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Assessment of genetic relationships among *Secale* taxa by using ISSR and IRAP markers and the chromosomal distribution of the AAC microsatellite sequence

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Abstract: Inter-simple sequence repeat (ISSR) and inter-retrotransposon amplified polymorphism (IRAP) techniques were used to investigate the genetic diversity among species of the genus *Secale* L. Chromosomal distribution of the AAC simple sequence repeat was examined by means of the fluorescent in situ hybridization technique. Comparative analysis revealed differences in the distribution of analyzed sequences in the genomes of *Secale* species. Using ISSR and IRAP data, a similarity matrix was constructed based on Dice's coefficient, and the unweighted pair-group method with arithmetic mean clustering was used to create dendrograms. *Secale sylvestre* Host and *Secale strictum* C.Presl were separated from a cluster consisting of the rest of the *Secale* taxa. The similarity for *Secale sylvestre* and *Secale strictum* reached over 85%. For *Secale strictum* subsp. *africanum* (Stapf) K.Hammer and *Secale strictum* subsp. *kuprijanovii* (Stapf) K.Hammer, which were clustered together, the similarity reached a value of 75%. The remaining taxa formed a large cluster, in which the highest value of similarity was assigned to *Secale cereale* L. and the *Secale vavilovii* Grossh. 121 inbred line (80%). The distribution patterns of the AAC repeat in chromosomes support these results. The pattern found in *Secale vavilovii* resembles that of *Secale cereale* and its subspecies. The most unique pattern of AAC distribution was observed in *Secale sylvestre*.

Key words: Fluorescent, hybridization, inter-simple sequence repeat, microsatellites, rye, taxonomy, phylogeny

1. Introduction

In most cereals, coding sequences account for less than 20% of the genome (Flavell et al., 1977; Barakat et al., 1997); the remaining part is composed mainly of repetitive sequences, among which microsatellites and retrotransposons are of particular importance. Retrotransposons are ubiquitous and abundant components of grass genomes, constituting a major fraction of repetitive sequences (approximately 10%–60% of the genome). In many species there is a positive correlation between the copy number of retrotransposons and genome size (Pearce et al., 1996; Vicient et al., 2001; Schulman et al., 2004). It is thought that even among individuals within one population there are differences in copy numbers of a given retrotransposon. Retrotransposons are excellent tools for detecting genetic diversity as they are major generators of genomic changes. Sequences generated from retrotransposon-based molecular markers are often more polymorphic than sequences generated from random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), or restriction fragment length polymorphism (RFLP). Detection of inter-retrotransposon amplified polymorphisms (IRAPs) became a retrotransposon-based fingerprinting technique. IRAP markers are generated by amplification of sequences, embedded between 2 retrotransposons, using outward-facing primers annealing to long terminal repeat (LTR) target sequences. This marker system was used for the first time in barley, based on the *BARE-1* retrotransposon (Kalendar et al., 1999; Vicient et al., 1999). It was also used in genetic studies of the diversity or phylogenetic relationships of *Oryza* L. (Branco et al., 2007), *Pisum* L. (Smykal, 2006), *Diospyros* Thunb. (Guo et al., 2006), *Musa* L. (Teo et al., 2002), and *Crocus* L. (Alavi-Kia et al., 2008).

Microsatellite markers exist throughout all known genomes, in both coding and noncoding regions (Tóth et al., 2000), although it has been proven that these sequences are generally rare in exons (Hancock, 1995). They play an important role in various studies, especially studies of genome structure. Microsatellites were developed in the early 1990s (Condit & Hubbell, 1991; Zhang et al., 2005). In plants, simple sequence repeat (SSR) markers have proven to be valuable tools for genotyping and population genetics (Condit & Hubbell, 1991; Akkaya et al., 1992; Zhao and Kochert, 1992; Liu et al., 1996; Milbourne et al., 1997, 1998; Gupta & Varshney, 2000; Doğan et al., 2011).

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They are helpful for genome organization studies and for identification of chromosomes (Schmidt & Heslop-Harrison, 1996; Cuadrado et al., 2000).

