8 was added (0.4 g per 1 g plant tissue) to eliminate polyphenols. The buffer was made up to 20% sucrose to provide density for loading into slots. Enzymes of superoxide dismutase (SOD), cathodal acid phosphatase (ACP), diaphorase (DIA) and catalase (CAT) were electrophoretically resolved on polyacrylamide slab gels utilizing a separating gel of 7.5% and a spacer of 3% with a slightly modified tris-glycine discontinious system of DAVIS 1964. The electrophoretic system of REISFELD & al. 1962 was employed to re-

Table 1.
Collection localities for fescues examined in this study. N denotes the number of individuals/population used in enzyme electrophoresis.

Species	N	Locality	Voucher (SOM)
F. hercegovinica	30	Strouma river valley, Kresna gorge	Co-425
	20	Rila Mt., the valley of river Blagoevgradska Bistritza, around village of Bistritza	Co-430
F. oviniformis	25	Eastern Rhodopes, near village of Zhulti chal	Co-426
	18	Eastern Rhodopes, around village of Goljamo Kamenjane	Co-427
	21	Eastern Rhodopes, Kazak village	Co-428
F. thracica	29	Western Rhodopes, in the vicinity of Asenovgrad, locality Korudere	Co-431
	24	Western Rhodopes, around Martziganitza chalet	Co-432
F. hirtovaginata	41	Rila Mt., the valley of Bistritza river, locality Samokovishteto	Co-433

solve the cathodal isoforms of ACP with spacer of a 3% and a 7.5% separating gel. The lenght of the separating gel was 5 cm for DIA, ACP, CAT and 7 cm for SOD. The following volumes of supernatant were loaded into each slot: 30 µl for SOD, 40 µl for ACP, 50 µl for DIA and 10 µl (tenfold diluted) for CAT. Electrophoresis was conducted until indicator dyes (bromphenol blue and pyronin G) reached the end of the gel (1 gel lenght) for ACP, 1.25 lenghts for SOD, 1.5 lenghts for DIA and 3 lenghts for CAT. Gels were stained according to the procedures of WOODBURY & al. 1970 for CAT and KOROCHKIN & al. 1970 for ACP. The gels for DIA were incubated in 30 ml 0.1 M tris-HCl buffer (pH 8.0), containing 16 mg reduced nicotine amide adenine dinucleotide, 8 mg nitroblue tetrazolium (NBT), 0.4 mg 2,6-dichlorophenolindophenol Na salt at 37° C. Reaction mixture for SOD consisted of 10 mg NBT, 3 mg riboflavine, 75 mg EDTA (dissolved separately) in totally 100 ml of 0.05 M tris-HCl buffer, pH 8.2. Gels were incubated at 25° C in dark for 30 min. then rinsed twice with water and illuminated until colourless bands on blue background were developed. Each isoform was assigned a number according to its gel migration in mm from the start (PEREZ DE LA VEGA & ALLARD 1984).

All four enzyme systems displayed activity and more or less legible bands. Due to uncertainties concerning the subunit structure of some enzymes, e. g. DIA (KEPHART 1990, GULDAHL & al. 2001) and difficulties with the interpretation of banding patterns in polyploid fescues (WILSON 1999), phenetic analysis of isoenzyme variation was preferred. Mean frequencies of isoforms (electrophoretic bands) and isoenzyme phenotype (electrophoretic spectra) were calculated for each species. Using isoform and isoenzyme phenotype (isophenotype) frequency data separately, mean values of D_{CD} (Coefficient of Divergence, CLARK 1952, see STUESSY 1990: 75) 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 / isoenzyme phenotypes for each enzyme, x_{ij} and x_{ik} – the frequency of i-th isoform / isoenzyme phenotype 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 P_{jx} and P_{jy} are the frequencies of j-th isoform/isoenzyme phenotype in species x and y and n is number of isoforms/isoenzyme phenotypes at each enzyme. Thus, two data sets, one based on isoform frequencies and second on isophenotype frequencies were generated.

Results

Superoxide dismutase. In total, seven isoforms of SOD (Table 2), and seven isoenzyme phenotypes (Table 3, Fig. 1), were detected in the studied group. All isoforms were common for the four fescues examined. Two isoforms, 7 and 44, were fixed (frequency of 1.00) throughout the group, whereas isoforms 19, 25 and 57 were nearly fixed or invariant in different species. Pair-wise comparisons among the species resulted in value of coeficient D_{CD} equal to 0.07 when *F. hirtovaginata* and *F. hercego-*

Table 2.
Mean isoform frequencies of SOD in the studied populations of the four fescues examined

Species	Isoforms						
	7	19	25	29	37	44	57
F. hercegovinica	1.00	0.90	1.00	0.66	0.33	1.00	1.00
F. oviniformis	1.00	1.00	1.00	0.40	0.60	1.00	0.80
F. thracica	1.00	0.90	0.90	0.80	0.50	1.00	1.00
F. hirtovaginata	1.00	0.90	1.00	0.80	0.20	1.00	1.00

Table 3.
Mean frequencies of isoenzyme phenotypes of SOD in the studied populations of the four fescues examined

