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IDENTIFICATION OF FINE-LEAVED SPECIES OF GENUS *FESTUCA* BY MOLECULAR METHODS

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Abstract

Festuca (L.) is a taxonomically complex genus of family *Poaceae*. The fine-leaved species of fescue are well adapted to grow in sandy and dry habitats, therefore, they can be used for establishment of lawns of minimal maintenance as well as recultivations of damaged soils. Breeding for the new varieties to meet these purposes requires reliable methods for identification of the species. The discrimination of fine-leaved fescue species based on morphological features is rather difficult, therefore reliable molecular marker would greatly facilitate it and eliminate the need to wait till floral organs are fully formed. Seven fine-leaved species of genus *Festuca* collected in Lithuania, namely, *F. ovina*, *F. trachyphylla*, *F. polesica*, *F. psammophila*, *F. sabulosa*, *F. pseudovina* and *F. wolgensis* were investigated at the Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry. The ISSR markers, seed storage proteins and isozymes were tested for their ability to distinguish between the fine-leaved species of the genus *Festuca.* Seed storage protein and ISSR fingerprint profiles could be used to distinguish between fine-leaved species of *Festuca*, except for closely related *F. sabulosa* and *F. polesica* species. Isozyme fingerprints did not contain sufficient number of species specific bands and were not feasible to discriminate between species.

Key words: Fine-leaved species, *Festuca*, *Poaceae*, Breeding, ISSR finger print.

Introduction

Genus *Festuca* (L.) is one of the taxonomically complex genus within family *Poaceae*. Many scientists have tried to revise the taxonomic structure of the genus, however, so far there is no agreement regarding ranking of certain taxa. The same taxa are classified as subspecies or even forms by some botanists and as species by others (Tzvelev, 1976; Markgraf-Dannenberg, 1980; Pawlus, 1985). Very close similarity of morphological features between related species is the main reason of this controversy. Flora of the Baltic States (Krall *et al*., 2004) indicates that there are fourteen species of genus *Festuca* found in the natural plant communities of Lithuania, of which the most difficult to identify according to the morphological features are the seven fine-leaved species: *F. ovina* L., *F. trachyphylla*, (Hack.) Krajina, *F. polesica* Zapał., *F. psammophila* (Hackel ex Čelak.) Fritsch, *F. sabulosa* (Anderson) H. Lindb., *F. rupicola* Heuff. and *F. duvalii* (St-Yves) Stohr. During the research of the species of this group *F. rupicola* and *F. duvalii* referred to in Flora of the Baltic States (2004) were not found in the habitats indicated, however, two new species were identified -*F. pseudovina* Hack. exWiesb. and *F. wolgensis* P. Smirnov (Stukonis & Bednarska, 2007).

These climatic changes can have a negative impact on grasslands. Different grass species exhibit different levels of resilience towards extreme fluctuations in precipitation, namely prolonged summer droughts. Drought resistance is important quality for turf grasses as they lose their ornamental qualities during dry periods (Kanapeckas *et al*., 2008). Most of the fine-leaved species of fescue genus are the dominants of plant communities in sands. As the climate is getting warmer and drier, these species could be widely used for the establishment of lawns of minimal maintenance as well as for the greening of dunes, coastal areas or roadsides and the recultivation of damaged soils. The previous EU Common Catalogue of Varieties of Agricultural Plant Species contained only the varieties of one fine-leaved species –*Festuca ovina* s. L. Currently *F. ovina*, *F. trachyphylla* and *F. tenuifolia* Sibth. are registered as separate species. Thereby, breeding for new varieties poses the necessity to identify the species correctly. The identification based on morpho-anatomical features before the formation of generative shoots and floral organs is very difficult. This obstacle can be avoided with the help of molecular markers (Shinwari *et al*., 1994, 1994a, 1994b).

Different molecular marker systems have been applied to distinguish between *Festuca* species. Fjellheim *et al*. (2001) used RAPD markers in combination with morphological analysis to distinguish among 4 closely related species in the *Festuca brachyphylla* complex: *F. baffinensi* Polunin, *F. trichophylla* (Ducros ex Gaudin) K. Richter, *F. hyberborea* Holmen ex Fred. and *F. edlundiae* S. Aiken). Genetic differentiation and admixture among *Festuca idahoensis* Elmer, *F. roemeri* (Pavlick) E.B. Alexeev, and *F. ovina* have been analysed using AFLP, ITS and chloroplast DNA markers. AFLP markers provided highest resolution, yet this technique is time and labour consuming, therefore it was suggested that ITS sequences might provide a more efficient means of discriminating between taxa (Jones *et al*., 2008). Biochemical analysis and particularly the electrophoretic analysis of seed proteins has been used for the identification of species, subspecies and varieties in *Lathyrus* L. (Sammour *et al*., 2007; Emre *et al*., 2010); *Onobrychis* Mill. (Emre *et al*., 2007), *Trifolium* L. (Paplauskienė & Dabkevičienė, 2012) and *Caralluma* (Mehmood *et al.* 2010).

