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ANALYSIS OF GENETIC SIMILARITY OF *Festuca rubra* L. AND *Festuca nigrescens* LAM. SUB-POPULATIONS OF THE SOUTH-EASTERN PART OF POLAND

Agnieszka Dąbrowska[1], Bogusław Sawicki[2]

[1]Botanical Garden, Maria Curie-Skłodowska University, Sławinkowska 3, 20-810 Lublin, Poland
[2]Department of Tourism and Recreation, University of Life Sciences, Akademicka 15, 20-950 Lublin, Poland
e-mail: dabrowskaa@vp.pl

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Abstract

The investigation of genetic similarity involved *Festuca rubra* L. and *Festuca nigrescens* Lam. ecotypes. The study plants originated from sub-populations in the south-eastern part of Poland. The analysis of the genetic similarity was performed by the PCR technique with the use of semi-specific primers targeting 12–18 bp long plant gene sequences from the exon targeting (ET) and intron targeting (IT) group. The investigations were carried out using 21 primers. In total, 735 DNA fragments were obtained, out of which the individual primers amplified from 16 (ET 10/18mer) to 56 (ET 11/18mer) fragments. On average, one primer yielded 32.38 polymorphic products from 11 (IT 28/12mer) to 56 (ET 11/18mer). In total, 680 (91.62%) polymorphic products were obtained. The primers used generated PCR products which exhibited high polymorphism. In the case of the 18-nucleotide primers, the number of amplified polymorphic fragments was more than 96%.

Based on the results obtained, it was found that Nei's genetic similarity for the *Festuca rubra* and *Festuca nigrescens* sub-populations analysed was high enough to correspond to the sub-species status. The genetic similarity between ecotypes of the species is closely related to the site of occurrence of the genotypes studied. The highest similarity was found between ecotypes growing in the immediate vicinity of each other.

Key words: *Festuca rubra*, *Festuca nigrescens*, genetic similarity, PCR semi-specific primers

INTRODUCTION

The genus *Festuca* contains about 300 species. In Flora Europaea, Markgraf-Dannenberd (1980) divided them into several sections. Undoubtedly, the most numerous is the section *Festuca ovinae* Hackel. (129 out of the 170 described species), which includes most of the fine-leaved species from the northern temperate zone. The group of *F. rubra* sensu Hackel. (with its 21 species) derives from this section, with fourteen species combined in a group called *F. rubra* aggregate (*cretacea*, *cyrnea*, *diffusa*, *juncifolia*, *nevadensis*, *nigrescens*, *oelandica*, *pseudotrichophylla*, *pyrenaica*, *richardsoni*, *rivularis*, *rothmaleri*, *rubra*, *trichophylla*). Species that are part of complexes are characterised by substantial morphological similarity (Sawicki, 1999; Sawicki et al. 2001; Dąbrowska, 2011) and simultaneously they may exhibit a varied level of genetic diversity (Šmarda et al. 2008).

The botanical literature presents a variety of taxonomic concepts of the two *Festuca rubra* L. and *Festuca nigrescens* Lam. taxa. In the study conducted by Markgraf-Dannenberd (1980), they have a status of species with numerous sub-species, i.e. *Festuca rubra* L. subsp.: *arenaria*, *asperifolia*, *juncea*, *litoralis*, *pruinosa*, *rubra*, *thessalica*, and *Festuca nigrescens* Lam. subsp.: *microphylla*, *nigrescens*. According to Hubbard (1973), these species are regarded as lower-rank taxa: *Festuca rubra* L. subsp. *rubra* and *Festuca rubra* L. subsp. *commutata* Gaud.

In Poland and abroad, *Festuca rubra* and *Festuca nigrescens* belong to a group of grasses that have the greatest economic importance. They are mainly turf grasses used, besides the establishment of lawns, for reinforcing slopes, roadsides, motorway verges and areas located in harsh habitat conditions. They are also suitable for restoration of degraded areas (Patrzałek, 2000). The species occur in plant communities from the class *Molinio-Arrhenatheretea*, characterised by semi-natural and anthropogenic turf meadow and pasture associations widespread across the Eurosiberian region (Matuszkiewicz, 2007).

