Systematic relationships among some *Festuca* species (*Poaceae*) from the Ukraine as revealed by isoenzymes

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Abstract. The species *Festuca valesiaca*, *F. rupicola, F. galiciensis*, *F. macutrensis* (*F. valesiaca* agg.), and *F. pallens* (*F. glauca* agg.) were analyzed by polyacrylamide gel electrophoresis (PAGE), in order to evaluate their systematic relationships. Affinities among them were assessed by coefficient of divergence D. Averaged for four enzyme markers, the mean values of coefficient D for comparison of *F. pallens* with the species of *F. valesiaca* group were the highest (0.34–0.45) and indicated the remote position of the former species within the studied group. Mean values of D for comparison within *F. valesiaca* agg. were lower and suggested close mutual affinities. The results are generally in concordance with the recent systematics of the narrow-leaved fescues, on the basis of morphological and anatomical features.

Key words: *Festuca,* isoenzymes, PAGE, systematic relationships

Introduction

Festuca L. is among the largest genera of *Poaceae* and, according to different authors, encompasses from 300 (Tzvelev 1976) to more than 500 species worldwide (Catalan & al. 2004). This is one of the most complicated genera of grasses. The species concept in *Festuca* was drastically changed over time. A century ago, relatively few widely defined taxa were described. Lately, the species concepts have changed to a narrow defined series of taxa. As a result of these processes, the number of species has increased from 28 (Hackel 1882) to 170 species in the taxonomic treatments of European fescues in *Flora Europaea* (Markgraff-Dannenberg 1980).

There are few studies of the genus in the Ukraine (Tzvelev 1976; Tveretinova 1977; Bednarska 2007, 2014, 2017). It was established that most narrow-leaved fescues greatly overlap in their morphological and anatomical traits, including in diagnostic ones. Hence, other approaches are needed to reveal the systematic structure and phylogenetic relationships of fine-leaved fescues. Series of isoenzyme studies into fescues (Livesey & Norrington-Davis 1991; Aiken & al. 1993; Aiken & al. 1994; Aiken & Lefkovitch 1995; Angelov 2002; Angelov & Bednarska 2016; Angelov & Bednarska in press) have been carried out, in order to reveal the boundaries between the species by means of isoenzyme markers.

This study includes five taxa of *Festuca* from the Ukraine: *F. pallens* Host, *F. valesiaca* Gaud., *F. rupicola* Heuff., *F. macutrensis* Zapał., and *F. galiciensis* Bednarska. The last four taxa belong to *F. valesiaca* agg. sensu Alegro & Sostaric (2006), or *F. valesiaca* group (Pils 1984; Arndt 2008). These taxa are characterized by rough leaves, 3–5 sclerenchyma strands in the leaf cross section, thickest at the margins and middle of the leaf, veins 5–7, and all lemmas awned (Arndt

2008). About 10 species of this group occur in the Ukraine. Most of them are significantly differentiated by ecological and geographical barriers, for exmple *F. callieri* (Hack.) Markgr.-Dann. – rock/steppe species of the Crimean mountains, *F. saxatilis* Schur – species of the Carpathian rocky outcrops, *F. arietina* Klok. – species of psammophytic meadows on the flood plain of NE Ukraine and Belarus, etc. Moreover, the territory of the Western Podolian Upland (West Ukraine) is among the richest in regard to the number of taxa belonging to this group. Of these, *F. rupicola* (2n=42), *F. macutrensis* (2n=28, 42) and *F. galiciensis* (2n=28) form a sophisticated complex of polyploid species (Tveretinova 1977; Bednarska 2014, 2017). Their distribution areas overlap on this territory, along with that of diploid *F. valesiaca* s.str. (2n=14. That region is characterized by its hilly terrain combined with mosaic outcrops of limestone, gypsum cliffs and wide valleys in the depressions with black soil and rendzina. Similar diversity of biotopes has influenced diversity of *F. valesiaca* agg. populations in the region and variety of their morphological and anatomical features. This resulted at some overlapping of their diagnostic traits, which leads to problems in precise identification of the respective taxa (Bednarska 2014, 2017).