The objective of this study was to establish the feasibility to distinguish between the fine-leaved species of the genus *Festuca* by use of ISSR markers, seed storage proteins and isozyme systems.

Materials and Methods

The populations of fine-leaved species of genus *Festuca* (42 in total) for the research were collected in different physico-geographical regions of Lithuania (Table 1). A fescue collection was established from the collected seeds in the trial fields of Lithuanian Research Centre for Agriculture and Forestry, of which plant leaves or seeds were used in the research, Akademija, Kėdainiai district. The seeds for the investigations of storage proteins were taken from the populations multiplied in isolation in the trial fields. Twenty five populations were used for seed storage protein analysis, 22 – for ISSR analysis and 16 – for isozyme analysis.

To achieve the protein electrophoretic spectra of fescue species different methodologies were applied: 1) a protein analysis adapted for grasses (Gardiner & Forde, 1988); 2) *Festuca pratensis* Huds. protein analysis in the acidic system suggested by Perchuk (1992); and 3) wheat ω-gliadin compositions (Johansson, 1995). The protein extraction buffer for all methodologies was prepared according to Johansson (1995). The electrophoresis was carried out in 12% PAA (polyacrylamide gel) (Kraic *et al*., 1995). The dyeing of proteins was carried out according to Kraic *et al*. (1995) method. The coefficient of genetic relatedness was calculated using Gao *et al*. (2005) formula.

Genomic DNA was isolated from young leaf tissue according to protocol of Doyle & Doyle (1990). Thirty leaves of each Festuca species were taken. Twenty three simple repetitive sequence primers were tested for ISSR analysis (Pivorienė *et al*., 2008): UBC 852, UBC 827, UBC 810, UBC 848, UBC 822, UBC 825, UBC 824, 104H, UBC 823, UBC 856, UBC 857, UBC 858, UBC 847, UBC 860, 77H, G03, G04, 105H, G08, UBC 811, UBC 855, 78H, 155H. Six of them were selected for genetic similarity evaluations: UBC 857 – $(AC)_8$YG, UBC 823 – $(TC)_8$C, UBC 824 – $(TC)_8$G, UBC 825 – $(AC)_8$T, UBC 827 – $(AC)_8$ G and UBC 822 – $(TC)_8$A. PCR was carried out in 15 µl reaction mixture, containing 1 x PCR buffer (Thermo Scientific, Lithuania) 1.5 mM $MgCl_2$, 0.2 µM of primer, 0.2 mMdNTP, 0.8 U *Taq* polymerase (Thermo Scientific, Lithuania) and 50 ng of DNA. PCR thermal profile: 95°C - 2 min, 40 cycles 95°C - 30 s, 50°C - 60 s, 72°C - 30 s, followed by 6 min extension at 72°C. Amplification products were separated by electrophoresis on 1.5% agarose gel containing ethidium bromide. GeneRuler DNA Ladder Mix (Thermo Scientific, Lithuania) was used as size standard. Only those bands which amplified in at least two PCR repeats were scored. Both seed storage protein and ISSR profiles were scored for presence (1) or absence (0) of bands. A similarity matrix was constructed NTSYS-pc version 2.2 of software package (*Exeter Software*, Setauket, NY) (Rolf, 2005). Cluster analysis of the matrix values was performed by employing the unweighted pair-group method with arithmetic mean (UPGMA) (Sneath & Sokal, 1973) provided in the SAHN program of NTSYS-pc and a dendrogram was produced using Tree Plot.

Young plant leaves were used for the isoenzyme extraction. Peroxidase (POD) isoenzymes samples were run on a starch gel and stained following the method of Safonovs (Safonov & Safonova, 1971). The electrophoretic profiles of other enzymes: esterase (EST), glutamate oxalate transferase (GOT), leucinaminopeptidase (LAP) and phosphoglucoisomerase (PGI) were developed by using other methods as well (Safonov & Safonova, 1971; Pasteur *et al*., 1988).