The aim of the study was to assess genetic similarity in two species: *Festuca rubra* and *Festuca nigrescens*, using the PCR method and semi-specific primers.

MATERIALS AND METHODS

Plant material

Genetic analyses were performed on plant material collected from *Festuca rubra* L. and *Festuca nigrescens* Lam. sub-populations growing in the south-eastern part of Poland. Samples were collected from 28 ecotypes (14 *Festuca rubra* ecotypes and 14 *Festuca nigrescens* ecotypes) (Table 1). The ecotypes investigated grew at different distances from each other. The mean distance between the ecotypes of *F. rubra* ranged between 18 km (between ecotypes R-14 and R-15) and 286 km (R-7 and R-17); in the case of *F. nigrescens* ecotypes, the distance was in the range from 7 km (N-3 and N-4) to 251 km (N-9 and N-19).

Table 1
Characteristics of 14 *Festuca rubra* ecotypes and 14 *Festuca nigrescens* ecotypes in the investigated habitats

Origin	Abbreviation of ecotype	Plant community	Geographical factors	
			latitude (°N)	longitude (°E)
		Festuca rubra		
Leszno	R-7	*Alopecuretum pratensis*	49°51’ 20’’	22°56’ 74’’
Wola Uhruska	R-10	*Poa-pratensis-Festuca rubra*	51°19’ 29’’	23°38’ 04’’
Tyszowce	R-11	*Poa-pratensis-Festuca rubra*	50°36’ 89’’	23°43’ 90’’
Krasnystaw	R-12	*Festuco-Cynosuretum*	50°59’ 83’’	23°10’ 28’’
Zastawek	R-13	*Poa-pratensis-Festuca rubra*	52°00’ 25’’	23°33’ 84’’
Serniki	R-14	*Poa-pratensis-Festuca rubra*	51°26’ 85’’	22°42’ 00’’
Sarny	R-15	*Poa-pratensis-Festuca rubra*	51°35’ 25’’	22°00’ 27’’
Wrzosów	R-16	*Poa-pratensis-Festuca rubra*	51°42’ 44’’	22°34’ 43’’
Węgrów	R-17	*Poa-pratensis-Festuca rubra*	52°34’ 50’’	21°30’ 45’’
Augustówka	R-18	*Festuco-Cynosuretum*	51°58’ 94’’	21°30’ 36’’
Hedwiżyn	R-20	*Poa-pratensis-Festuca rubra*	50°35’ 15’’	22°48’ 25’’
Dobcza	R-21	*Deschampsietum caespitosae*	50°12’ 91’’	22°45’ 50’’
Czerniejów	R-22	*Cirsietum rivularis*	51°06’ 96’’	22°36’ 16’’
Chodel	R-23	*Lolio-Cynosuretum*	51°06’ 74’’	22°08’ 65’’
		Festuca nigrescens		
Wysokie	N-1	*Festuco-Cynosuretum*	50°45’ 19’’	23°12’ 50’’
Sawin	N-2	*Deschampsietum caespitosae*	51°16’ 71’’	23°26’ 05’’
Grabowiec	N-3	*Lolio-Cynosuretum*	51°55’ 53’’	22°42’ 40’’
Żuków	N-4	*Festuco-Cynosuretum*	51°36’ 60’’	23°24’ 65’’
Przytoczno	N-5	*Poa-pratensis-Festuca rubra*	51°37’ 03’’	22°16’ 98’’
Suchawa	N-6	*Festuco-Cynosuretum*	51°29’ 91’’	23°24’ 43’’
Leszczanka	N-8	*Deschampsietum caespitosae*	51°56’ 73’’	23°01’ 84’’
Sokółka	N-9	*Cirsietum rivularis*	52°36’ 69’’	21°53’ 55’’
Gródek	N-19	*Alopecuretum pratensis*	50°28’ 80’’	23°39’ 99’’
Godziszów	N-24	*Lolio-Cynosuretum*	50°45’ 65’’	22°28’ 66’’
Annopol	N-25	*Poa-pratensis-Festuca rubra*	50°53’ 00’’	21°51’ 98’’
Bochotnica	N-26	*Cirsietum rivularis*	51°20’ 99’’	22°01’ 69’’
Bełżyce	N-27	*Poa-pratensis-Festuca rubra*	51°10’ 26’’	22°17’ 02’’
Andrzejówka	N-28	*Cirsietum rivularis*	50°35’ 36’’	22°36’ 89’’

