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THE USEFULNESS OF RAPD AND AFLP MARKERS FOR DETERMINING GENETIC SIMILARITY IN RYE (*SECALE* L.) SPECIES AND SUBSPECIES

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In this work we searched for genetic similarities in twelve wild rye species and subspecies and a control (*S. cereale* ssp. *cereale*, cv. Walet), using RAPD and AFLP markers. AFLP is useful for distinguishing homo- and heterozygotes but is not recommended for evaluation of codominant markers. We assessed the usefulness of the applied methods for examining genetic similarity in rye. RAPD yielded four groups of genetic similarity, with similarity values between 0.32 and 0.81. AFLP markers distinguished two groups of genetic similarity, ranging from 0.49 to 0.79.

Key words: *Secale* L., molecular markers, AFLP, RAPD, genetic similarity.

INTRODUCTION

Molecular markers are used to determine genetic similarity by scientists in different fields. Scientists dealing with plant breeding became interested in genetic similarity more than a decade ago (Broda and Wojciechowska, 2004; Sztuba-Solińska, 2005). The use of molecular markers can accelerate breeding work by rapidly determining the degree of genetic similarity between studied organisms as well as the genetic stability of progeny resulting from interspecific crossing (Chrząstek and Paczos-Grzęda, 2003). Molecular markers can also help in selection of maternal components to produce the desired result with minimal input of funds and labor (Paczos-Grzęda et al., 2005).

Wild species and subspecies of rye are very valuable material for plant breeding. The use of molecular markers offers a way to better characterize and describe plant species. It can determine the range of genetic similarity between species, explicitly indicating the possibility of crossing between genotypes (Kuczyńska et al., 2003).

In this work we evaluate the usefulness of RAPD markers (randomly amplified polymorphic DNA) and AFLP markers (amplification fragment of length polymorphism) as methods for determining similarities between wild species and subspecies of rye.

MATERIAL AND METHODS

The experiment was established in the greenhouse of the Department of Genetics and Plant Breeding of the Poznan University of Life Sciences, using 12 wild species and subspecies of the genus *Secale* L: *S. cereale* ssp. *afghanicum*, *S. cereale* ssp. *ancestrale*, *S. cereale* ssp. *dighoricum* 5685, *S. cereale* ssp. *dighoricum* 17785, *S. cereale* ssp. *segetale*, *S. strictum*, *S. strictum* ssp. *africanum*, *S. strictum* ssp. *anatolicum*, *S. strictum* ssp. *ciliatoglume*, *S. strictum* ssp. *kuprianovii*, *S. vavilovii* and *S. sylvestre*. The control species was cultivated rye *S. cereale* ssp. *cereale*, cv. Walet. Twenty plants of each were used.

RAPD METHOD

Genomic DNA from *Secale* L. species and subspecies was isolated by Thompson and Henry's (1995) method, with modifications. Polymerase chain reactions (PCR) were carried out in 12.5 µl of the following mixture: 9.75 µl deionized water, 1 M Tris HCl (0.125 µl), pH 8.3; 25 mM $MgCl_2$ (1.0 µl); 0.0625 µl BSA; 2 mM dNTP (0.625 µl); starter – 5p mol/µl (0.25 µl); Taq polymerase – 5U/µl (0.1875 µl); and 0.5 µl DNA extract at 25 ng/µl concentration.

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TABLE 1. Characteristics of amplification products generated by the applied RAPD markers

Starter	Sequence	Number of polymorphic fragments of DNA	Size of DNA fragments [bp] min-max
OPA 01	CAGGCCCTTC	1	315
OPA 02	TGCCGAGCTG	4	298-370
OPA 07	GAAACGGGTG	1	760
OPA 09	GGGTAACGCC	7	396-2036
OPA 12	TCGGCGATAG	11	280-1633
OPA 15	TTCCGACCC	9	360-500
OPA 20	GTTGCGATCC	8	360-1018
OPB 10	CTGCTGGGAC	8	680-2036
OPB 11	GTAGACCGT	15	390-2200
OPB 17	AGGGAACGAG	2	396-1150
OPC 18	TGAGTGGGTG	1	515
OPD 16	AGGGCGTAAG	9	298-1400
OPG 19	GTCAGGCAA	4	450-750
OPH 13	GACGCCACAC	5	700-1750
OPI 02	GGAGGAGAGG	4	360-650
OPJ 12	GTCCCGTGGT	3	1000-1100
OPJ 20	AAGCGGCCTC	1	370

Electrophoresis was carried out in 100 ml 1.5% agarose gel with 1 vl ethidium bromide (0.25 µg/ml). Among 40 tested starters, 17 were utilized for further analyses (Tab. 1).