Festuca pallens belongs to the species with glaucous, smooth leaves, sclerenchyma in the form of complete and unthickened ring, and 7–11 veins. According to the East-European tradition, this species belongs to the group of *F. glauca* agg. (Bednarska 2003). There were two reasons for it to be included in the study: first, for comparing the diagnostic capacity of isozyme markers within one species aggregate, and among the different aggregates; second, for checking the hypothesis of possible involvement of *F. pallens* in the origin of *F. galiciensis* – an endemic species to Podolian Upland. A number of characters of *F. galiciensis* make it somewhat similar to *F.pallens*: habitat (xerothermic gypsum outcrops), intensive waxy leaves and corresponding glaucous-blue color, weak pubescence of all parts of the plant, more pronounced and long hairs on the adaxial leaf blade surface, frequent occurrence of broad bands or continuous sclerenchyma. In our opinion, these characteristics suggest that *F. pallens* could be one of the parental species of *F. galiciensis*.

The purpose of the study was to reveal the isoenzyme variation and systematic relationships among the above-listed species of genus *Festuca*.

Material and methods

Young seedlings (30–35 seedlings per each species, a month old) derived from natural populations of the above – mentioned species were individually studied (Table 1). The enzymes acid phosphatase (ACP), malate dehydrogenase (MDH), peroxidase (PER), and catalase (CAT) were examined. Anodal isoforms were resolved on 7.5 % polyacrylamide slab gels (Davis 1964). Cathodal PER was run on 7.5 % polyacrylamide

Table 1. Locality list of the studied taxa of genus Festuca from the Ukraine.

Taxon	Number LWKS	Locality
F. valesiaca	1598	Zhytomyr region, Berdychiv region, Berdychiv town 24.07.2010 Leg. O.Orlov 49°54'0" 28°34'0"
	1533	Zakarpattia region, Vynohradiv district, village Klynovetska Gora 19.06.2011 Leg. leg. I.Bednarska, I.Danylyk, R.Kish
F. macutrensis locus classicus	1622	Lvov region, Brody district, village Sukhovolya Mountain Makitra 01.07.2013 leg. I.Bednarska, 50°2'17.32" 25°14'53.75"
F. galiciensis sp. nova locus classicus	1672	Ivano-Frankovsk region, Rohatyn district, v. Kuropatnyky (glaucous plants) 19.07.2014 leg. I.Bednarska, 49°17'2.47" 24°40'8.10"
F. rupicola	1673	Ivano-Frankovsk region, Rohatyn district, v. Kuropatnyky (green plants) 19.07.2014 leg. I.Bednarska, 49°17'1.98" 24°40'8.87"
F. rupicola f. glaucescens	1678	Ivano-Frankovsk region, Rohatyn district, v. Fraga (glaucous plants) 19.07.2014 leg. I.Bednarska, 49°28'2.47" 24°26'49.50"
F. rupicola	1679	Ivano-Frankovsk region, Rohatyn district, v. Fraga (green plants) 19.07.2014 leg. I.Bednarska, 49°28'2.47" 24°26'49.50"
F. rupicola f. glaucescens	1674	Ivano-Frankivsk region, Galych district, village Podilla (glaucous plants) 19.07.2014 leg. I.Bednarska, 49°16'33.46" 24°44'28.45"
F. rupicola	1675	Ivano-Frankovsk region, Galych district, village Podilla (green plants),19.07.2014 leg. I.Bednarska 49°16'30.88" 24°44'29.95"

slab gels (Reisfeld & al. 1962). Gels were 6 cm long. Catalase was run on 6% gels for 8 hours at 150 V. The following staining recipes were used: ACP (Korochkin & al. 1977), MDH (Shaw & Prasad 1970), PER (Przybylska & al. 1982), CAT (Woodbury & al. 1970). Each isoform was designated by a number reflecting its migration (in mm) from the origin.