DNA extraction and amplification

Young leaves from 14 *F. rubra* ecotypes and 14 *F. nigrescens* ecotypes were collected and frozen before DNA extraction. The DNA was prepared according to the procedure of Davis et al. (1986). 0.5 g of plant tissue was used for extraction. The quantity of DNA was evaluated fluorometrically according to the procedure described in the TKO fluorometer manual (Hoefer Sci.). DNA was diluted to 10 ng/µl.

The polymerase chain reactions were carried out using a Uno II thermocycler (Biometra). The 20 µl reaction mixture contained: 15 ng of genomic DNA template, 1.0–1.2 µM of primer, 200 µM each of dATP, dCTP, dGTP and dTTP, 2 mM $MgCl_2$, 1.0 unit of Taq polymerase (MBI, Fermentas) and an appropriate reaction buffer. Amplification was carried out in two steps: in the first seven cycles, the annealing temperature was 50°C for 15-base primers and 60°C for 18-base primers. This was followed by further 33 cycles in which the annealing temperature was, respectively, 54°C or 64°C for 15- or 18-base primers. In all the cycles, denaturation was carried out for 40 sec. at 95°C, annealing for 1 min., and amplification for 2 min. at 72°C. The amplification products were separated by electrophoresis on 1.5% agarose gels stained with ethidium bromide.

Data analysis

The photographs were analysed using Fragment NT (Molecular Dynamics). The presence or absence of a band was regarded as a single trait, which was given the value of 1 or 0, respectively. The zero-one matrix obtained in this way was used for calculation of genetic similarity coefficients between pairs of all the phenotypes analysed, employing the formula devised by Nei and Li (1979): $SI = 2N_{XY}/(N_X+N_Y)$, where N_{XY} is the number of bands common for both compared genomes X and Y, while N_x and N_y denote the number of bands present in genotype X and genotype Y. The calculations done used version 7.1 of the STATISTICA programme (StatSoft Inc. 2007). The matrix of similarity indices (SI) was used for constructing a dendrogram with the UPGMA method (Unweighted Pair Group Method with Arithmetic Average) using version 7.1 of the STATISTICA programme (StatSoft Inc. 2007).

RESULTS

The presence of semi-specific sequences at the intron-exon junction of plant genes facilitated designing primers with complementary 9- and 7-nucleotide sequences. The sequences supplemented with oligonucleotides with random sequences of 3, 6 and 9 bases in length yield sets of 12, 15 and 18-nucleotide exon targeting (ET) and intron targeting (IT) primers (Table 2). A high proportion of polymorphic bands were obtained; they facilitated the precise determination of the genetic similarity between the study ecotypes.

The primers selected for the analysis amplified 735 DNA fragments in total (Table 2). From 16 (ET 10/18mer) to 56 (ET 11/18mer) DNA fragments were obtained, which is on average 35 per primer. The size of identified products ranged between 260 and 2900 base pairs (Fig. 1 and 2). The individual primers generated between 11 (IT 28/12mer) and 56 (ET 11/18mer) polymorphic fragments. In total, 680 (91.62%) polymorphic products were obtained, with the mean of 32.38 per primer. The best results were obtained using 18 nucleotide ET primers; the number of polymorphic DNA fragments was 90–100% (Table 2).