AFLP METHOD

DNA isolation was carried out with the Dneasy™ Plant Mini Kit (QIAGEN®) according to the manufacturer's instructions. The isolated DNA was digested for 3 h at 37°C using restrictive enzymes EcoR I and Msel I (Vos et al., 1995). Ligation employed 12 µl ligation solution adapter, 0.5 µl T-4 DNA ligase and 25 µl DNA obtained after digestion. After ligation and pre-amplification the final dilution of DNA (1.5 µl) was made by adding 73.5 µl TE buffer to the mixture. The mixture for selective amplification included 2.5 µl DNA matrix mix 1 (0.09 µl EcoR I primer, 0.61 µl deionized water, 1 µl Msel I primer, 0.8 µl dNTP) and mix 2 (7.3 µl deionized water, 2 µl 10×PCR buffer, 0.6 µl $MgCl_2$, 0.1 µl Taq polymerase). The following touchdown PCR program was used in all selective amplifications: 1 min in 94°C, 13 touchdown cycles at 94°C, 65°C for 30 s (decreasing temperature by 0.7°C/cycle) and 72°C for 60 s and 25 cycles at 94°C for 30 s and 72°C for 60°C. Electrophoresis of the amplification products was carried out in 5% polyacrylamidic gel. The amplification products were visualized with 0.2% silver nitrate. Twenty starter combinations were tested for the studied rye species and subspecies. Ten of the combinations generated polymorphism and were selected for analysis (Tab. 2).

To estimate genetic similarity (GS) with the RAPD and AFLP markers, similarity coefficients were calculated according to the formula:

$$GS_{ab} = 2\ N_{ab} /(N_a + N_b\ ),$$

where Nab denotes the number of alleles present in both object a and object b, N_a is the number of alleles present in object a, and N_b is the number of alleles present in object b (Nei and Li, 1979). Based on the calculated coefficients the objects were grouped hierarchically by the unweighted pair group method of arithmetic means (UPGMA). The grouping results are presented as a dendrogram.

RESULTS

RAPD METHOD

The 17 starters we used generated 93 polymorphic bands. The mean number of polymorphic DNA fragments obtained per starter was 5.1. Starter OPB 11 generated the highest number of polymorphic amplification products (15 bands). Polymorphic DNA fragment size ranged from 280 to 2200 bp (Tab. 1). Relations between the studied species are presented in a dendrogram constructed on the basis of similarity indexes (Fig. 1). Table 2 gives the coefficients of genetic similarity between all analyzed objects.

The studied species and subspecies formed three similarity groups (Fig. 1). The first included two species, *S. cereale* ssp. *cereale* and *S. sylvestre*,

TABLE 2. Characteristics of amplification products generated by the applied AFLP markers

