ISSN 0206-5657. Вісник Львівського університету. Серія біологічна. 2018. Випуск 79. С. 29–37
Visnyk of the Lviv University. Series Biology. 2018. Issue 79. P. 29–37

УДК 581.1+582.54

ISOENZYME VARIATION AND GENETIC AFFINITIES AMONG FIVE *FESTUCA* SPECIES OF SECTION *AULAXYPER* DUMORT.

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Despite a considerable number of taxonomical and biosystematic studies of genus *Festuca* L. in Europe, there are few papers discussing phylogeny and systematic of genus *Festuca* as well the evolution of its different groups. The most important among them are the studies of N. Tzvelev, who proposed three sections within type subgenus *Festuca*: *Variae* Hack., *Aulaxyper* Dumort. and *Festuca*. There are many studies on species belonging to section *Festuca*, including chemosystematic ones, but the species of section *Aulaxyper* are rather neglected. For this reason we choose *F. rubra* L., *F. nigrescens* Lam., *F. picturata* Pils., *F. amethystina* L. and *F. heterophylla* Lam., which belong to section *Aulaxyper*. The aim of the study was to examine the isoenzyme variation and to evaluate the genetic affinities among the above-listed species of genus *Festuca*.

Ten natural Bulgarian populations were examined. The isoforms of enzymes glutamate-oxaloacetate transaminase, malate dehydrogenase, glutamate dehydrogenase, isocitrate dehydrogenase and 6-phosphogluconate dehydrogenase were resolved by polyacrylamide gel electrophoresis. Based on mean allelic frequencies/locus/taxon, genetic identities (I) values for all pair-wise comparisons among the studied species were calculated.

The group of *F. rubra* s.l. is more primitive compared to *F. ovina* s.l. and has sheaths closed nearly to the mouth while the sheaths of species of section *Festuca* are closed to the base. *Festuca amethystina* occupies an intermediate position as its sheaths are closed for 1/3-1/2 of their length. This character indicates for its specific position within section *Aulaxyper* and isolates it from the rest taxa of the group. Our results confirmed its peculiar position within section *Aulaxyper. Festuca heterophylla* at the same time includes both primitive traits which are specific for the ancient species of genus *Festuca*, and the number of highly-specialized characters. The obtained molecular data confirm a peculiar position of *F. heterophylla* within genus *Festuca*. On the contrary, the closely related to *F. rubra* polyploids, namely *F. nigrescens* and *F. picturata* should be considered as more recent "new" species. *Festuca amethystina* showed the greatest divergence and should be considered also as an ancient species.

Keywords: Festuca, isoenzymes, variation, systematic relationships

Introduction

Festuca (fescue) is a genus of flowering plants belonging to the grass family, Poaceae (subfamily Pooideae). Because of its complicated taxonomy, it is not clear how many true species belong to the genus, but estimates range from over 400 to over 500. Since Hackel's *Monographia Festucarum Europaearum* [16] and the studies of many festucologists from the first several decades of 20-th century, Markgraf-Dannenberg [20] proposed a contemporary treatment of this genus in *Flora Europaea*. Since *Flora Europaea*, series of taxonomical and biosystematic studies on critical groups of *Festuca* were carried out [9–11, 26–28].

However there are few papers discussing phylogeny and systematic of genus *Festuca* as well as the evolution of its different groups. Most important among them are the studies of N. Tzvelev [29, 30]. His main approach is comparative analysis of morphological and anatomical traits (primitive/advanced) of different taxa within *Festuca.* Recently N. Tsvelev [31] proposed three sections: *Variae* Hack., *Aulaxyper* Dumort. and *Festuca* within type subgenus *Festuca*. There are many studies on species belonging to section *Festuca,* including chemosystematic ones [1–3, 15] but the species of section *Aulaxyper* are rather neglected. For this reason we choose species belonging to section *Aulaxyper* (type *F. rubra*) which is among the most primitive sections of thin-leaves fescues. These species 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), with less prominent ribs on adaxial surface**,** as well sheaths closed to no more than 1/3.