Species	Isoenzyme phenotypes						
	1	2	3	4	5	6	7
F. hercegovinica	0.33	0.50	0.00	0.00	0.17	0.00	0.00
F. oviniformis	0.20	0.20	0.40	0.00	0.00	0.20	0.00
F. thracica	0.33	0.40	0.17	0.00	0.00	0.00	0.10
F. hirtovaginata	0.20	0.70	0.00	0.10	0.00	0.00	0.00

vinica were compared. The highest value (D_{CD}=0.23) was obtained in the comparison between the former and *F. oviniformis*. Isoenzyme phenotypes 1 and 2 were shared by all species investigated. Isophenotype 3 was common for *F. oviniformis* and *F. thracica,* while phenotype 6 was unique for the former species. The rare isophenotypes 4 and 7 were diagnostic for *F. hirtovaginata* and *F. thracica*, respectively. Isophenotype 5 was observed in *F. hercegovinica* only. The coefficient I_h varied from 0.44 (*F. oviniformis*

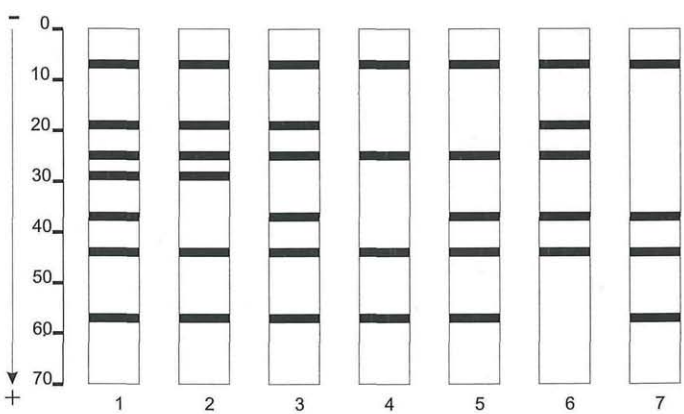

Fig. 1. Schematic isoenzyme phenotypes of SOD for the *Festuca* species examined. The scale is in mm, the start is at the top.

vs. *F. hirtovaginata*) to 0.90 in the comparison between the latter and *F. hercegovinica*. Values of D_{CD} ranged from 0.12 (*F. thracica* vs. *F. hercegovinica*) to 0.30 (*F. oviniformis* vs. *F. hirtovaginata*).

Cathodal acid phosphatase. Isoenzyme phenotypes 1 and 2 were common for the whole group, whereas isophenotypes 3 and 4 were specific for the species pairs *hirtovaginata / hercegovinica* and *oviniformis / thracica*, respectively (Fig. 2.). The values of Ih varied from 0.36 in the case of *F. oviniformis* vs. *F. hirtovaginata* to 0.80 when the latter was compared to *F. hercegovinica*. Isoform 11 was invariant throughout the group, while isoform 17 had frequency between 0.20 in *F. oviniformis* and 0.55 in *F. hirtovaginata*. Isoform 22 was shared by the species pair *hirtovaginata / hercegovinica*. Isoform 24 was diagnostic for *oviniformis / thracic*a. The coefficient I_h ranged between 0.82 and 0.98 in pair-wise comparisons among the taxa examined. The values of D_{CD} varied from 0.11 (*F. hercegovinica* vs. *F. hirtovaginata*) to 0.35 when the latter species was contrasted to *F. oviniformis*.

Diaphorase. The isoenzyme phenotypes of diaphorase are shown in Fig. 3. The most frequent isoenzyme phenotype 1 was shared by *F. hirtovaginata*, *F. hercegovinica* and *F. thracica*, whereas isophenotype 2 was diagnostic for the first species. *F. oviniformis* possessed two specific isopenotypes, 3 and 4, with frequencies of 0.75 and 0.25, respectively. The species *F. thracica* and *F. hercegovinica* were indistinguishable (I_h=1.00, D_{CD}=0.00) in regard to DIA isophenotypes, while *F. oviniformis* was quite different (I_h=0.00 for each pair-wise comparison) from the rest of taxa examined. Except for *F. oviniformis*, isoform 32 was invariant across the studied group. Isoform 37 was also fixed in all four taxa. Isoform 16 was specific for *F. oviniformis*, while isoform 20 was unique for *F. hirtovaginata*. Comparisons of *F. oviniformis* with the other three taxa resulted in values of I_h between 0.75 and 0.76. The coefficient D_{CD} ranged from

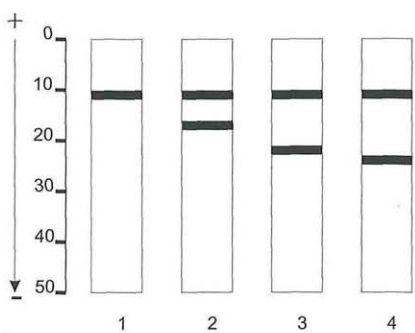

Fig. 2. Schematic isoenzyme phenotypes of ACP for the *Festuca* species examined. The scale is in mm, the start is at the top.