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

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

where, N is the number of isoforms for each enzyme, x_{ij} and x_{ik} are the frequencies of i^{-th} isoform in taxa j and k. An index of group affinity (GA) was calculated for each taxon as a sum of its D values.

Results and discussion

Acid phosphatase. A total of eight isoforms of the enzyme were observed in the studied species (Table 2). Isoform 12 was monomorphically fixed across the entire group. Isoforms 5 and 9 were invariant (frequency of 1.00) in the species of *F. valesiaca* agg. The remaining isoforms occurred with different frequency in the populations of all examined species. The values of coefficient D for the pair-wise comparisons within *F. valesiaca* agg. varied within the range 0.17–0.29 and indicated close mutual affinities between the respective taxa. On the contrary, a comparison of *F. pallens* with the species of *F. valesiaca* group resulted in higher values of coefficient D (0.35–0.45) – an indication of a more distant position of the former species.

Malate dehydrogenase. Six isoforms of MDH were resolved electrophoretically in the examined *Festuca* taxa (Table 3). Isoform 24 was common and invariant for all species. With the exception of *F. pallens*, isoforms 4, 18 and 20 were monomorphically fixed in the species of *F. valesiaca* group. Isoform 27 was absent in *F. pallens*. The values of coefficient D for the pair-wise comparisons among the members of *F. valesiaca* agg. varied within a narrow range (0.14–0.21) and suggested close affinities. Coefficient D ranged from 0.30 (*F. pallens* vs *F. macutrensis*) to 0.46, when the former species was contrasted to *F. valesiaca*. It could be concluded that *F. pallens* occupied a remote position as judged by the enzyme marker MDH.

Anodal peroxidase. A total of six isoforms of the enzyme were detected in the studied taxa (Table 4). The invariant isoform 30 was shared by all studied species. Isoform 33 was common and fixed in the taxa of *F. valesiaca* agg. Isoform 15 was not found in both *F. galiciensis* and *F. pallens*, while isoform 12 was absent from *F. rupicola* and *F. macutrensis*. The species *F. pallens* proved to be most distant from *F. rupicola* (D = 0.48) and *F. macutrensis* (D = 0.52), whereas the species of *F. valesiaca* agg. were almost equidistantly positioned from each other.

Cathodal peroxidase. A total of six isoforms of the enzyme occurred in the studied species of genus *Festuca* (Table 5). Isoforms 22 and 25 were monomorphically fixed across the entire group. With the exception of *F. galiciensis* and *F. pallens*, isoform 29 was also invariant. Similarly, isoform 32 was common

Table 2. Isoform frequencies of acid phosphatase in the studied species of genus *Festuca*.

	Isoform							
Species	**5**	**9**	**12**	**23**	**25**	**29**	**31**	**33**
F. valesiaca	1.00	1.00	1.00	1.00	0.24	0.21	0.47	1.00
F. rupicola	1.00	1.00	1.00	0.95	0.68	0.36	0.35	1.00
F galiciensis	1.00	1.00	1.00	0.65	0.36	0.92	0.42	0.72
F. macutrensis	1.00	1.00	1.00	0.75	0.75	0.28	0.38	1.00
F. pallens	0.75	0.75	1.00	0.24	0.08	0.39	0.11	0.24

Table 3. Isoform frequencies of malate dehydrogenase in the studied species of genus *Festuca*.

	Isoform					
Species	**4**	**5**	**18**	**20**	**24**	**27**
F. valesiaca	1.00	0.63	1.00	1.00	1.00	1.00
F. rupicola	1.00	0.27	1.00	1.00	1.00	1.00
F galiciensis	1.00	0.40	1.00	1.00	1.00	0.50
F. macutrensis	1.00	0.33	1.00	1.00	1.00	0.65
F. pallens	0.80	0.59	0.90	0.90	0.90	0.00

Table 4. Isoform frequencies of anodal peroxidase in the studied species of genus *Festuca*.