Table 1. Description of the collection sites.

No/species	Place of collecting	No/species	Place of collecting
S-27 *F. sab.*	55°32′27.14″-21°5′59.52″	38 *F. ovi.*	55°41′34.23″-22°29′0.52″
87 *F. sab.*	56°2′44″-21°4′32.36″	49 *F. ovi.*	55°31′50.63″-21°14′11.44″
93 *F. pol.*	54°6′41″-24°30′10.36″.	51 *F. ovi.*	55°30′55.29″-21°14′45.93″
98 *F. pol.*	54°9′43.54″-24°11′17.21″	56 *F. ovi.*	56°18′50.09″-22°39′11.98″
3 *F. trach.*	56°2′4.99″-22°55′44.11″	82 *F. ovi.*	56°2′11.5″-25°14′43.8″
7 *F. trach.*	54°25′49.3″-25°9′24.88″	88 *F. ovi.*	55°51′58.27″-21°3′49.44″
S-14 *F. trach.*	55°47′33.61″-23°51′53.62″	89 *F. ovi.*	55°39′23.79″-22°17′24.7″
S-16 *F. trach.*	56°13′5.95″-24°46′17.48″	91 *F. ovi.*	54°10′47.56″-25°39′1.94″
S-20 *F. trach.*	55°40′3.2″-24°7′23.3″	100 *F. ovi.*	54°10′25.94″-25°4′5.55″
S-23 *F. trach.*	56°22′10.08″-23°16′2.91″	101 *F. ovi.*	55°32′14.89″-21°6′45.83″
25 *F. trach.*	55°55′41.93″-23°21′24.19″	104 *F. ovi.*	55°14′26.28″-24°9′37.86″
S-26 *F. trach.*	56°2′45.71″-21°48′1.3″	106 *F. ovi.*	55°31′36.44″-24°4′55.27″
27 *F. trach.*	55°4′19.62″-22°35′57.14″	107 *F. ovi.*	54°25′0.95″-22°55′8.39″
42 *F. trach.*	54°22′6.78″-24°19′22.9″	147 *F. ovi.*	53°59′3.57″-24°6′20.58″
45 *F. trach.*	54°6′13.71″-23°51′16.06″	10 *F. psa.*	54°9′26.48″-24°11′13.32″
52 *F. trach.*	54°13′8.3″-24°32′43.34″	12 *F. psa.*	54°8′38.62″-24°12′36.03″
92 *F. trach.*	53°59′58.94″-24°17′51.93″	59 *F. psa.*	54°9′1.53″-24°11′11.26″
96 *F. trach.*	54°3′28.23″-24°25′26.21″	60 *F. psa.*	54°9′38.68″-24°10′37.25″
99 *F. trach.*	54°3′3.58″-23°23′58.2″	97 *F. psa.*	54°9′50.22″-24°9′45.16″
290 *F. trach.*	54°50′46.31″-25°28′43.85″	11 *F. wolg.*	54°8′48.16″-24°11′52.61″
26 *F. ovi.*	55°32′36.82″-21°6′39.44″	13 *F. pseud.*	54°8′44″-24°11′55.07″

F. sab. – F. sabulosa, *F. pol. – F. polesica*, *F. trach. – F. trachyphylla*, *F. ovi.– F. ovina*, *F. psa.– F. psammophilla*, *F. wolg. – F. wolgensis*, *F. pseud. – F. pseudovina*

Results and Discussion

The genetic similarity of the fine-leaved species and populations of genus *Festuca* was evaluated by three methods.

Seed storage protein electrophoresis: Three protocols of the seed storage protein extraction and electrophoresis were tested. Protocol adapted for grasses by Gardiner & Forde (1988) and protein electrophoresis in the acid system protocol, suggested by Perchuk (1992) for *Festuca pratensis* Huds. Protein analysis yielded poor results - the obtained fragments in 45-35 KDa area were diffusive and difficult to evaluate. The best results were achieved having applied Johansson's protocol for establishing wheat ω-gliadin composition (Johansson, 1995). Having applied this method for the analysis of 25 populations of seven *Festuca* species, 22 fragments of different electrophoretic mobility were scored in the electrophoretic profile of seed storage proteins. The populations of *F. psammophila* species differed from other species as there were no Rf 0.52 fragment in its protein electrophoretic profiles. *F. ovina* and *F. trachyphylla* populations were rather polymorphic and their protein profiles were very similar, except that Rf 0.76 fragment was characteristic for *F. trachyphylla*, but was not found in the electrophoretic profiles of *F. ovina* populations.