The present study includes *F. rubra* L., *F. nigrescens* Lam. (syn. *F. rubra* subsp. *fallax* (Thuill.) Nym.), *F. picturata* Pils. (syn. *F. violacea* subsp. *picta* (Kit.) Hegi), *F. amethystina* L. and *F. heterophylla* Lam. They belong to the section *Aulaxyper* Dumort. of the type subgenus *Festuca*. The comparative characteristics of the species [32, 33] are given in Table 1.

Table 1

Comparative characteristics of the species [32, 33]

Species	Life form	Non-flowering shoots	Flowering shoots
F. rubra	Laxly caespitose, with rhizomes, extravaginal shoots	(0,5-)0,6–1,2 mm, flat; sheaths closed nearly to the mouth	30–80 cm; under a panicle glabrous; ovary glabrous, rarely with solitary hairs 2n=14, 28, 42, 56
F. nigrescens	Densely caespitose, most of shoots intravaginal	0,4–0,7 mm, flat; sheaths closed to the mouth	30–80 cm, under a panicle glabrous; ovary glabrous 2n=42
F. picturata	Densely caespitose, all or most of shoots extravaginal	(0,4-)0,5–0,75 mm, flat; sheaths closed nearly to the mouth	(20-)30–40(-50) см, under a panicle densely shortly hairy; ovary usually sparsely hairy at apex 2n=14
F. amethystina	Densely caespitose, extravaginal shoots from few (subsp. *amethystina*) to numerous (subsp. *orientalis*)	0,4–0,6 mm, glabrous or nearly glabrous, pruinose; sheaths purple-violet, closed for 1/3-1/2 of their length	30–60 cm; under a panicle glabrous to slightly scabrid; ovary glabrous or subglabrous 2n=28
F. heterophylla	Densely caespitose, most of shoots intravaginal	(0,3-)0,4–0,6 mm, scabrid; sheaths closed, glabrous or slightly scabrid	(50-)60–120 cm, cauline leaves 2-3 mm wide; ovary densely hairy at apex 2n=28, 42

Within the group *F. rubra* is the most polymorphic and widely spread. This is a laxly caespitose species, usually with more or less long rhizomes, distributed almost throughout Europe. The rest of species have combined type of reproduction with different combinations of intra/extravaginal shoots. A common feature of these species is also their origin and distribution is related to mountain systems of Europe. For example, *F. nigrescens* is a densely caespitose species, which firstly was occurring in high mountain meadows. At present it is used as a component in seed mixtures for artificial meadows. It widened its distribution but made difficult the distinction between *F. nigrescens* and *F. rubra*. Its distribution range encompasses S., W. & C.

Europe, reaching to S. Sweden. *Festuca picturata* is rather densely caespitose plant. It is usually calcicole, occurring in E. Alps; Carpathians and the mountains of Bulgaria. *Festuca amethystina* and *F. heterophylla* are a densely caespitose species. The first one occupies dry places in Alps, C. Europe and Balkan peninsula, while the second species prefers habitats mainly in woods and it is distributed from S. England and Poland southwards to N.W.Spain and Greece.

Isoenzymes are valuable genetic markers. Their most significant advantage is the simple genetic basis of their polymorphism. Being proteins, they can directly reflect alterations in the genome. Electrophoretic methods for isoenzyme analysis testified their value to resolving systematic and evolutionary problems on species and subspecies level [6, 14]. In the last two decades several isoenzyme studies of subarctic/arctic [1–3, 15] and temperate zone fescues [19] were conducted in attempt to investigate species delimitation based on isoenzyme markers.

The aim of the study was to examine the isoenzyme variation and to evaluate the genetic affinities among the above-listed species of genus *Festuca*.

Materials and Methods

Living plants (25–30 individuals/population) belonging to 10 natural Bulgarian populations were examined (Table 2). Vouchers are deposited at the Herbarium of Institute of Biodiversity and Ecosystem Research in Sofia (SOM).

