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Research Article

ISOENZYME VARIATION AND SYSTEMATIC RELATIONSHIPS AMONG *FESTUCA ASPERIFOLIA*, *F. PERISTEREA*, *F. DIFFUSA* AND *F. VALESIACA* (POACEAE)

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Summary: The isoenzyme variation of *Festuca asperifolia*, *F. peristerea*., *F. diffusa* and *F. valesiaca* was examined by means of four molecular markers. Systematic relationships among the above mentioned taxa were assessed by calculating the coefficient of divergence D. The mean values of coefficient D averaged over the four enzymes varied in a rather wide range from 0.15 to 0.50 for all pair-wise comparison among the studied species of genus *Festuca*. The values of coefficient D for species *F. diffusa* and *F. peristerea* were an indication that these species were tightly bound within the studied group, while *F. asperifolia* was somewhat distantly positioned. The species *F. valesiaca* was most distantly related within the group. Thus, the studied species could be arranged according to their decreasing genetic affinities as follows: *F. diffusa, F. peristerea, F. asperifolia, F. valesiaca.* The results are in good correspondence with data derived from morphology, anatomy, ecology and paleobotany. It could be concluded that the present isoenzyme study contributes further to more distinct discrimination between mesomorphic and xeromorphic lines and supports the contemporary concepts in taxonomy and systematics of genus *Festuca.*

Keywords: *Festuca*, species, isoenzyme variation, systematic relationships.

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INTRODUCTION

Festuca L. is taxonomically and systematically one of the most complicated genera in Poaceae. Due to overlapping of morphological and anatomical characters, including diagnostic ones in genus *Festuca,* there are problems in identification and uncertainties in its taxonomy. So, there is a need to apply different new approaches, including biochemical ones, to reveal the systematic structure and relationships among the respective taxa.

The present study includes four species of genus *Festuca,* namely, *F.*

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asperifolia St.-Yves, *F. peristerea* (Vetter) Markgr.-Dann., *F. diffusa* Dumort. and *F. valesiaca* Schleich. ex Gaudin.

The species *F. asperifolia* is mesoxerophyte, calcicole. It occurs in grassy and stony habitats. It is a rare species as only two populations from Vitosha Mountain are known. *F. peristerea* is mesophyte and occupies grassy and stony habitats. It is sparsely distributed in Bulgaria – several small populations in Pirin Mountain and Western Rhodopes. The species *F. diffusa* is mesophyte that occurs in grassy and stony habitats on silicates. It is rare species with several small populations in Vitosha Mountain. *F. valesiaca* is xerophyte and occupies grassy, shrubby and stony habitats on both silicates and limestone. It is widely distributed throughout Bulgaria up to 1100 m. above sea level.

Isoenzymes are more useful and reliable 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 a method of evaluating genetic differences and systematic relationships within taxonomically complicated plant groups. Several studies based on isoenzyme markers were conducted in attempt to investigate species delimitation and relationships in genus *Festuca* (Livesey and Norrington-Davis, 1991; Aiken et al., 1993; Aiken et al., 1994; Aiken and Lefkovitch, 1995; Guldahl et al., 2001; Angelov, 2003; Angelov and Bednarska, 2018).

The purpose of this study was to reveal the isoenzyme variations and systematic relationships among the above-mentioned four species of genus *Festuca*.

MATERIAL AND METHODS

Randomly chosen set of enzymes with different metabolic functions and degree of variability were used to represent more adequately the species genome. Thus, anodal and cathodal peroxidase (EC 1.11.1.7, PER), superoxide dismutase,(EC 1.15.1.1, SOD) and amylase, (EC 3.2.1.1 (AMY) were analyzed individually (25-30 living plants/population) in natural populations (2-4 populations/species) collected in different floristic regions of Bulgaria (Table 1). Anodally migrating isoforms were resolved on 7.5% polyacrilamide slab gels as was earlier described (Davis, 1964). Isoforms of cathodal PER were run on 7.5% polyacrilamide acidic slab gels (Reisfeld et al. 1962). The length of gels was 11 cm for AMY and SOD, 7 cm for cathodal PER and 6 cm for anodal PER. The following staining recipes were used: AMY (Reisfeld et al. 1962), PER (Przybylska et al., 1982), SOD (Baur and Schorr, 1969).

Systematic relationships among the above mentioned taxa of genus *Festuca* were assessed by calculating the coefficient of divergence D (Stuessy, 1990) according to the following formula:

$$D = \sqrt{\frac{1}{N}\sum_{i=1}^{N}\left(x_{ij} - x_{ik}\right)^2}$$

where, N is the number of isoforms for each enzyme, x_{ij} and x_{ik} are the mean frequencies of i-th isoform in taxa j and k. The mean values of coefficient D

averaged over the four enzymes surveyed were calculated. Additionally, an index group distance (GD) was calculated for each species as a sum of its coefficient D values.