Starter combination	Sequence	Number of polymorphic fragments of DNA	Size of DNA fragments [bp] min–max
E-ACC M-CTA	5' E-GACTGCGTACCAATTCACC 3' 5' M-GATGAGTCCTGAGTAACTA 3'	39	102–1500
E-AGG M-CTC	5' E-GACTGCGTACCAATTCAGG 3' 5' M-GATGAGTCCTGAGTAACTA 3'	37	105–1000
E-ACA M-CTA	5' E-GACTGCGTACCAATTCACA 3' 5' M-GATGAGTCCTGAGTAACTA 3'	42	106–900
E-ACT M-CAA	5' E-GACTGCGTACCAATTCACT 3' 5' M-GATGAGTCCTGAGTAACAA 3'	42	106–1100
E-ACG M-CTA	5' E-GACTGCGTACCAATTCACG 3' 5' M-GATGAGTCCTGAGTAACTA 3'	48	113–1100
E-AGC M-CTC	5' E-GACTGCGTACCAATTCAGC 3' 5' M-GATGAGTCCTGAGTAACTC 3'	51	100–860
E-AGC M-CAA	5' E-GACTGCGTACCAATTCAGC3' 5' M-GATGAGTCCTGAGTAACAA 3'	44	107–900
E- ACT M-CTA	5' E-GACTGCGTACCAATTCACT 3' 5' M-GATGAGTCCTGAGTAACTA 3'	25	104–301
E- ACC M-CTC	5' E-ACTGCGTACCAATTCACC 3' 5' M-ACTGCGTACCAATTCCTC 3'	24	109–230
E-AGG M-CAA	5' E-ACTGCGTACCAATTCAGG 3' 5' M-ACTGCGTACCAATTCCAA 3'	54	108–800

with a similarity index of 0.68, and one subspecies, *S. sylvestre*. ssp. *ciliatoglume*, with 0.60 similarity to the other two (Tab. 1). The second group included two subspecies of *S. cereale*, showing 0.58 genetic similarity: *S. cereale* ssp. *dighoricum* 17785 and *S. cereale*. ssp. *segetale* (Tab. 3). The third group, the most diversified one, was formed by the remaining species and subspecies. It included three subgroups: first subgroup – *S. strictum* ssp. *anatolicum*, *S. strictum* ssp. *dighoricum* 5687 and *S. vavilovii*; second subgroup – *S. strictum* and *S. strictum*. ssp. *kuprianovii*; third subgroup – *S. cereale*. ssp. *afghanicum, S. cereale*. ssp. *ancestrale* and *S. strictum.* ssp. *africanum*. Within this group, similarity ranged from 0.81 between *S. strictum.* ssp. *dighoricum* 5687 and *S. vavilovii*, to 0.62 between *S. strictum.* ssp. *ancestrale* and *S. strictum*. ssp. *africanum* (Tab. 2). Between them the three groups separated by marker analyses had similarity indexes of ~0.47 (Fig. 1).

AFLP METHOD

The ten starters used in AFLP analysis generated 404 polymorphic bands. The mean number of polymorphic bands generated by one combination was 40.4. The starter combination E – AGG, M – CAA generated the greatest number of polymorphic application products – 54. Polymorphic DNA fragment size ranged between 100 and 1500 bp (Tab. 3). Relations between the studied species are presented in a dendrogram constructed on the basis of similarity indexes (Fig. 2). Table 4 gives the coefficients of genetic similarity between all analyzed objects.

The studied species and subspecies created two main groups of similarity; two of them did not directly join any of the created groups. The first group had two smaller subgroups. In the first subgroup were *S. cereale* ssp. *ancestrale* and *S. strictum* ssp. *africanum*, with 0.72 genetic similarity (Tab. 4). The second subgroup included *S. cereale* ssp. afghanicum, *S. cereale* ssp. *dighoricum* 17785, *S. cereale* ssp. *segetale*, *S. cereale* ssp. *dighoricum* 5687 and *S. vavilovii*. Within this subgroup, genetic similarity ranged from 0.70 between *S. cereale*. ssp. *afghanicum* and *S. cereale* ssp. *segetale* to 0.79 between *S. cereale*. ssp. *dighoricum* 5687 and *S. vavilovii* (Tab. 4). Both of those subgroups were joined with the subspecies *S. strictum* ssp. *ciliatoglume* at 0.68 similarity. The second group included three species and subspecies: *S. strictum*, *S. strictum* ssp. *anatolicum* and *S. strictum* ssp. kuprianovii. In this group, genetic similarity ranged from 0.64 between *S. strictum* ssp. *anatolicum* and

S. strictum ssp. *kuprianovii* to 0.76 between *S. strictum* and *S. strictum* ssp. *kuprianovii* (Tab. 4). *S. sylvestre* was not directly linked to any of these groups, though indirectly it showed 0.62 similarity to all those species and subspecies. Nor was the control species *S. cereale* ssp. *cereale*, cv. Walet found directly in any genetic similarity group. The control species showed ~0.46 similarity to all wild species and subspecies (Fig. 2).