Table 2

Species and populations' localities

Species	Populations' localities
F. rubra	Rila Mt., in the vicinity of Vada chalet
	Stara Planina Mt., around Chumerna charcoal mine
F. nigrescens	Rila Mt., Parangalitsa reserve
	Rhodopes Mt., Kupena reserve
F. picturata	Vitosha Mt., around Aleko chalet, Platoto
	Rila Mt., in the vicinity of Dodov vrah peak
F. heterophylla	Vitosha region, around Iskar dam
	Rila Mt., in the vicinity of Treshtenik chalet
F. amethystina	Rila Mt., around Gorna Cadiitsa peak
	Rila Mt., along Bistritsa river, 2–3 km westwards Ivan Vazov chalet

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 10 000 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 B. Davis [7]. 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 C. Shaw & R. Prasad [25] for MDH and GDH, J. Przybylska *et al.* [23] for GOT, N. Henderson [17] for 6PGDH and F. Yeh & D. O΄Malley [35] for IDH.

Zones of enzyme activity that varied independently of other such zones were considered to be coded by single gene loci. According to D. Crawford [6], 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 by (b), and so on. Based on mean allelic frequencies/locus/taxon, genetic identities (I) were calculated [21].

Genetic affinities among the studied *Festuca* species were presented graphically as a dendrogram produced from Nei's identities matrix using STATISTICA 7.0. An index of group affinity (GA) was calculated for each taxon as a sum of its I values.

Results and Discussion

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* [8, 22] and *Triticum* [18, 24]. The enzymes MDH and 6-PGDH are dimers coded by three genes in maize and *Secale* [8, 22]. Two gene loci and dimeric subunit structure was proposed for IDH in barley [5].

The patterns of variation observed in the studied species of genus *Festuca* conform to the above-mentioned genetic models. The studied populations of each taxon were electrophoretically similar. Hence, the data for a taxon were pooled and mean frequencies were calculated. Mean allelic frequencies in the studied species are presented in Table 3. Totally, four enzymes, putatively coded by eleven gene loci, namely, 6-PGDH 1, 2, 3, GOT 1, 2, 3, MDH 1, 2, 3 and IDH 1, 2 were scored. Most of alleles were shared by all studied species – an indication for their close relationships. Excepting *F. heterophylla,* the studied species were monomorphically fixed for allele c of gene locus 6-PGDH 2. The species *F. rubra*, *F. nigrescens*, *F. picturata* were invariant for allele c of gene locus 6-PGDH 3, while *F. amethystina* and *F. heterophylla* were fixed for alleles a and b, respectively. Similarly, *F. rubra*, *F. nigrescens* , *F. picturata* were monomorphic for allele a of locus GOT 1. Excepting *F. heterophylla,* the examined species were monomorphically fixed for allele a of gene locus MDH 1. All studied species but *F. heterophylla* were invariant for allele a of MDH 3. Excluding *F. amethystina*, all species were fixed for allele b of locus IDH 1.The former was invariant for allele a. The same pattern was observed in respect to locus IDH 2.

Table 3

Mean allele frequencies in the studied species of genus *Festuca*

Locus	Alelle	*F. rubra*	*F. nigrescens*	*F. picturata*	*F. amethystina*	*F. heterophylla*
6PGDH 1	a	0.42	0.25	0.50	1.00	1.00
	b	0.58	0.75	0.50	0.00	0.00
6PGDH 2	a	0.00	0.00	0.00	0.00	0.00
	b	0.00	0.00	0.00	0.00	1.00
	c	1.00	1.00	1.00	1.00	0.00
6PGDH 3	a	0.00	0.00	0.00	1.00	0.00
	b	0.00	0.00	0.00	0.00	1.00
	c	1.00	1.00	1.00	0.00	0.00
GOT 1	a	1.00	1.00	1.00	0.20	0.84
	b	0.00	0.00	0.00	0.80	0.16
GOT 2	a	0.65	0.46	0.68	0.20	0.16
	b	0.35	0.54	0.32	0.80	0.84
GOT 3	a	1.00	1.00	0.47	0.00	0.00
	b	0.00	0.00	0.53	0.42	1.00
	c	0.00	0.00	0.00	0.58	0.00
MDH 1	a	1.00	0.97	1.00	1.00	1.00
	b	0.00	0.03	0.00	0.00	0.00
MDH 2	a	0.63	0.33	0.73	0.43	0.59
	b	0.37	0.67	0.27	0.57	0.41
MDH 3	a	1.00	1.00	1.00	1.00	0.50
	b	0.00	0.00	0.00	0.00	0.50
IDH 1	a	0.00	0.00	0.00	1.00	0.00
	b	1.00	1.00	1.00	0.00	1.00
IDH 2	a	0.00	0.00	0.00	1.00	0.00
	b	1.00	1.00	1.00	0.00	1.00