	Isoform					
Species	**12**	**14**	**15**	**27**	**30**	**33**
F. valesiaca	0.29	0.85	0.18	1.00	1.00	1.00
F. rupicola	0.00	1.00	0.12	0.85	1.00	1.00
F galiciensis	0.75	0.90	0.00	0.65	1.00	1.00
F. macutrensis	0.00	1.00	0.08	1.00	1.00	1.00
F. pallens	0.80	0.45	0.00	0.39	1.00	0.91

Table 5. Isoform frequencies of cathodal peroxidase in the studied species of genus *Festuca*.

Species	Isoform					
	17	22	25	29	32	38
F. valesiaca	1.00	1.00	1.00	1.00	1.00	0.87
F. rupicola	0.46	1.00	1.00	1.00	1.00	0.43
F galiciensis	0.28	1.00	1.00	0.85	1.00	0.62
F. macutrensis	0.36	1.00	1.00	1.00	0.55	0.48
F. pallens	0.72	1.00	1.00	0.12	0.42	0.00

and fixed in *F. valesiaca, F. rupicola* and *F. galiciensis.* Pair-wise comparisons of *F. pallens* with taxa of *F. valesiaca* agg. resulted in high values of coefficient D (0.44–0.58) and confirmed the distant position of the former species within the examined group.

Catalase. One isoform (15) of the enzyme was electrophoretically resolved and shared by all examined *Festuca* taxa.

Averaged for all examined enzymes, the mean values of coefficient D for comparison of *F. pallens* with species of *F. valesiaca* group were the highest (0.34–0.45) and indicated the remote position of the former species within the studied group (Table 6). Mean values of D for comparisons within *F. valesiaca* agg. were lower and suggested close mutual affinities. Index GA contributed further to revealing the systematic relationships within the examined group. Lower values of index GA mean closer affinity for a given taxon, and vice versa, higher values indicate a greater distance. The species *F. pallens* proved to be the most distantly positioned within the group, as its index GA (1.71) was the highest. The values of index GA within *F. valesiaca* agg. varied within a narrow range (0.96–1.11) and indicated close and almost equidistant positions among the respective taxa.

Despite significant differences between the species aggregates, some similarity should be noted between *F. pallens* and *F. galiciensis*. Bearing in mind the morphological and anatomical data (Bednarska 2014), as well as the florogenesis of the region where it was described, we have assumed that *F. galiciensis* is of hybridogeneous origin and *F. pallens* is one of its parents. The presented isoenzyme data do not exclude such possibility.

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

Species	Coefficient D				
	1	2	3	4	5
1. F. valesiaca	0.00				
2. F. rupicola	0.16	0.00			
3. F galiciensis	0.26	0.23	0.00		
4. F. macutrensis	0.23	0.12	0.22	0.00	
5. F. pallens	0.46	0.47	0.39	0.39	0.00

A second species which deserves special attention is *F. macutrensis*. Similarly to *F. galiciensis*, it is characterized by fusion of the sclerenchyma strands forming an entire or an interrupted sclerenchyma ring. Because of its leaf anatomical structure, *F. macutrensis* is also suggested to be of hybridogeneous origin (Tzvelev 1976). However, it is assumed that a species of *F. ovina* agg. with an entire ring is one of its parents. As for the second ancestral species, a comparative analysis of the diagnostic traits population variability of *F. macutrensis* (2n=4x=28; 2n=6x=42) and *F. rupicola* (2n=6x=42) practically showed full resemblance of their morphological traits and significant overlapping of the anatomical leaf traits (Bednarska 2011, 2014). The presented isoenzyme data also confirmed the high level of similarity and, together with other available data, evidenced a close affinity between these two species.