Comparison of the genetic similarity values for the wild species and subspecies indicated that the two methods gave different similarity ranges but grouped the species and subspecies in similar ways. *S. cereale* ssp. *dighoricum* 17785 and *S. cereale* ssp. *segetale*, *S. cereale* ssp. *dighoricum* 5687 and *S. vavilovii, S. strictum* and *S. strictum* ssp. *kuprianovii*, and *S. cereale* ssp. *ancestrale* and *S. strictum* ssp. *africanum* were grouped together by both methods, as were *S. sylvestre*, *S. cereale* ssp. *cereale* and *S. strictum.* ssp. *ciliatoglume*. Subspecies *S. cereale* ssp. *afghanicum* and *S. strictum* ssp. *anatolicum* joined other groups depending on the method applied.

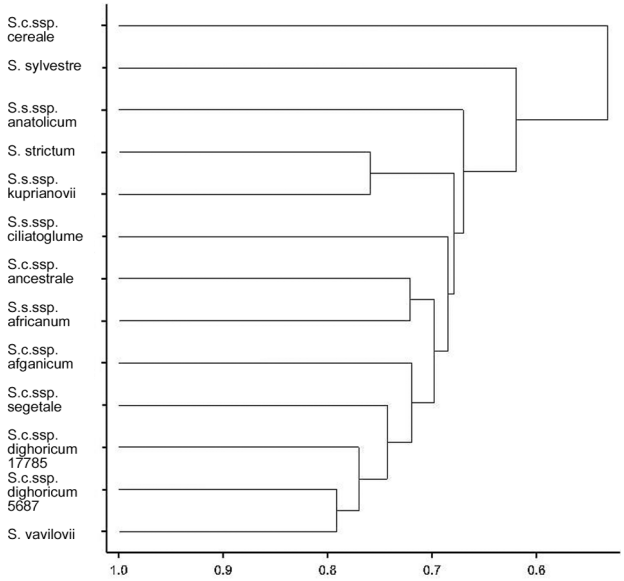

Fig. 1. Genetic similarity dendrogram of wild oat species and subspecies, from RAPD marker analysis.

DISCUSSION

Scientists frequently use more than one method to analyze genetic similarity. The purpose of such a procedure, besides comparison of methods, is to minimize the errors that may burden the methods. For example, Vaillancourt et al. (2008) applied SCAR and ISSR to analyze genetic similarity in rye and wheat. Chrząstek et al. (2006) used AFLP and pedigree methods to study genetic similarity in oat. Hallden et al. (1994) used RFLP and RAPD methods to determine similarities between rape lines. Williams and StClair (1993) also used RFLP and RAPD methods to search for genetic similarities between wild and cultivated forms of tomato.

In selecting suitable methods to study genetic similarity, their effectiveness, replicability and costs need to be considered. Molecular markers useful for genetic similarity analysis are characterized by the number of the polymorphic amplification products the particular starters can generate. Studies indicate that AFLP markers generate more amplification products than RAPD markers do. RAPD markers generate from 2 (Shang et al., 2006) to 43 (Matos et al., 2001) polymorphic amplification products, while AFLP markers can generate from 9 (Bednarek et al., 2003) to 82 (Chwedorzewska et al., 2006), or even 86 (Chikmawati et al., 2005). Our experience with these markers was similar: AFLP markers generated ten times more amplification products than RAPD markers did. The mean number of products generated by RAPD (5) and AFLP (40) was in the range reported by others. The number of polymorphic products would seem to indicate the superiority of AFLP over RAPD. Sztuba-Solińska (2005) argued that a greater number of amplification products is proof of the higher precision of the method. Some investigators, however, suggest that the number of amplification products may indicate a shortcoming of a given method (Koch and Jung 1997), as a multitude of products generated by a given method may cause some important polymorphic products to