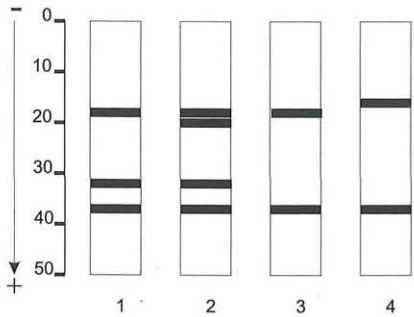

Fig. 3. Schematic isoenzyme phenotypes of DIA for the *Festuca* species examined. The scale is in mm, the start is at the top.

0.14 between *F. hercegovinica* and *F. hirtovaginata* to 0.68 between the former species and *F. oviniformis*.

Catalase. Two isoforms, 13 and 17, and three isophenotypes were observed. The isophenotype consisting of isoform 13 was shared by *F. oviniformis, F. thracica* and *F. hirtovaginata. F. hirtovaginata* and *F. hercegovinica* possessed in common the isophenotype formed by isoform 17. The isophenotype 13/17 was specific for *F. oviniformis* and *F. thracica.*

Mean values of the coefficients I_h and D_{CD} based on the frequencies of isoenzyme phenotypes, are presented in Table 4. The lowest value for I_h was obtained when *F. oviniformis* and *F. hirtovaginata* were contrasted. Other pair-wise comparisons among the studied taxa resulted in two to threefold higher values of I_h. The highest values for D_{CD} were found in comparisons of *F. oviniformis* with the rest of taxa. Comparisons among the remaining taxa resulted in values of D_{CD} that were two to five times lower.

Table 4.

Mean values of coefficients I_h and D_{CD} based on the frequencies of isoenzyme phenotypes, which were calculated for enzymes SOD, DIA and ACP

Coefficient	Phenotypic identity I_h					Coefficient of Divergence D_{CD}				
Species (Sp)	Sp	1	2	3	4	Sp	1	2	3	4
F. hercegovinica	1	x				1	x			
F. oviniformis	2	0.35	x			2	0.46	x		
F. thracica	3	0.83	0.42	x		3	0.07	0.37	x	
F. hirtovaginata	4	0.88	0.23	0.74	x	4	0.20	0.38	0.21	x

Mean values of the coefficients I_h and D_{CD} based on isoform frequency data are shown in Table 5. The values of I_h varied within a narrow range. Considering D_{CD}, its lowest value was obtained in the comparison between *F. thracica* and *F. hercegovinica. Festuca oviniformis* exhibited the highest values of D_{CD} in pair-wise comparisons with the rest of taxa examined.

Table 5.
Mean values of coefficients I_h and D_{CD} based on isoform frequencies which were calculated for enzymes SOD, DIA and ACP

Coefficient	Phenotypic identity I_h					Coefficient of Divergence D_{CD}				
Species (Sp)	Sp	1	2	3	4	Sp	1	2	3	4
F. hercegovinica	1	x				1	x			
F. oviniformis	2	0.87	x			2	0.37	x		
F. thracica	3	0.94	0.90	x		3	0.07	0.30	x	
F. hirtovaginata	4	0.98	0.85	0.93	x	4	0.11	0.35	0.11	x

Discussion

It is evident from the two data sets that the taxa examined could be discriminated by isoenzymes. It is worth mentioning that *F. oviniformis* is the most distinct taxon in both data sets. Moreover, this species possessed three unique isophenotypes followed by *F. hirtovaginata* with two unique isophenotypes. One specific isophenotype each for *F. thracica* and *F. hercegovinica* was observed. In contrast to the latter two species, each of *F. oviniformis* and *F. hirtovaginata* had also one unique isoform.

Similar patterns of isoenzyme variation have been found in other studies of fescues. Isoenzymes were used to assess species boundaries in North American representatives of the *F. ovina* complex (AIKEN & 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 & al. 2001). AIKEN & al. 1994, 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, WILSON 1999).

Enzymatically, *F. oviniformis* seems to be the most distinct species within the studied group. This species, like most of the recently recognized *Festuca* taxa, occupies a narrow ecological niche. Moreower, it is characterized by a single chromosome number. *F. oviniformis* is a calcifuge and occur on sepentinites only. It is noteworthy that *F. thracica* and *F. hirtovaginata*, which have been separated on the basis of subtile morphological differences, were isoenzymatically well-characterized. Thus, the isoenzyme data support the current practice among fescue reseachers to accept closely similar taxa at the species level. *F. thracica* may in fact be more closely related to *F. hercegovinica*. However, taking into account all data available, it could be concluded that *F. thracica, F. hercegovinica* and *F. hirtovaginata* are more or less equidistant from each other.

The four fescues examined belong the group of *F. ovina*. They are characterized by morphological and anatomical adaptations to extremely xeric conditions. These fescues exhibits subtile morphological differences. They differ mainly in anatomical characters observed by cross-sectioning of the leaves, but their identification is difficult. Isoenzyme data presented here provide evidence that the four taxa are genetically well defined. These results are in concordance with those reported in the before-mentioned studies of other fescue species. Thus, isoenzyme data generally support a narrow species concept in fescues.

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