Genetic identities values for all pair-wise comparisons among the studied species are given in Table 4. The values of coefficient I varied from 0.98 (*F. rubra* vs. *F. nigrescens*) to 0.40 when *F. heterophylla* was contrasted to *F. amethystina.* The species *F. rubra*, *F. nigrescens*, *F. picturata* were genetically tightly related, while but *F. heterophylla* demonstrated isolation within the group. *Festuca amethystina* was the most distant taxon (Fig 1). Index of group affinity contributed further to revealing the relationships within the examined group of genus *Festuca.* Lower values of index GA mean greater distance for a given taxon, and vice versa, higher values indicate a closer affinity within the group. The values of index GA for *F. rubra* and *F. picturata* (3.10) as well for *F. picturata* (3.01) are an indication for their close affinity within the studied group. *F. heterophylla* (GA=2.30) was relatively distant while *F. amethystina* (GA=1.93) proved to be the most isolated within the group. In short, the examined species could be arranged by their decreasing affinity and increasing genetic divergence as follows: *F. rubra*, *F. nigrescens*, *F. picturata*, *F. heterophylla*, *F. amethystina.*

Table 4

Genetic identities (I) for all pair-wise comparisons among the studied species of genus *Festuca*

Species		Genetic identity (I)				
		1	2	3	4	5
1	*F. rubra*	1.00	0.98	0.97	0.53	0.62
2	*F. nigrescens*	0.98	1.00	0.94	0.49	0.6
3	*F. picturata*	0.97	0.94	1.00	0.51	0.68
4	*F. amethystina*	0.53	0.49	0.51	1.00	0.4
5	*F. heterophylla*	0.62	0.60	0.68	0.40	1.00