The data in literature on the protein electrophoretic profiles relates only to *F. ovina* and *F. trachyphylla* species (Bulinska-Radomska & Lester, 1986). In their research these authors established three different protein fragments which distinguish *F. ovina* and *F. trachyphylla* species. The electrophoretic analysis of seed storage proteins was successfully used in investigating seven *Lathyrus* L. species. The differences among species were observed and all seven species were clearly identifiable from the protein patterns (Emre *et al*., 2010). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of storage endosperm proteins was used for electrophoretic characterization of genotypes and as indicators of a population status in the genus *Elymus* (Lipin & Agafonov, 2007). The genetic similarity in the protein electrophoretic spectrum of three fine-leaved species of fescue (five and more populations were investigated) is presented in Fig. 1.

The dendrogram shows that the populations of individual species were distinguished quite clearly. The greatest differences were observed among *F. trachyphylla* populations according to the genetic distance of the protein electrophoretic spectrum, the smallest differences – among *F. psammophila* populations. This result was not unexpected as other features, especially morphological and anatomical, show greater polymorphism of *F. trachyphylla* species (Stukonis *et al*., 2007). The greatest genetic distance was typical for population No. 290 which was collected the farthermost from other locations in the collection of these species populations. Five populations of *F. ovina* species (56, 49, 88, 89, 106) had identical profiles and profile 38 of the population differed from them minutely. All these populations are prevalent in the north-western and Central Lithuania. The analogous data was achieved researching *Lathyrus sativus* L. populations, collected in different geographical locations. A high genetic variability among the accessions of different geographical regions and low variability among the accessions of the same region were observed (Sammour *et al*., 2007). However, similar profiles can be found among the populations growing in geographically far-off habitats.

ISSR analysis: The genetic similarity of the fine-leaved species and populations of fescue (*Festuca*) genus was assessed by inter simple sequence repeat (ISSR) analysis. According to this method the fine-leaved species and their populations of fescue (*Festuca*) genus grown in Lithuania were identified (Armonienė *et al*., 2010).

The efficiency of the selected six ISSR primers varied. The largest number of ISSR fragments was synthesized by primer UBC 822 and the smallest number - by primer UBC 827 (13 and 6 fragments respectively). On average 9.5 fragments were achieved with one primer. The fragment size among different primers was similar and varied from 410 to 1150 bp. ISSR markers were characterized by high polymorphism – 98% of fragments were polymorphic. Fifty seven fragments were obtained in total, on the basis of which the dendrogram was drawn (Fig. 2).

The clearest division was observed between *F. sabulosa* and *F. polesica* (two populations of each) group and other fescue species. This study proves that *F. sabulosa* and *F. polesica* are related species. *F. trachyphylla* (7 populations) was also clearly different from other species. Meanwhile, *F. ovina* (six populations) and *F. wolgensis* (one population) fell into one group. Presumably, that was determined by several reasons: too small number of primers could have been selected, DNA markers do not always reflect morphological differences and thirdly, the species researched were quite related genetically. *F. trachyphylla* populations were not distinguished clearly in terms of a habitat and the distance among single populations. Two *F. ovina* populations found in Western part of Lithuania were genetically closer related than others found in other districts of Lithuania. *F. psammophila* populations were very close genetically. The genetic similarity of this species can be explained by the fact that the populations were collected in the same region.

Enzyme system research of fine-leaved species of fescue (*Festuca*) genus: Together with the mentioned two methods for the identification of the fine-leaved species of fescue (*Festuca)* the third one - the enzyme system - is also used (Bennett *et al*., 2002; Malaviya, 2004). This method was applied to research the electrophoretic spectra of seven isozymes. The largest number of fragments (14) was obtained from the investigated ferment systems of fine-leaved fescue within esterase (EST) electrophoretic spectrum (Table 2).

A part (42.8%) of EST fragments was common for all fine-leaved species of fescue genus. Eleven fragments were obtained in EST electrophoretic profiles of seven *F. ovina* populations; of which Rf 0.34, 0.52, 0.81, 0.83 and 0.85 were established in individual *F. ovina* populations. Populations of *F. trachyphylla* had identical EST spectra among them. The fragment 0.73 of *F. trachyphylla* enables to distinguish the latter from *F. ovina, F. wolgensis* and *F. sabulosa* species. Two populations of *F. psammophila* were researched which differed from each other in fragment 0.76. Fragment 0.88 was established in the EST profile of *F. wolgensis* which is characteristic only to this population. The population of *F. sabulosa* species had no specific fragments. Its profile was very similar to *F. wolgensis* only fragment 0.85 was present in its spectre instead of fragment 0.89.