The results of the genetic similarity analysis obtained with the PCR method were used in constructing the matrix of genetic similarity indices (SI) (Table 3). Genetic similarity among all the *Festuca* genotypes studied was on average 0.658 (0.584–0.802). The range of genetic similarity for the *F. rubra* ecotypes and *F. nigrescens* ecotypes was 0.600–0.764 (0.677) and 0.586–0.802 (0.681), respectively. The similarity between the two species was on average 0.639 (0.584–0.713). Among the ecotypes of *Festuca* species studied, those that grew in the immediate vicinity of each other exhibited the highest genetic similarity, while the lowest similarity was reported between the ecotypes growing in distant sub-populations.

Based on the similarity matrix SI, a dendrogram was constructed with the UPGMA method (Fig. 3). Two cluster groups were distinguished. The first group was composed of two sub-clusters, the first of which comprised the *F. nigrescens* ecotypes; the other one consisted of ecotypes of the two species studied. The second cluster group comprised *F. rubra* ecotypes only.

Table 2
The characteristics of primers and the number of amplified DNA used in the PCR analysis of genetic similarity of *Festuca rubra* and *Festuca nigrescens* ecotypes

Primers	Semi-specific sequence (5' – 3')	Number of fragments			Percentage of polymorphic fragments
		Total	Polymorphic	Monomorphic	
ET 1/18mer	ACTTACCTGAGGCGCGAC	40	39	1	97.50
ET 2/18mer	ACTTACCTGCTGGCCGGA	40	38	2	95.00
ET 4/18mer	ACTTACCTGCCTGCCGAG	39	36	3	92.31
ET 5/18mer	ACTTACCTGGCACGCCTC	47	43	4	91.50
ET 6/18mer	ACTTACCTGCCTACGCGG	35	35	0	100.00
ET 7/18mer	ACTTACCTGAGGCTGCCG	40	37	3	92.50
ET 9/18mer	ACTTACCTGGCCGAGAGG	30	29	1	96.67
ET 10/18mer	ACTTACCTGGTCCGTGGG	16	16	0	100.00
ET 11/18mer	ACTTACCTGCCGCATCCG	56	56	0	100.00
ET 12/18mer	ACTTACCTGGGGCCGATC	31	28	3	90.32
ET 14/15mer	ACTTACCTGCCACGC	35	33	2	94.28
ET 15/15mer	ACTTACCTGAGGCGC	35	32	3	91.43
ET 16/15mer	ACTTACCTGGCCAGC	34	31	3	91.18
ET 18/15mer	ACTTACCTGGCACCG	35	30	5	85.71
ET 31/15mer	ACTTACCTGGGCCAG	38	37	1	97.37
ET 19/12mer	ACTTACCTGGGC	40	37	3	92.50
ET 20/12mer	ACTTACCTGCCG	26	21	5	80.77
ET 21/12mer	ACTTACCTGGGG	34	31	3	91.18
ET 22/12mer	ACTTACCTGCCC	34	31	3	91.18
ET 23/12mer	ACTTACCTGGCG	33	29	4	87.88
IT 28/12mer	GGAACACCTGCA	17	11	6	64.70
Total		735	680	55	-
Mean		35	32.38	2.62	91.62