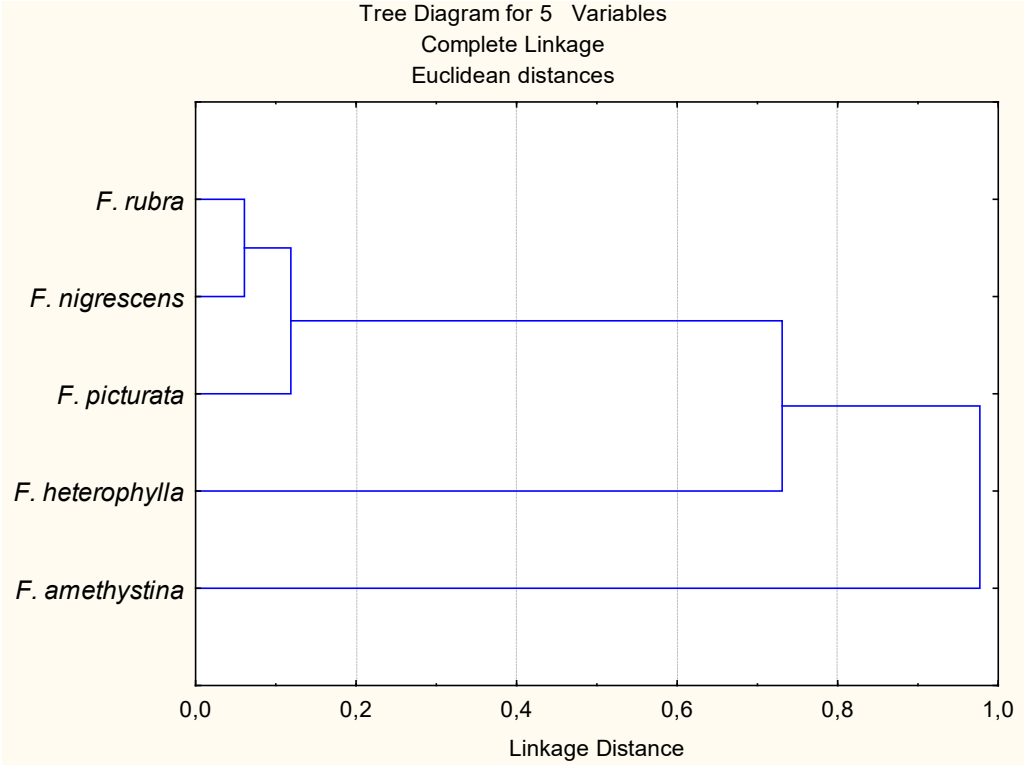

Fig. 1. The dendrogram of Cluster analysis for studied species of genus *Festuca* based on I coefficient (Genetic identities, Tabl. 4)

The results of the study afforded an opportunity to shed light on the hypothetical evolution of section *Festuca*. Before discussing the topic, some considerations should be taken into account. First, analyzing progenitor-derivative species' pair as an example of rapid speciation, L. Gottlieb [12, 13] found that they possess an identical or very similar isoenzyme structure. A contrasting pattern of divergence was found in plants where speciation is thought to have taken place by a gradual geographic mode. For example, S. Warwick and L. Gottlieb [34] showed that isoenzyme divergence paralleled the degree of divergence in morphology and ecology. These findings mean: the more two taxa diverge in their evolutionary history, the more differences accumulate on a molecular level, resulting in a gradually decreasing homology between their isoenzyme structure [6]. Generally speaking, genetic divergence reflects evolutionary distance between the taxa. Second, similarity of isoenzyme structure implies a common origin and progenitors. When considering a group of related taxa, it is reasonable to assume that each taxon's genome is shared partly by the rest ones. Such groups possess a common ancestral gene pool from which the separate lines (taxa) have originated. One should expect that the genome of more ancient "old" taxa will demonstrate a greater divergence from the common gene pool compared to the more recent "new" taxa within the group.

Bearing in mind the above discussed topic, an attempt will be made to analyze the evolutionary history of the studied group. Using morphological, cytological, ecological and paleobotanical methods, N. Tsvelev [30] proposed a hypothesis about evolution of genus *Festuca*. Type subgenus *Festuca* is supposed to have polytopic and paraphyletic origin connected with the Alps' stage of orogenesis. Its prototypes were growing in open high mountains habitats. The main trends of evolution were xeromorphogenesis and cryomorphogenesis. The high mountains' species *F. picturata* is closely related to *F. rubra.* It is considered as an ecologic and geographic race of Alps' *F. violacea* and it is treated as its subspecies in some floras. The Carpathian and Balkan's species *F. amethystina* is also connected with Alps' orogenesis and it is considered as a transition to more xeromorphic species of *Festuca.*

It should be mentioned that the degree of integration of vegetative shoots' sheaths (Tabl. 1) has not only diagnostic value but is an indication for the relationships among narrow-leaved fescues. The group of *F. rubra* s.l. is more primitive compared to *F. ovina* s.l. and has sheaths closed nearly to the mouth while the sheaths of species of section *Festuca* are closed to the base [4]. *Festuca amethystina* occupies intermediate position as its sheaths are closed for 1/3–1/2 of their length. This character indicates for its specific position within section *Aulaxyper* and isolates it from the rest taxa of the group. Our results confirmed its peculiar position within section *Aulaxyper.*

Festuca heterophylla has unique morphological trait (ovary hairy at apex) which is specific for the ancient species of genus *Festuca*. This character differentiates it from the rest taxa of the group. But *F. heterophylla* has a number of highly-specialized characters: transition to intravaginal reproduction, reduction of veins number of vegetative shoots, reduction of ribs'number on leaves, width reduction of vegetative leaves to 0.3–0.5 mm in diameter. Such combination of traits is quite unique and confirms the peculiar position of *F. heterophylla* within genus *Festuca*.