RESULTS AND DISCUSSION

Superoxide dismutase

Eleven isoforms of SOD were observed in the studied species of genus *Festuca* (Table 2). Monomorphically-fixed isoforms 28, 39, 45, 70 and 73 were common for the whole species group. Except for *F. valesiaca,* isoform 61 was also monomorphic throughout the studied group. The values of coefficient D fluctuated from 0.02 (*F. diffusa* vs *F. peristerea*) to 0. 45 when the species *F. valesiaca* and *F. peristerea* were contrasted.

Amylase

Totally six isoforms of the enzyme were electrophoretically resolved in the examined species (Table 3). Isoform 13 was species-specific for *F. peristerea* while isoform 16 was found in *F. diffusa* only. Except for *F. valesiaca*, isoform 18 was also common for the whole group. Similarly, isoform 38 was observed in all studied taxa but absent in *F. valesiaca*. The values of coefficient D for pair-wise comparisons among *F. diffusa*, *F. peristerea* and *F. asperifolia* varied in a comparatively narrow range – from

Table 1. Species and populations examined.

Species	Population locality
F. asperifolia	Vitosha Mt., around Selimitsa chalet
	Vitosha Mt., around Ostritsa chalet
F. peristerea	Pirin Mt., around Hvoynati vrah peak
F. diffusa	Vitosha Mt., around Kamen dyal peak
F. valesiaca	Vitosha Mt., in the vicinity of Knyazhevo
	Sredna gora Mt., around the village of Petrich
	Ponor Mt., around Belidie han

Table 2. Isoform frequencies of superoxide dismutase in the studied populations of *F. asperifolia*, *F. peristerea, F. diffusa* and *F. valesiaca.*

Species	Isoform										
	24	28	31	36	39	45	50	56	61	70	73
F. asperifolia	1.00	1.00	1.00	0.45	1.00	1.00	0.54	1.00	1.00	1.00	1.00
F. peristerea	0.00	1.00	0.00	0.00	1.00	1.00	0.42	1.00	1.00	1.00	1.00
F. diffusa	0.00	1.00	0.00	0.00	1.00	1.00	0.22	1.00	1.00	1.00	1.00
F. valesiaca	0.75	1.00	0.65	1.00	1.00	1.00	0.34	1.00	0.85	1.00	1.00

Table 3. Isoform frequencies of amylase in the studied populations of *F. asperifolia*, *F. peristerea, F. diffusa* and *F. valesiaca.*

Species	Isoform					
	13	**16**	**18**	**20**	**38**	**46**
F. asperifolia	0.00	0.00	1.00	0.00	0.50	0.50
F. peristerea	0.34	0.00	1.00	0.00	0.00	1.00
F. diffusa	0.00	0.25	1.00	0.00	0.00	1.00
F. valesiaca	0.00	0.00	0.00	0.82	1.00	0.00

0.23 (*F. diffusa* vs *F. peristerea*) to 0.38 when the latter species and *F. asperifolia* were compared. In contrast, pair-wise comparisons of *F. valesiaca* with the above mentioned three species resulted in very high values of coefficient D – from 0.66 (*F. asperifolia* vs *F. valesiaca*) to 0.74 when the latter species was contrasted with *F. peristerea.*

Anodal peroxidase

Eight isoforms of the enzyme were electrophoretically resolved in the studied populations of all species (Table 4). A group of three isoforms, namely 39, 43, 48 were monomorhically fixed throughout the studied group. Except for *F. valesiaca,* isoform 22 was observed in the studied group. The species *F. valesiaca* possessed two unique isoforms (17 and 20) and shared the monomorphic isoform 19 with *F. asperifolia*. The values of coefficient D varied in a wide range (0.05-0.51). The species *F. diffusa* and *F. peristerea* proved to be most closely positioned (D=0.05), while *F. valesiaca* was most distant related with the species pair *diffusa- peristerea* (D=0.51).

Cathodal peroxidase

In total, ten isoforms of cathodal PER were electrophoretically resolved in the studied species of genus *Festuca* (Table 5). Most of them, namely 28, 31, 36, 39, 44 were monomorhically fixed and shared by all studied species. Excluding *F. asperifolia,* isoform 22 was also monomorphic throughout the studied group. Two species-specific isoforms (12 and 17) were detected in *F. asperifolia.* The values of coefficient D indicated that the species *F. valesiaca* and *F. peristerea*

Table 4. Isoform frequencies of anodal peroxidase in the studied populations of *F. asperifolia*, *F. peristerea, F. diffusa* and *F. valesiaca.*

Species	Isoform							
	17	**19**	**20**	**22**	**23**	**39**	**43**	**48**
F. asperifolia	0.00	1.00	0.00	0.48	0.00	1.00	1.00	1.00
F. peristerea	0.00	0.00	0.00	1.00	0.20	1.00	1.00	1.00
F. diffusa	0.00	0.00	0.00	1.00	0.25	1.00	1.00	1.00
F. valesiaca	0.22	0.00	0.12	0.00	0.00	1.00	1.00	1.00