Regarding the abundance of repetitive sequences in rye genome (Smith & Flavell, 1977), it is no wonder that SSRs have been often used in studies of *Secale* L. species (Cuadrado & Schwarzacher, 1998; Cuadrado & Jouve, 2002; Achrem & Rogalska, 2006). It was found that the patterns of some microsatellite sequences' distributions enable the identification of individual rye chromosomes. Another microsatellite sequence-based marker system used in rye is inter-simple sequence repeat (ISSR), also called the *Secale cereale* L. inter-microsatellite (SCIM) (Camacho et al., 2005). ISSR markers are generated from nucleotide sequences located between 2 microsatellite priming sites inversely oriented on opposite sides. Primers used in ISSR analysis can be based on any of the SSR motifs (di-, tri-, tetra-, or pentanucleotides) anchored at the 3' or 5' end by 2–4 arbitrary nucleotides (Zietkiewicz et al., 1994).

Extensive diversity of distribution and copy number variation are the features that characterize both microsatellites and retrotransposons. These types of sequences are prone to rearrangements that make them useful in studies of genetic divergence and phylogenetic of rye. The goal of this study was to assess genetic similarity among selected *Secale* taxa. Interspecific genetic variation was analyzed by means of ISSR and IRAP techniques and chromosomal distribution of the AAC repeat.

2. Materials and methods

2.1. Plant material

The following taxa of *Secale* were included in the study: *Secale cereale* L. 'Dankowskie Zielonkawe', *Secale cereale* subsp. *segetale* Zhuk., *Secale cereale* subsp. *afghanicum* Vav., *Secale sylvestre* Host., *Secale strictum* (C.Presl) C.Presl, *Secale strictum* subsp. *kuprijanovii* (Stapf) Hammer, and *Secale strictum* subsp. *africanum* (Stapf) Hammer (obtained from the Botanical Garden of the Polish Academy of Science, Warsaw). From *Secale vavilovii* Grossh., 8 accessions were considered. Three inbred lines of *Secale vavilovii* Grossh. (225 żcz, 121, and 116), obtained by self-pollination, showed anthers and caryopses with color modifications. In addition, a pronounced heterochromatin band was present on chromosome 2R (Rogalska et al., 1993; Achrem et al., 2010).

2.2. ISSR and IRAP procedure

Genomic DNA was isolated from coleoptiles (1 g per sample) of the etiolated rye seedlings by modified CTAB protocol (Murray & Thompson, 1980). The quantity and quality of DNA samples were determined spectrophotometrically (SmartSpec Plus, Bio-Rad). Thirty ISSR and 20 IRAP primers were tested, out of which 13 ISSR and 4 IRAP primers were selected for further study (Table 1). IRAP primers were designed with the FastPCR program (Kalendar et al., 2009). Four IRAP primers used in this study were designed based on the

Table 1. List of ISSR and IRAP primer sequences and their annealing temperatures (Ta).

No.	Primer	5′→3′ sequence	Ta
1	$(AAC)_6G$	AACAACAACAACAACAACG	43 °C
2	$(AC)_{10}TA$	ACACACACACACACACACACTA	50 °C
3	$AC(AC)_{10}G$	ACACACACACACACACACACACG	52 °C
4	$(ACAG)_5C$	ACAGACAGACAGACAGACAGC	50 °C
5	$(CAG)_6A$	CAGCAGCAGCAGCAGCAGA	52 °C
6	$(CTC)_6G$	CTCCTCCTCCTCCTCCTCG	55 °C
7	$(CTGT)_5G$	CTGTCTGTCTGTCTGTCTGTG	52 °C
8	$(GGAA)_5T$	GGAAGGAAGGAAGGAAGGAAT	52 °C
9	$(GTA)_6C$	GTAGTAGTAGTAGTAGTAC	40 °C
10	$(GTC)_6A$	GTCGTCGTCGTCGTCGTCA	50 °C
11	$(GTT)_6A$	GTTGTTGTTGTTGTTGTTA	42 °C
12	$(TCT)_6G$	TCTTCTTCTTCTTCTTCTG	42 °C
13	$(TGA)_6A$	TGATGATGATGATGATGAA	40 °C
14	*Angela*	GAGGCTCACTAGGGACACAGT	53 °C
15	*Sukkula*	GTCACGCCCAAGATGCGACC	55 °C
16	*Bilby*	GTGCTTGGCGGTTAGCCTCGGCAT	60 °C
17	*Cassandra*	TGCGCACTTTGTCCTCACTCA	52 °C