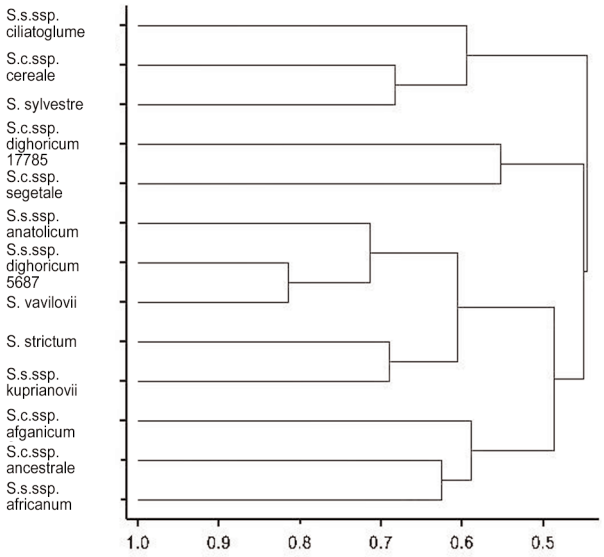

Fig. 2. Genetic similarity dendrogram of wild oat species and subspecies, from AFLP marker analysis.

TABLE 3. Coefficients of genetic similarity between species and subspecies of rye for RAPD method

Species	*S. c. ssp. cereale*	*S. c. ssp. afghanicum*	*S. c. ssp. ancestrale*	*S. c. ssp. dighoricum* 5685	*S. c. ssp. dighoricum* 17785	*S. c.ssp. segetale,*	*S. strictum*	*S. s.ssp. africanum*	*S. s. ssp. anatolicum*	*S. s. ssp. ciliatoglume,*	*S.s. ssp. kuprianovii*	*S. vavilovii,*
S. c. ssp. cereale	1											
S. c. ssp. *afghanicum*	0.32	1										
*S. c.*ssp. *ancestrale*	0.46	0.55	1									
S. c. ssp. *dighoricum* 5685	0.52	0.44	0.47	1								
S. c. ssp. *dighoricum* 17785	0.51	0.37	0.34	0.51	1							
S. c. ssp. *segetale*	0.44	0.46	0.35	0.51	0.56	1						
S. strictum	0.46	0.45	0.35	0.6	0.52	0.41	1					
S. s. ssp. *africanum*	0.43	0.62	0.62	0.51	0.51	0.45	0.54	1				
S. s. ssp. *anatolicum*	0.48	0.52	0.36	0.68	0.58	0.45	0.69	0.58	1			
S.s. ssp. *ciliatoglume*	0.57	0.52	0.61	0.49	0.44	0.46	0.33	0.41	0.39	1		
S.s. ssp. *kuprianovii*	0.49	0.43	0.57	0.54	0.47	0.37	0.69	0.58	0.59	0.38	1	
S. vavilovii	0.49	0.48	0.48	0.81	0.52	0.37	0.64	0.54	0.74	0.4	0.58	1
S. sylvestre	0.68	0.3	0.42	0.5	0.39	0.36	0.39	0.39	0.42	0.61	0.53	0.5

be lost if they are too closely integrated with the monomorphic amplification products closest to them. Matos et al. (2001) showed that the picture of genetic similarity can differ depending on the number of polymorphic bands generated by a given method. They argued that the more polymorphic products are obtained, the smaller is the similarity between the objects. Much work supports that. In oat, Drossou et al. (2004) obtained the same range of genetic similarity by RAPD and AFLP despite the difference in the number of polymorphic amplification products, as did Puecher et al. (2001) in grasses. In the work of Chrząstek et al. (2006), Nowosielski et al. (2002) and Seman et al. (2003), the use of AFLP gave higher similarity coefficients than RAPD did for the same objects, despite the higher number of polymorphic products generated by AFLP.

When we want to select one of the available methods, we must consider the degree of its complication and the labor input necessary for the required analyses. From this point of view, the RAPD method appears to be a cheaper, quicker and less complicated one, and this was stressed in work by Colombo et al. (2000).