Table 5. Isoform frequencies of cathodal peroxidase in the studied populations of *F. asperifolia*, *F. peristerea, F. diffusa* and *F. valesiaca.*

Species	Isoform									
	12	**17**	**22**	**28**	**31**	**36**	**39**	**44**	**47**	**50**
F. asperifolia	0.18	0.42	0.85	1.00	1.00	1.00	1.00	1.00	0.75	1.00
F. peristerea	0.00	0.00	1.00	1.00	1.00	1.00	1.00	1.00	0.64	0.00
F. diffusa	0.00	0.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
F. valesiaca	0.00	0.00	1.00	1.00	1.00	1.00	1.00	1.00	0.58	0.00

were most closely positioned (D=0.05), with respect to cathodal PER. On the contrary, the former species was most distantly related to *F. asperifolia* (D=0.35) and *F. diffusa* (D=0.38) in regard to this isoenzyme marker.

The mean values of coefficient D averaged over the four enzymes surveyed are shown in Table 6. The values ranged in a rather wide range from 0.15 (*F. diffusa* vs *F. peristerea*) to 0.50 when the former species was compared with *F. valesiaca.* In should be noticed that all pair-wise comparisons of *F. valesiaca* with the other three species resulted in very high mean values of coefficient D – twice higher than the lowest one.

The index of group distance contributed further to revealing the relationships within the examined group of genus *Festuca.* Lower values of index GD show closer affinity for a given taxon, and vice versa, higher values indicate a greater distance within the group. The values of index GD for species *F. diffusa* (GD=0.95) and *F. peristerea* (GD=0.98) were an indication that these species were tightly bound within the studied group, while *F. asperifolia* (GD=1.05) was somewhat distantly positioned. The species *F. valesiaca* (GD=1.30) was most distantly related within the group. Thus, the studied species could be arranged according their decreasing genetic affinities as follows: *F. diffusa, F. peristerea, F. asperifolia, F. valesiaca.* These findings correspond well with morphological, anatomical and ecological data.

The species *F. diffusa, F. peristerea, F. asperifolia* are members of the *F. rubra* group. Taxonomically they belong to the section *Aulaxyper* Dumort. of the type

Table 6. Mean values of coefficient D for each pair-wise comparison among the studied species of genus *Festuca.*

	Species	Coefficient D			
		1	**2**	**3**	**4**
1	*F. asperifolia*	x			
2	*F. peristerea*	0.39	x		
3	*F. diffusa*	0.30	0.15	x	
4	*F. valesiaca*	0.50	0.44	0.50	x

subgenus *Festuca* (Tzvelev 2010), which is among the most primitive sections of thin-leaves fescues. These species exhibit specific set of morphological and anatomical traits. They are characterized with extravaginal shoots, often flat leaves, specific anatomy of vegetative leaves (multifaceted leaf cross-sections, more than 3 sclerenchyma strands, deep grooves between ribs on adaxial surface), in certain species ovary hairy at apex, sheaths closed to the mouth. For comparison, the evolutionary more advanced section *Festuca* is characterized with hairless ovary, lack of extravaginal shoots, very thin leaves (sclerenchyma as subepidermal layer or 3 strands**),** less prominent ribs on adaxial surface and sheaths closed to no more than 1/3.

Using morphological, cytological, ecological and paleobotanical methods, Tsvelev (1972) proposed a hypothesis about the evolution of genus *Festuca*. Type subgenus *Festuca* is supposed to have polytopic and paraphyletic origin connected with the Alps stage of orogenesis. Its prototype species (presumably diploids) were growing in wet high mountains habitats. The main trends of evolution were xeromorphogenesis and cryomorphogenesis. More advanced polyploid species migrated and adapted to lower more dry habitats. So, two main lines in the evolutionary history of *Festuca* could be observed: mesomorphic and xeromorphic ones (Kozuharov, 1985). The species *F. diffusa* is diploid, occupies wet mountain habitats. Similarly, *F. peristerea* is diploid, high-mountain species. It exhibits a set of morphological traits indicating that it is one of the old members of the *F. rubra* group (Kozuharov, 1985). The species *F. asperifolia* is diploid, mesoxerophyte and morphologically and ecologically occupies an intermediate position between *F. rubra* group and *F. valesiaca* group (Kozuharov, 1985). The species *F. valesiaca* includes widespread polyploid xerophytes occupying dry grassy, shrubby and stony habitats on both silicates and limestone. It demonstrates high ecological plasticity.

Considering the results of this study, a good correspondence with data derived from morphology, anatomy, ecology and paleobotany could be observed. The diploid mesophyte species *F. diffusa* and *F. peristerea* are most closely related, while *F. asperifolia* occupies an intermediate position between *F. rubra* group and *F. valesiaca* group. The species *F. valesiaca* as a typical representative of a xeromorphic evolutionary line is most distantly related within the studied group. It could be concluded that the present isoenzyme study contributes further to more distinct discrimination between mesomorphic and xeromorphic lines and supports the contemporary concepts in taxonomy and systematics of genus *Festuca.*

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