following retrotransposons: *Bilby*, *Cassandra*, *Sukkula*, and *Angela*. All primers were synthesized at the Institute of Biochemistry and Biophysics (Polish Academy of Sciences, Warsaw). Polymerase chain reactions (PCRs) were performed in 25 µL of reaction mixture containing 150–400 ng of DNA, 1X buffer (Novazym), 4.4–7.5 mM $MgCl_2$, 0.2–0.4 mM dNTP (Fermentas), 1–2 µM primer, and 1.25–5 U polymerase (RED AllegroTaq, Novazym); concentrations were optimized individually for each primer. The PCR temperature profile was as follows: 95 °C for 5 min; 35 cycles with 94 °C for 40 s, 37 °C to 60 °C for 1 min, and 72 °C for 2 min; and ending at 72 °C for 5 min. Annealing temperature (Table 1) was based on the primer's melting point. PCR was performed using the Peltier Thermal Cycler (Bio-Rad). Amplification products were separated in 2% agarose gel in 1X TBE buffer. Ethidium bromide was added to the gel to a final concentration of 0.1 µg/mL. Electrophoresis was carried out with an electrophoresis system (Sub-Cell Model 192, PowerPac HV, Bio-Rad) in 1X TBE buffer at 80 V for approximately 5–7 h. The gel images were captured with the Gel Doc XR system (Bio-Rad). Bands were scored and analyzed with Quantity One software (Bio-Rad). The size of the products was determined by comparison with a DNA ladder (GeneRuler, Fermentas).

2.3. Data analysis

The bands were transformed into a binary character matrix with "1" for presence and "0" for absence of a band at a particular position. Pairwise similarity matrices were computed based on the Dice coefficient with FreeTree software (Pavlícek et al., 1999). Dendrograms were created with the unweighted pair-group method with arithmetic mean (UPGMA) for cluster analysis. The dendrograms were visualized with the TreeView program (Page, 1996). Polymorphic information content (PIC) coffecients were calculated according to Ghishlain et al. (1999) for dominant marker systems, i.e. $PIC = 1–p^2–q^2$, where p is the band frequency and q is no band frequency. The diversity index (DI) was calculated, which is equivalent to the average PIC value. Marker index (MI) was calculated as EMR × DI, where EMR (effective multiplex ratio) is the number of polymorphic markers generated per assay.