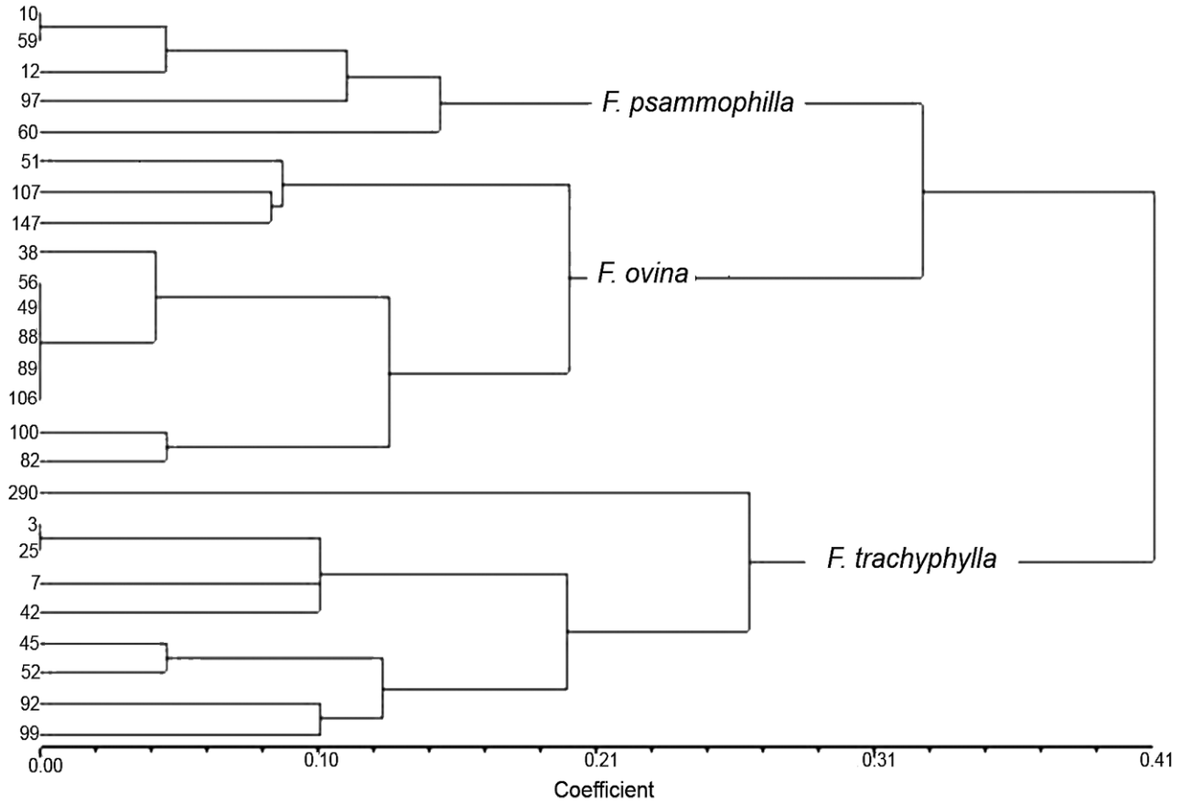

Fig. 1. Genetic similarity in protein electrophoretic spectrum of fine-leaved species and populations of fescue (*Festuca*) genus.