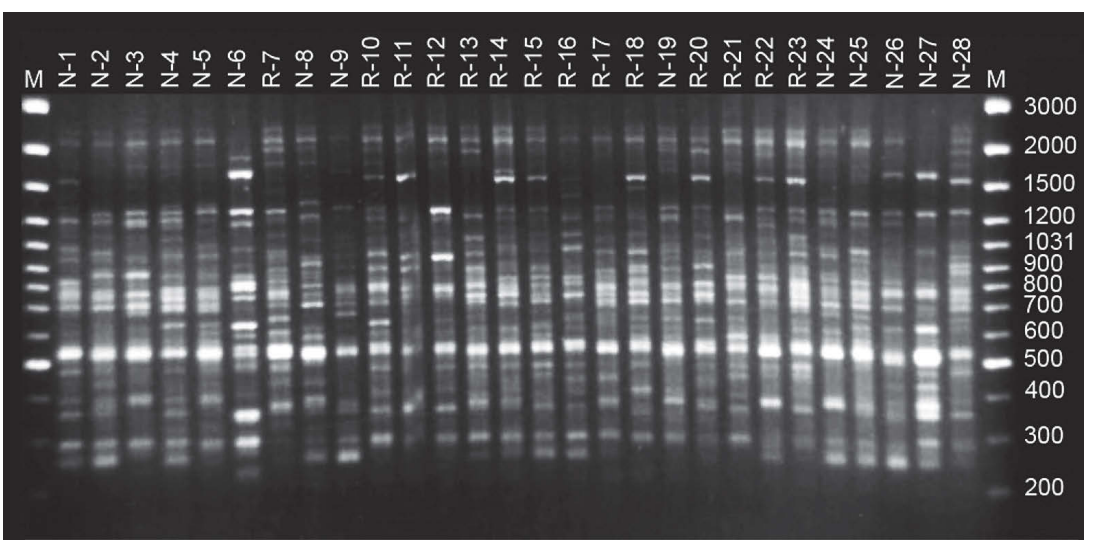

Fig. 1. Amplification of the DNA of 14 *Festuca rubra* ecotypes (lanes 7, 10–18, 20–23) and 14 *Festuca nigrescens* ecotypes (lanes 1-6, 8, 9, 19, 24–28) using the ET 9/18 primer: 5'(ACTTACCTGGCCGAGAGG)3'. M – size standard DNA.

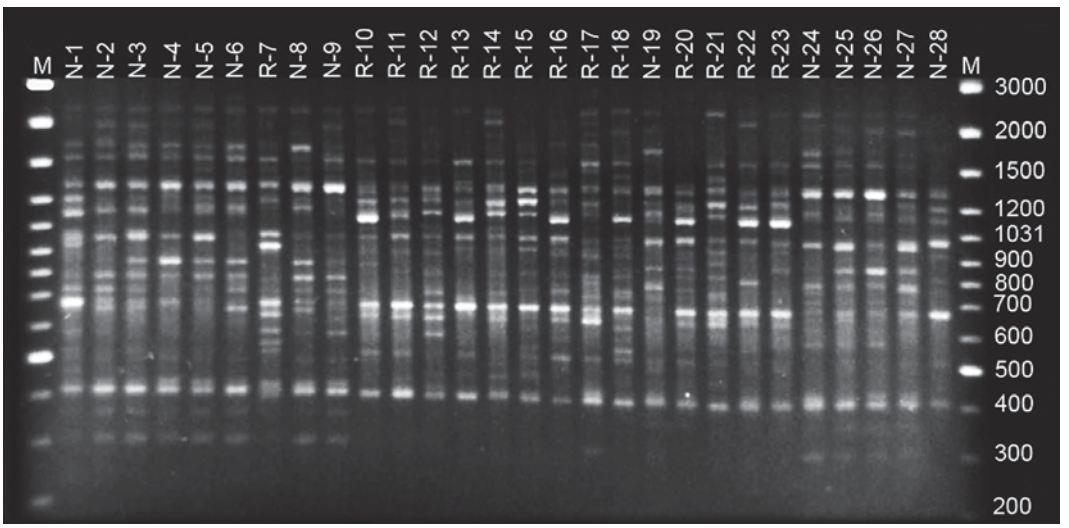

Fig. 2. Amplification of the DNA of *Festuca rubra* ecotypes (lanes 7, 10-18, 20-23) and 14 ecotypes *Festuca nigrescens* (lanes 1–6, 8, 9, 19, 24–28) using the ET 19/12 primer: 5'(ACTTACCTGGGC)3'. M – size standard DNA.