2.4. Fluorescent in situ hybridization (FISH)

Mitotic chromosome preparations were obtained from root tips of germinating seeds. Roots were treated with 0.5% colchicine (Sigma) for 3 h, then fixed in ethanol and acetic acid (3:1, 4 °C) for 1 day. Roots were macerated with 2% pectinase (from *Aspergillus niger*, Sigma) for 2 h at 32 °C. Root tips were excised and squashed in a drop of 45% acetic acid on a glass slide. The cover slips were removed after freezing over dry ice, and the slides were air dried overnight. Preparations were incubated in DNase-free RNase (10 µg/mL) (Sigma) in 2X SSC buffer (0.03 M Na citrate, 0.3 M NaCl) for 1 h at 37 °C. The slides were then washed twice in 2X SSC buffer for 5 min at 37 °C, incubated in 0.006% pepsin (Sigma) for 15 min at 37 °C, washed twice in 2X SSC buffer for 5 min at 37 °C, and dehydrated in a graded ethanol series (70%, 90%, and 99.8%) at room temperature. Molecular probe $(AAC)_5$ was synthesized and labeled with biotin. The probe was mixed with a hybridization mixture [50% formamide (Sigma), 10% dextran sulfate (Sigma), 0.1% SDS, 2X SSC buffer, 0.3 µg/µL salmon sperm DNA (Roche)] to a final concentration of 1 ng/µL. The probe and target chromosomal DNAs were denatured simultaneously on a slide at 83 °C for 5 min, and hybridization was carried out overnight at 37 °C. Posthybridization washes were performed 3 times each for 5 min in 30% formamide and 2X SSC buffer at 43 °C and 2X SSC buffer at 43 °C. Slides were incubated in DB buffer (4X SSC, 0.2% Tween 20) at 37 °C for 5 min. Slides were incubated in 5% BSA (Sigma) at room temperature for 30 min. Signal detection was carried out using avidin-FITC (5 µg/mL) (Sigma). Signal amplification was performed by alternate incubation in antiavidin antibody (5 µg/mL) (Vector) and avidin-FITC, after each incubation slide was washed 3 times in DB buffer for 5 min at 37 °C. Chromosomes were counterstained with DAPI (1 µg/mL) (Sigma) for 15 min. Slides were mounted in antifade solution (20 mM Tris-HCl, pH 8.0, Sigma; 90% glycerol, Sigma; 2.3% DABCO, Sigma) and analyzed with the epifluorescence microscope ECLIPSE E600 (Nikon). Images of about 20 well-spread metaphase plates per taxon were captured using the Genus software (Applied Imaging Int.).

3. Results

3.1. ISSR and IRAP results

A total of 253 PCR products were generated by 13 ISSR primers, of which 225 (88.9%) were polymorphic. Each primer gave from 7 to 36 bands with an average of 19.5 bands per primer within a size range from 179 to 1655 bp (Table 2). PIC values for individual primers were in the range of 0.216–0.380 with an average DI of 0.294 (Table 2). The MI for ISSR was 5.097. Genetic similarity for 8 taxa ranged from 0.37 to 0.82 (Table 3). The highest genetic similarity occurred between *Secale cereale* and *Secale vavilovii* (225 żcz), while the lowest was between *Secale vavilovii* (225 żcz) and *Secale strictum*. The results indicate that *Secale strictum* and *Secale sylvestre* were the most divergent from other species (genetic similarity from 0.37 to 0.51). The genetic relationships in *Secale* as estimated using UPGMA cluster analysis of the genetic similarity matrix are shown in Figure 1. Three clusters could be distinguished. *Secale strictum* and *Secale sylvestre* are clustered into first group, *Secale strictum* subsp. *kuprijanovii* and *Secale strictum* subsp. *africanum* into second group, and the third group is

Table 2. Characteristics of ISSR marker polymorphisms.

No.	Primer	No. of fragments		% Polymorphic fragments	PIC	Product size range (bp)
		Total	Polymorphic			
1	$(AAC)_6G$	23	21	91.3	0.271	426–1532
2	$AC(AC)_{10}G$	19	17	89.5	0.323	291–981
3	$(ACAG)_5C$	17	17	100.0	0.357	354–1141
4	$(CTC)_6G$	20	18	90.0	0.316	219–1092
5	$(GTA)_6C$	7	7	100.0	0.380	308–582
6	$(GTC)_6A$	14	13	92.8	0.308	268–969
7	$(TCT)_6G$	19	16	84.2	0.251	384–1596
8	$(CAG)_6A$	36	33	91.7	0.274	188–1253
9	$(GGAA)_5T$	27	24	88.9	0.305	179–1600
10	$(CTGT)_5G$	20	17	85.0	0.292	287–1655
11	$(AC)_{10}TA$	23	20	87.0	0.315	284–1134
12	$(GTT)_6A$	19	14	73.7	0.216	445–1514
13	$(TGA)_6A$	9	8	88.9	0.293	323–990
Mean		19.5	17.3	89.5	0.294	-
Min–Max		7–36	7–33	73.7–100.0	0.216–0.380	179–1655

Table 3. Dice coefficient similarity matrix based on the ISSR marker polymorphisms.