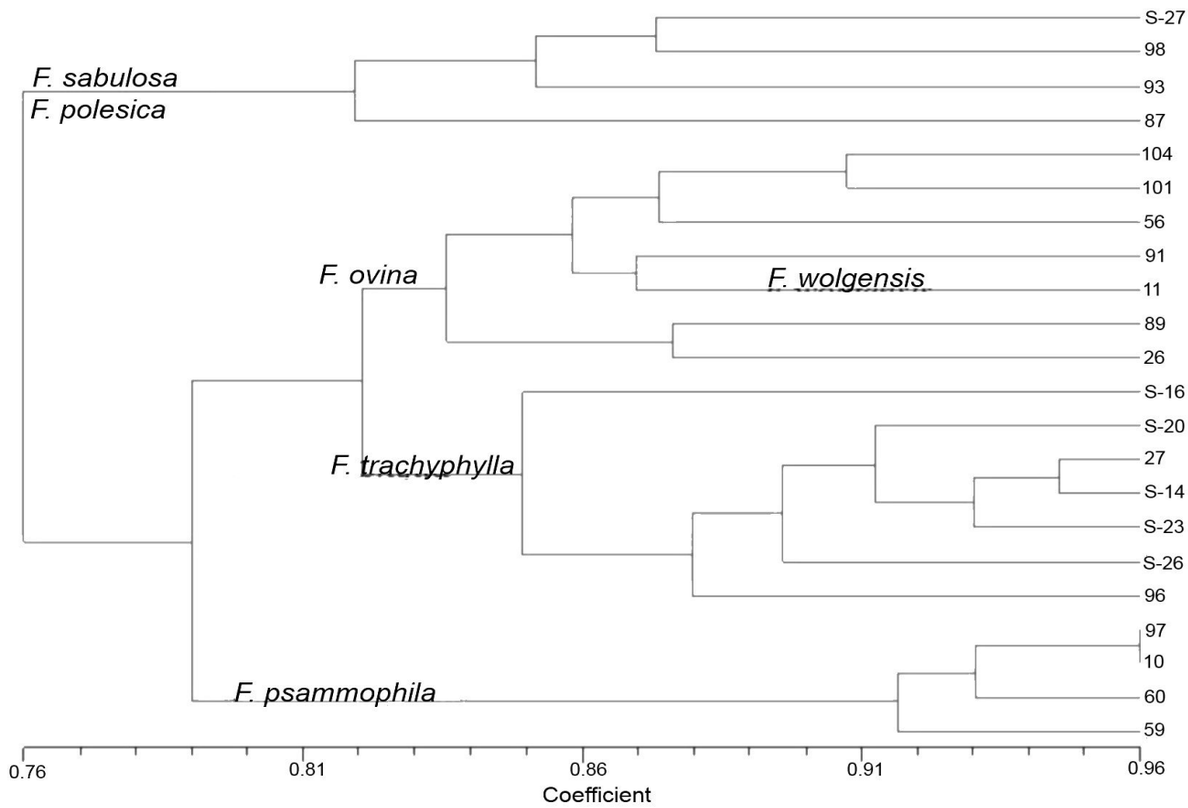

Fig. 2.Genetic similarity of fine-leaved species of fescue (*Festuca*) according to 6 ISSR primers.

Table 2. Rf fragments of esterase (EST) electrophoretic spectrum of genus fescue (*Festuca*) fine-leaved species and frequency of their repetition.

Rf fragments	Number of researched *Festuca* populations					
	ovina **n – 7**	***trachyphylla*** **n – 4**	***psammophila*** **n – 2**	***pseudovina*** **n – 1**	***wolgensis*** **n – 1**	***sabulosa*** **n –1**
0.23	100	100	100	100	100	100
0.30	100	100	100	100	100	100
0.34	43	0	0	0	0	0
0.52	29	0	100	100	0	0
0.60	100	100	100	100	100	100
0.64	100	100	100	100	100	100
0.69	100	100	100	100	100	100
0.73	0	100	100	100	0	0
0.76	0	0	50	0	0	0
0.78	100	100	100	100	100	100
0.81	43	0	0	0	0	0
0.83	43	0	0	0	0	0
0.85	29	0	100	100	0	100
0.88	0	0	0	0	100	0

Another investigated isozyme was PGI. Seven fragments were established in its electrophoretic profile. Unfortunately, due to insufficient clearness of certain fragments their interpretation was complicated to carry out. One fragment was obtained in the electrophoretic profile of GOT of all fescue species. While investigating LAP electrophoretic profiles one common fragment for all species was obtained as well. ACP (acetic phosphatase), PO and SOD (superoxide dismutase) electrophoretic profiles were blurred and difficult to assess.

To conclude, the electrophoretic profiles of seed storage proteins and ISSR markers can be applied for distinguishing between the fine-leaved species of genus *Festuca* and their populations. Both molecular marker systems provided a clear distinction between *F. psammophilla*, *F. ovina* and *F. trachyphylla*. However, to identify very closely related species a more sensitive system with higher distinguishing ability of molecular markers should be used. Isozyme profiles of the fine-leaved *Festuca* species did not contain sufficient number of species-specific bands and therefore were not applicable for species differentiation.

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