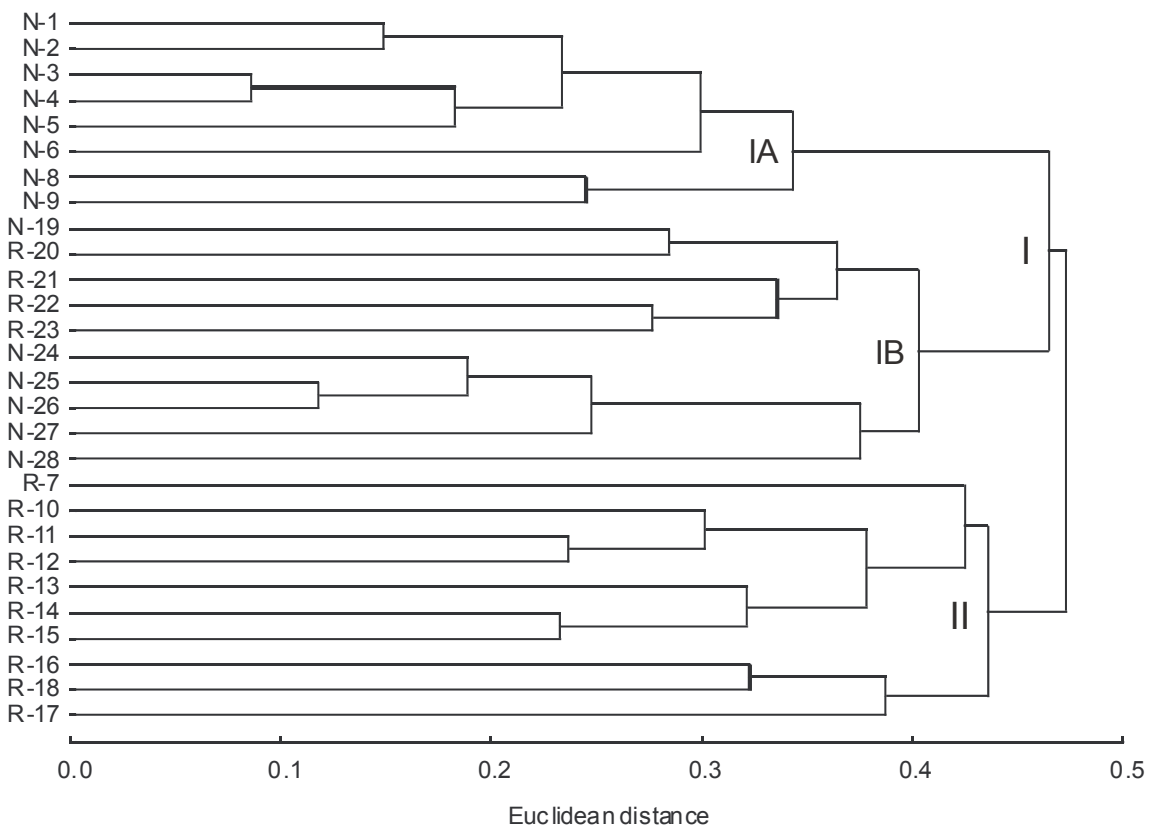

Fig. 3. UPGMA dendrogram of *Festuca rubra* ecotypes (R) and *Festuca nigrescens* ecotypes (N) based on Nei-Li's genetic similarity. For codes of *Festuca* samples, see Table 1.

Table 3
The matrix of Nei's similarity coefficients (SI) between *Festuca rubra* ecotypes and *Festuca nigrescens* ecotypes from the south-eastern part of Poland