	S. cereale	*S. vavilovii* 225 żcz	*S. vavilovii* 116	*S. vavilovii* 121	*S. cereal* subsp. *afghanicum*	*S. strictum* subsp. *africanum*	*S. strictum* subsp *kuprijanovii*	*S. cereale* subsp. *segetale*	*S. sylvestre*	*S. strictum*
S. cereale	1									
S. vavilovii 225 żcz	0.82	1								
S. vavilovii 116	0.74	0.79	1							
S. vavilovii 121	0.72	0.73	0.71	1						
S. cereale subsp. *afghanicum*	0.73	0.74	0.70	0.71	1					
S. strictum subsp. *africanum*	0.59	0.61	0.65	0.63	0.64	1				
S. strictum subsp. *kuprijanovii*	0.60	0.61	0.62	0.62	0.64	0.72	1			
S. cereal subsp. *segetale*	0.69	0.69	0.67	0.66	0.68	0.63	0.64	1		
S. sylvestre	0.44	0.41	0.44	0.43	0.47	0.51	0.50	0.47	1	
S. strictum	0.39	0.37	0.39	0.40	0.42	0.42	0.44	0.43	0.81	1

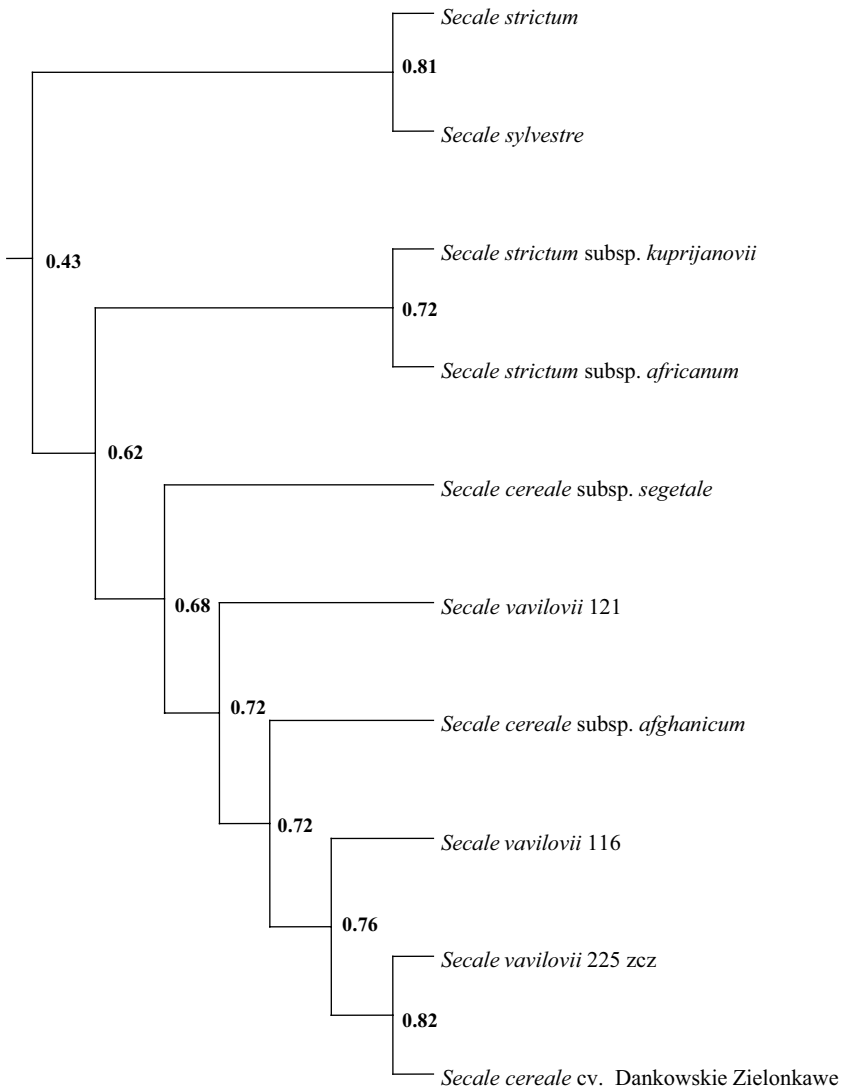

Figure 1. Dendrogram of *Secale* taxa constructed using UPGMA based on Dice similarity coefficients for the ISSR data set.

composed of *Secale vavilovii* inbred lines and *Secale cereale* along with its subspecies.