Analysis of the isoenzyme data showed that the examined taxa could be distinguished by these molecular markers. Several monomorphically-fixed isoform differences were detected. For example, isoforms 5 and 9 of ACP were invariant in *F. valesiaca*, *F. rupicola*, *F. galiciensis,* and *F. macutrensis,* and clearly differentiated them from *F. pallens*. Similarly, isoforms 4, 18, 22, and 24 of MDH were monomorphically-fixed in the members of *F. valesiaca* agg. and distinguished them from *F. pallens.* Furthermore, isoform 33 of anodal PER was monomorphic in the *F. valesiaca* group in contrast to *F. pallens.* On the other hand, certain isoforms were not found in some of the examined *Festuca* species. Thus, isoform 38 of cathodal PER was absent from *F. pallens,* while it occurred in the representatives of *F. valesiaca* agg. These specific combinations form distinct isoenzyme patterns which discriminate the respective species within the group. Similar patterns of variation were found in the studies of some Balkan representatives of genus *Festuca* (Angelov 2002). Distinct isoenzyme profiles and isoform frequency differences delimited discrete entities within the *F. ovina* complex (Aiken & al. 1993; Aiken & al. 1994; Aiken & Lefkovitch 1995).

Mean values of coefficient D for each pair-wise comparison among the studied species of *Festuca,* based on fourteen examined isoenzyme markers in the present study, as well as in Angelov & Bednarska

(2016;2017) are presented in Table 7. It could be seen that *F. valesiaca*, *F. rupicola* and *F. macutrensis* were most closely related, as the values of coefficient D for each pair-wise comparison among them were the lowest and varied within a narrow range. *F galiciensis* occupied a more distant position in respect to these three species. It tended to be comparatively closer to *F. pallens*. The relationships among the studied *Festuca* species are demonstrated graphically by a dendrogram based on coefficient D (Fig. 1; Table 7).

Summarizing the results, it became evident that the species *F. pallens*, as a representative of other species aggregate, occupied a more remote position in regard to the species belonging to *F. valesiaca* agg. It could be concluded that the results are generally in concordance with the recent systematics of narrow-leaved fescues, on the basis of their morphological and anatomical features.

Table 7. Mean values of coefficient D for each pair-wise comparison of the studied species of *Festuca* on the basis of fourteen examined isoenzyme markers.

Species	Coefficient D				
	1	2	3	4	5
1. F. valesiaca	0.00				
2. F. rupicola	0.17	0.00			
3. F galiciensis	0.28	0.26	0.00		
4. F. macutrensis	0.18	0.16	0.25	0.00	
5. F. pallens	0.38	0.34	0.31	0.35	0.00

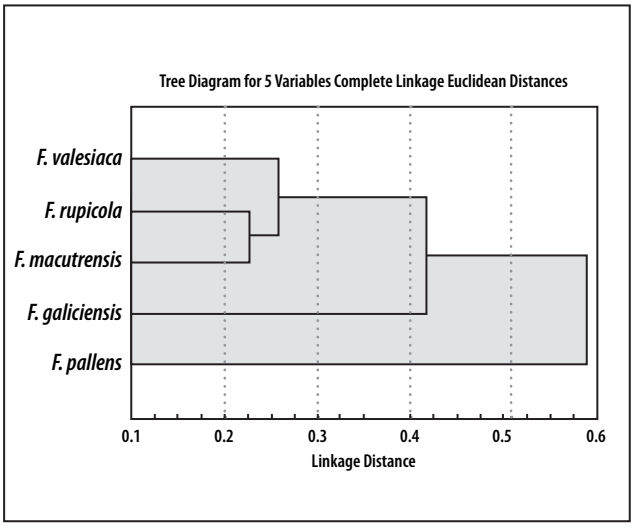

Fig. 1. Cluster analysis dendrogram of isoenzymatic data for five *Festuca* species (*F. valesiaca, F. rupicola, F. macutrensis, F. galiciensis,* and *F. pallens*) based on coefficient D (Table 7).

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