In selecting a method for studying genetic similarity we also need to consider the final result in the form of the similarity dendrogram. When the dendrograms obtained by both methods are the same, it is better to choose the quicker and less expensive method. If the dendrograms differ, as in Vaillancourt et al. (2008), Chrząstek et al. (2006), and Nowosielski et al. (2002), then the more exact, more replicable and more detailed method should be chosen. In our work a significant majority of the studied objects were grouped alike, regardless of the

TABLE 4. Coefficients of genetic similarity between species and subspecies of rye for AFLP method

Species	*S. c. ssp. cereale*	*S. c.* ssp. *afghanicum*	*S. c.* ssp. *ancestrale*	*S. c.* ssp. *dighoricum* 5685	*S. c.* ssp. *dighoricum* 17785	*S. c.*ssp. *segetale*,	*S. strictum*	*S. s.*ssp. *africanum*	*S. s.* ssp. *anatolicum*	*S. s.* ssp. *ciliatoglume*,	*S.s.* ssp. *kuprianovii*	*S. vavilovii*,
S. c. ssp. cereale	1											
S. c. ssp. *afghanicum*	0.49	1										
*S. c.*ssp. *ancestrale*	0.58	0.67	1									
S. c. ssp. *dighoricum* 5685	0.52	0.73	0.72	1								
S. c. ssp. *dighoricum* 17785	0.52	0.71	0.70	0.78	1							
S. c. ssp. *segetale*	0.52	0.71	0.70	0.73	0.76	1						
S. strictum	0.53	0.67	0.69	0.72	0.68	0.65	1					
S. s. ssp. *africanum*	0.56	0.67	0.72	0.7	0.68	0.67	0.70	1				
S. s. ssp. *anatolicum*	0.5	0.63	0.65	0.72	0.68	0.66	0.71	0.66	1			
S.s. ssp. *ciliatoglume*	0.52	0.67	0.68	0.71	0.69	0.68	0.67	0.64	0.64	1		
S.s. ssp. *kuprianovii*	0.54	0.63	0.66	0.74	0.67	0.64	0.76	0.67	0.64	0.64	1	
S. vavilovii	0.55	0.72	0.74	0.79	0.75	0.74	0.72	0.72	0.71	0.70	0.73	1
S. sylvestre	0.56	0.58	0.65	0.64	0.64	0.61	0.63	0.60	0.59	0.63	0.59	0.65

method used for similarity analyses. Only two subspecies (*S. cereale* ssp. *afghanicum*, *S. strictum* ssp. *anatolicum*) completely changed their place versus the groups in the similarity dendrogram. RAPD and AFLP yielded similar dendrograms of genetic similarity in Galeta et al.'s (2007) work on the *Asteraceae* family, and by Drossou et al.'s (2004) work on oat genotypes. In those studies the methods gave different ranges of genetic similarity and slightly different forms of the similarity dendrogram, but only two species in the work on *Asteraceae* and one species in the work on oat joined different groups depending on the method.

The choice of method must depend on the objects to be studied. There is no unanimity about which method is better. AFLP seemed better for similarity analysis in Puecher et al.'s (2001) work on grasses and Paran et al.'s (1998) study of pepper cultivars, but Paczos-Grzęda (2004) found RAPD and AFLP equally effective in her genetic similarity analysis of oat cultivars. Our results and the literature indicate that RAPD and AFLP are both useful methods for studying genetic similarity between rye species and subspecies.

CONCLUSIONS

1. Molecular markers of the AFLP method gave more polymorphic amplification products than RAPD molecular markers, so AFLP seems to be the more precise method.
2. RAPD and AFLP gave different genetic similarity ranges. The RAPD markers indicated less similarity (from 0.32 between *S. cereale* ssp. afghanicum and *S. cereale* ssp. *cereale* to 0.81 between *S. cereale* ssp. *diphoricum* and *S. vavilovii*) than AFLP did (from 0.49 between *S. cereale* ssp. *afghanicum* and *S. cereale*. ssp. *cereale* to 0.79 between *S. cereale* ssp. *dighoricum* 5687 and *S. vavilovii*).
3. The wild species and subspecies we used were grouped similarly regardless of the markers used for analysis, suggesting that RAPD and AFLP markers are equally suited for studying the genetic similarity of wild rye species and subspecies.

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