A total of 73 amplification products were generated by all IRAP primers, of which 86.8% were polymorphic. Products in a range from 109 bp to 3374 bp were included in the analysis. The total number of amplified DNA bands varied between 6 (primer *Cassandra*) and 29 (primer *Bilby*), with an average of 19 bands per primer. The average number of polymorphic bands per IRAP primer was 16.5. The value of the PIC coefficient ranged from 0.248 to 0.267 per individual assay, giving a DI of 0.265. The IRAP MI (4.376) was lower than the ISSR MI (5.097) (Table 4). Based on IRAP data, a similarity matrix and dendrogram were constructed. The dendrogram was constructed from the matrix of Dice similarity coefficients. The highest genetic similarity was observed among inbred lines of *Secale vavilovii* (0.92–0.90), whereas the lowest values of genetic similarity were found between *Secale cereale* subsp. *segetale* and *Secale sylvestre*, as well as between *Secale cereale* subsp. *segetale* and *Secale strictum*, at 0.47 and 0.48, respectively (Table 5). The dendrogram revealed the presence of 3 clusters. The first group consists of *Secale strictum* and *Secale sylvestre*, while the second group is composed of *Secale strictum* subsp. *kuprijanovii*, *Secale strictum* subsp. *africanum*, and *Secale cereale* subsp. *segetale*. Inbred lines of *Secale vavilovii*, *Secale cereale*, and *Secale cereale* subsp. *afghanicum* are clustered into the third group (Figure 2).

Finally, an UPGMA dendrogram was constructed based on cumulative (IRAP and ISSR) data. The dendrogram showed that *Secale* taxa are grouped into 3 clusters, exactly

Table 4. Characteristics of IRAP marker polymorphisms.

No.	Primer	No. of fragments		% Polymorphic fragments	PIC	Product size range (bp)
		Total	Polymorphic			
1	*Angela*	26	19	73.1	0.267	267–1850
2	*Bilby*	29	28	96.6	0.254	109–3374
3	*Cassandra*	6	6	100.0	0.350	392–1193
4	*Sukkula*	15	13	86.7	0.248	141–1179
Mean		19	16.5	89.1	0.265	-
Min–Max		6–29	6–28	73.1–100.0	0.248–0.267	109–3374

Table 5. Dice coefficient similarity matrix based on the IRAP markers polymorphism.

	S. cereale	*S. vavilovii* 225 żcz	*S. vavilovii* 116	*S. vavilovii* 121	*S. cereal* subsp. *afghanicum*	*S. strictum* subsp. *africanum*	*S. strictum* subsp. *kuprijanovii*	*S. cereale* subsp. *segetale*	*S. sylvestre*	*S. strictum*
S. cereale	1									
S. vavilovii 225 żcz	0.81	1								
S. vavilovii 116	0.85	0.91	1							
S. vavilovii 121	0.82	0.92	0.90	1						
S. cereale subsp. *afghanicum*	0.77	0.77	0.78	0.78	1					
S. strictum subsp. *africanum*	0.72	0.72	0.68	0.76	0.76	1				
S. strictum subsp. *kuprijanovii*	0.65	0.67	0.63	0.70	0.74	0.78	1			
S. cereal subsp. *segetale*	0.73	0.79	0.74	0.77	0.72	0.79	0.64	1		
S. sylvestre	0.54	0.51	0.54	0.55	0.51	0.53	0.52	0.47	1	
S. strictum	0.51	0.54	0.54	0.58	0.49	0.53	0.55	0.48	0.86	1

the same as in the case of ISSR (Figure 3). The stability of the clades is supported by high jackknife and bootstrap values (1000 resampling repetitions) (Figure 4).