In general, molecular data conform to the above stated hypothesis. In the light of the results, one can suppose that *F. heterophylla* is an ancient species because its genome diverged substantially from the common gene pool. On the contrary, the closely related to *F. rubra* polyploids, namely *F. nigrescens* and *F. picturata* should be considered as more recent "new" species. *Festuca amethystina* showed the greatest divergence and should be considered also as an ancient species.

Finally, the molecular data corresponded to the main points of the proposed hypothesis. The more ancient species *F. heterophylla* and *F. amethystina* demonstrated the greatest divergence on molecular level. Their divergence reflected the evolutionary time which had elapsed since the formation of the more advanced polyploids.

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Стаття: надійшла до редакції 25.09.18

доопрацьована 15.11.18

прийнята до друку 16.11.18

МІНЛИВІСТЬ ІЗОЕНЗИМІВ ТА ГЕНЕТИЧНА СПОРІДНЕНІСТЬ П'ЯТЬОХ ВИДІВ FESTUCA СЕРІЇ AULAXYPER DUMORT.

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Незважаючи на значну кількість таксономічних і біосистематичних досліджень роду *Festuca* L. в Європі, є вкрай мало статей, у яких би обговорювали філогенію та систематику роду *Festuca*, а також еволюцію її різних груп. Найбільш важливими серед них є дослідження Цвєльова, який запропонував поділ на три секції у межах типового підроду Festuca: *Variae* Hack., *Aulaxyper* Dumort. і *Festuca*. Є багато досліджень щодо видів, які належать до секції Festuca, включаючи хемосистематичні, тоді як видами секції *Aulaxyper* практично нехтують. З цієї причини ми обрали *F. rubra* L., *F. nigrescens* Lam., *F. picturata* Pils., *F. amethystina* L. і *F. heterophylla* Lam., які належать до останньої. Метою дослідження було вивчення мінливості ізоензимів і відповідна оцінка генетичної спорідненості серед вищезгаданих видів роду *Festuca*.

Було розглянуто десять природних популяцій із Болгарії. Ізоформи ферментів глутамато-оксалоацетатної трансамінази, малатдегідрогенази, глутаматдегідрогенази, ізоцитратдегідрогенази та 6-фосфоглуконатдегідрогенази були досліджені методом електрофорезу в поліакриламідному гелі. Виходячи зі середніх алельних частот / локусів на таксон, були розраховані значення генетичної ідентичності (I) для парних порівнянь усіх досліджуваних видів.

Група *F. rubra* s.l. є примітивнішою порівняно з *F. ovina* s.l. – їхні піхви замкнені практично по всій довжині, тоді як краї піхов видів секції *Festuca* зрослі тільки при основі. *Festuca amethystina* посідає проміжне місце, оскільки її піхви замкнені на 1/3–1/2 довжини. Ця ознака вказує на особливий статус виду в секції *Aulaxyper* і виділяє його з-поміж інших таксонів групи. Результати наших біохімічних досліджень повністю підтвердили особливе місце виду в секції *Aulaxyper*. *Festuca heterophylla* має водночас як примітивні риси, специфічні для давніх видів роду *Festuca*, так і численні ознаки високої спеціалізації. Отримані молекулярні дані підтверджують своєрідну позицію *F. heterophylla* у роді *Festuca*. На противагу їй, тісно пов'язані з політроїдною *F. rubra* такі види як *F. nigrescens* і *F. picturata* слід розглядати як більш пізні «молоді» види. *Festuca amethystina*, що показала найбільшу розбіжність з іншими, має розглядатись як один із давніх видів.

Ключові слова: Festuca, ізоферменти, мінливість, систематичні відносини