Ecotypes	N-1	N-2	N-3	N-4	N-5	N-6	R-7	N-8	N-9	R-10	R-11	R-12	R-13	R-14	R-15	R-16	R-17	R-18	N-19	R-20	R-21	R-22	R-23	N-24	N-25	N-26	N-27	Mean similarity
N-1																												0.669
N-2	0.778																											0.682
N-3	0.727	0.775																										0.671
N-4	0.731	0.787	0.802																									0.677
N-5	0.736	0.752	0.770	0.788																								0.668
N-6	0.697	0.714	0.722	0.719	0.747																							0.655
R-7	0.673	0.685	0.670	0.662	0.671	0.633																						0.642
N-8	0.678	0.721	0.686	0.718	0.696	0.705	0.684																					0.650
N-9	0.705	0.747	0.723	0.735	0.711	0.705	0.645	0.749																				0.661
R-10	0.686	0.708	0.679	0.680	0.695	0.686	0.697	0.682	0.711																			0.671
R-11	0.681	0.678	0.674	0.675	0.648	0.650	0.694	0.655	0.658	0.748																		0.664
R-12	0.669	0.654	0.645	0.652	0.636	0.640	0.677	0.642	0.657	0.720	0.764																	0.661
R-13	0.661	0.653	0.657	0.633	0.642	0.618	0.670	0.607	0.622	0.734	0.710	0.737																0.662
R-14	0.654	0.674	0.662	0.638	0.650	0.623	0.660	0.617	0.632	0.695	0.681	0.686	0.756															0.663
R-15	0.674	0.662	0.641	0.651	0.634	0.639	0.648	0.618	0.653	0.691	0.692	0.683	0.701	0.742														0.667
R-16	0.615	0.619	0.595	0.602	0.614	0.612	0.648	0.606	0.587	0.657	0.672	0.671	0.678	0.694	0.705													0.636
R-17	0.635	0.621	0.632	0.622	0.585	0.624	0.630	0.609	0.602	0.663	0.658	0.671	0.670	0.666	0.668	0.671												0.639
R-18	0.622	0.640	0.633	0.632	0.643	0.617	0.623	0.599	0.626	0.669	0.645	0.641	0.669	0.691	0.710	0.704	0.693											0.652
N-19	0.634	0.651	0.639	0.652	0.638	0.666	0.609	0.636	0.646	0.638	0.637	0.633	0.659	0.653	0.654	0.608	0.628	0.652										0.653
R-20	0.678	0.661	0.637	0.641	0.659	0.641	0.641	0.611	0.638	0.662	0.674	0.659	0.671	0.668	0.710	0.667	0.675	0.717	0.713									0.668
R-21	0.637	0.629	0.645	0.632	0.641	0.646	0.607	0.642	0.629	0.670	0.654	0.642	0.670	0.703	0.694	0.660	0.669	0.699	0.667	0.738								0.662
R-22	0.657	0.648	0.662	0.666	0.649	0.645	0.623	0.653	0.646	0.644	0.673	0.650	0.671	0.670	0.683	0.639	0.648	0.697	0.667	0.717	0.724							0.664
R-23	0.622	0.629	0.625	0.646	0.609	0.602	0.600	0.598	0.615	0.639	0.632	0.619	0.651	0.665	0.670	0.649	0.661	0.692	0.674	0.706	0.694	0.721						0.651
N-24	0.646	0.675	0.660	0.664	0.650	0.631	0.600	0.634	0.658	0.628	0.618	0.631	0.629	0.631	0.652	0.617	0.617	0.627	0.703	0.657	0.656	0.674	0.698					0.657
N-25	0.666	0.697	0.673	0.674	0.664	0.640	0.586	0.625	0.666	0.621	0.612	0.636	0.617	0.622	0.646	0.585	0.611	0.646	0.698	0.676	0.667	0.658	0.694	0.798				0.660
N-26	0.650	0.679	0.652	0.659	0.651	0.638	0.600	0.649	0.659	0.611	0.632	0.645	0.626	0.643	0.653	0.608	0.629	0.622	0.683	0.664	0.674	0.651	0.676	0.750	0.792			0.660
N-27	0.631	0.652	0.631	0.655	0.647	0.640	0.590	0.621	0.643	0.601	0.614	0.624	0.643	0.624	0.625	0.589	0.589	0.612	0.671	0.616	0.650	0.641	0.654	0.716	0.742	0.770		0.644
N-28	0.622	0.623	0.609	0.651	0.617	0.587	0.623	0.606	0.586	0.618	0.614	0.655	0.623	0.606	0.610	0.597	0.610	0.584	0.636	0.638	0.640	0.646	0.646	0.621	0.624	0.654	0.685	0.623

DISCUSSION

The advantages of the PCR method using semi-specific primers targeting plant gene sequences are particularly visible in the case of the genus *Festuca*. This is probably due to the high proportion of repetitive sequences, which represent more than 90% in the *Festuca* genome (Rafalski, 2004). An important feature of the 18-nucleotide primer-base PCR is high repeatability. According to the data presented by Brown (1986) and the suggestions of Weining and Langridge (1991), the PCR using primers with partially complementary sequences in the semi-conservative sequences of the intron-exon junction are a common system. In previous studies, the effect of these primers was tested in many grasses such as *Poa pratensis* (Rafalski, 2004), *Triticum aestivum* (Gaweł et al. 2002), *Secale cereale* (Rafalski et al. 2002). The results of investigations of these plants indicate that the method proposed can be used not only in the analysis of the diversification of crops, but also of the widely understood biodiversity of species, botanical varieties and populations of wild plants.