3.2. FISH results

AAC microsatellite sequence localization revealed differences in the chromosomal distribution of this repeat among *Secale* taxa. Hybridization signals were observed at centromeric regions of 7 chromosome pairs of all the taxa; however, the subtelomeric and interstitial clusters of the AAC simple sequence repeat were variously located (Figure 5). Identical hybridization patterns were observed in *Secale cereale* and *Secale cereale* subsp. *segetale* chromosomes (Figures 5 and 6) and were very similar to the pattern observed in the *Secale vavilovii* (225 żcz) inbred line (Figures 5 and 6). In *Secale cereale* chromosomes, 2 distinct hybridization signals were found on distal regions of 5RL and 6RL, which were absent on *Secale vavilovii* chromosomes. More differences in the AAC sequence distribution were found between *S. cereale* and *Secale cereale* subsp. *afghanicum* (Figure 5). Hybridization signals were

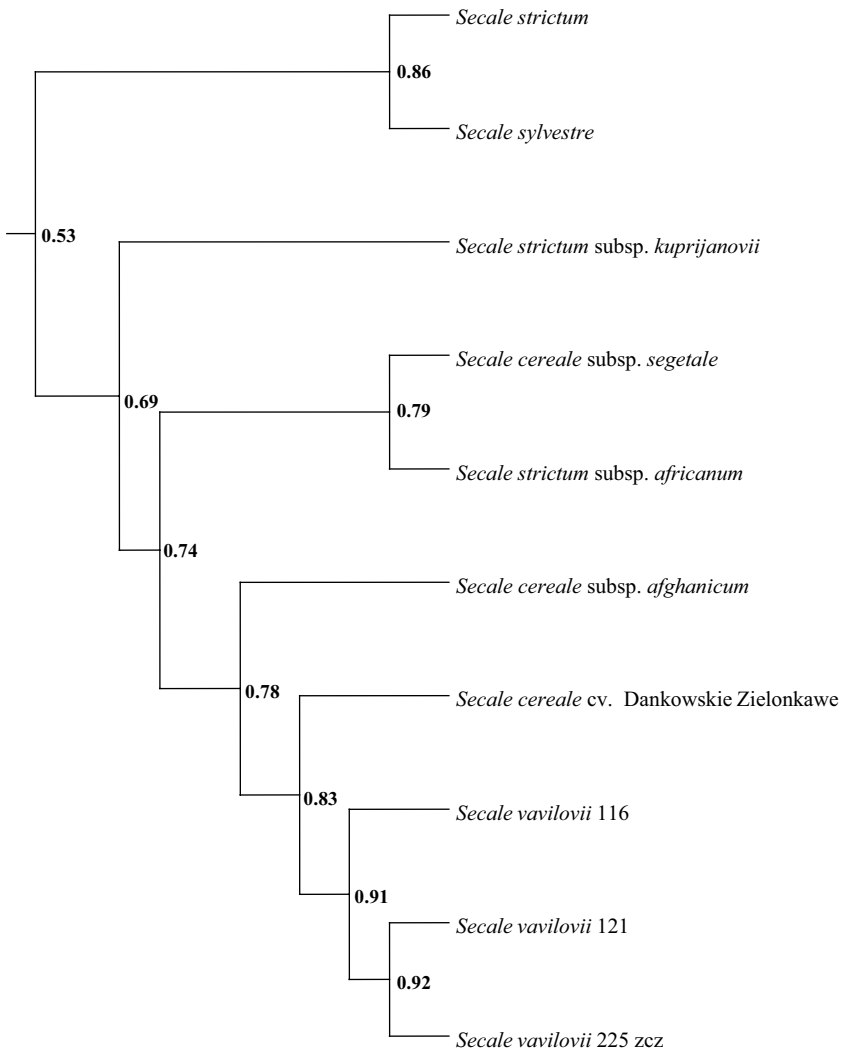

Figure 2. Dendrogram of *Secale* taxa constructed using UPGMA based on Dice similarity coefficients for the IRAP data set.