Given the values of similarity corresponding to different stages of evolution (Futuyma, 1987), it is possible to identify the stage that the analysed *Festuca* taxa have reached. *Festuca rubra* exhibits Nei's genetic similarity to *Festuca nigrescens* at a mean level of 0.639, which classifies the two taxonomic species as sub-species. Taking into account the fact that both species differ solely in caespitose, the presence of rhizomes, the number of ribs in the leaf blade, and the width of the cauline leaf (Dąbrowska, 2011), the classification of both taxa as sub-species seems to be well-founded.

The values of similarity between *F. nigrescens* and the species from the group *F. rubra* agg.: *F. nevadensis*, *F. rothmaleri*, and *F. trichophylla*, are lower and reach, respectively, 0.35, 0.17, 0.39 (Nova et al. 2006). The above-mentioned species differ in many morphological features (Markgraf-Dannenberd, 1980); therefore, they fulfil the criterion for biological species. The mean value of genetic similarity in the *F. rubra* population in eastern Poland was 0.677, which was comparable to the similarity value of 0.740 in a Hungarian population of this species (Majidi and Mirlohi, 2010). The results obtained in this study partially overlap with the results obtained by Nova and co-workers (2006) who found the similarity in Spanish *F. nigrescens* populations to be 0.650, whereas in south-eastern Poland it was 0.681.

According to Nova et al. (2006), genetic similarity among 31 *Festuca* spp. taxa occurring naturally in the Iberian Peninsula exhibited a wide range from 0.39 to 0.83.

CONCLUSIONS

1. The PRC method using semi-specific primers was shown to be useful in assessment of genetic similarity in the genus *Festuca*.
2. The *Festuca* species studied are characterised by a high degree of genetic similarity, which corresponds to the sub-species status.

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Autors' contributions

The following declarations about authors' contributions to the research have been made: genetic research, data analyses, writing of the manuscript: AD; collection of plant material BS.

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Analiza podobieństwa genetycznego sub-populacji *Festuca rubra* L. i *Festuca nigrescens* Lam. w południowo-wschodniej części Polski

Streszczenie

Przeprowadzono badania podobieństwa genetycznego sub-populacji *Festuca rubra* L. i *Festuca nigrescens* Lam. pochodzących z południowo-wschodniej części Polski. Analizę podobieństwa genetycznego przeprowadzono techniką PCR z zastosowaniem starterów semi-specyficznych ukierunkowanych na sekwencje genów roślinnych z grupy ET i IT o długości od 12 do 18 zasad. Badania przeprowadzono z udziałem 21 starterów. Łącznie uzyskano 735 fragmentów DNA, z czego poszczególne startery powielały od 16 (ET 10/18mer) do 56 (ET 11/18mer) odcinków. Średnio na starter przypadało 32.38 polimorficznych produktów od 11 (IT 28/12mer) do 56 (ET 11/18mer). Łącznie otrzymano 680 (91.62%) produktów polimorficznych. Stosowane startery generowały produkty PCR, wykazujące wysoki polimorfizm. W przypadku starterów 18-nukleotydowych liczba powielonych fragmentów polimorficznych wynosiła ponad 96%.

Na podstawie uzyskanych wyników stwierdzono, że podobieństwo genetyczne Nei'a dla badanych sub-populacji *Festuca rubra* i *Festuca nigrescens* jest na tyle wysokie, że odpowiada statusowi podgatunków. Podobieństwo genetyczne pomiędzy ekotypami tych gatunków ma ścisły związek z miejscem występowania badanych genotypów. Największe podobieństwo wystąpiło pomiędzy ekotypami rosnącymi najbliżej siebie.