less abundant on both centromeric and interstitial regions of *Secale cereale* subsp. *afghanicum* chromosomes than on *Secale cereale* chromosomes. Moreover, the chromosome arms 2RS, 4RS, 5RS, 6RS, 6RL, and 7RL did not have the interstitial signals that were present on chromosomes of *Secale cereale* (Figures 5 and 6). *Secale sylvestre* chromosomes showed the most distinct pattern of the AAC sequence distribution (Figure 5) of all *Secale* taxa. The AAC sequence motif occupied numerous chromosomal regions specific exclusively for *Secale sylvestre*. Significant hybridization signals were observed on short and long arms of 1R, 4R, and 5R chromosomes. This pattern of AAC clusters distribution in *Secale sylvestre* differed remarkably from the *Secale cereale* standard pattern. In *Secale strictum* and *Secale strictum* subsp. *kuprijanovii*, AAC clusters were localized to the same chromosomal regions (Figures 5 and 6). These 2 taxa presented an AAC distribution pattern similar to that observed for *Secale cereale*, although there were no subtelomeric signals on 1RL, 3RL, 5RL, and 6RL. On the other hand, clear distinctions between *Secale strictum* and *Secale strictum* subsp. *africanum* were apparent (Figures 5 and 6). The differences were found in pericentric regions. Moreover, in *Secale strictum* subsp. *africanum*, distal signals were observed exclusively on the short arm of 3R and the long arm of 1R chromosomes. A diversified pattern of AAC clusters distribution was found even among the *Secale vavilovii* inbred lines. However, while in inbreds 225 żcz and 121 the patterns were rather similar, they differed more from the pattern observed in inbred line 116. There were distal signals on 4RL and 7RL in line 225 żcz, which were absent from chromosomes in the 121 line (Figure 5). In some specimens of *Secale vavilovii* (116), a pericentric AAC

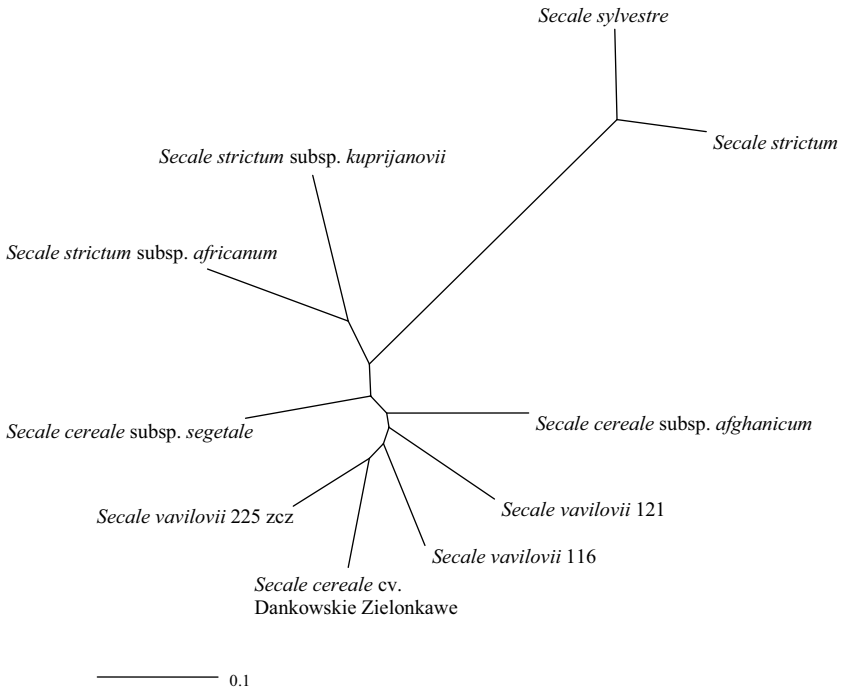

Figure 3. Cumulative dendrogram of *Secale* taxa constructed using UPGMA based on Dice similarity coefficients for the ISSR and IRAP data sets.

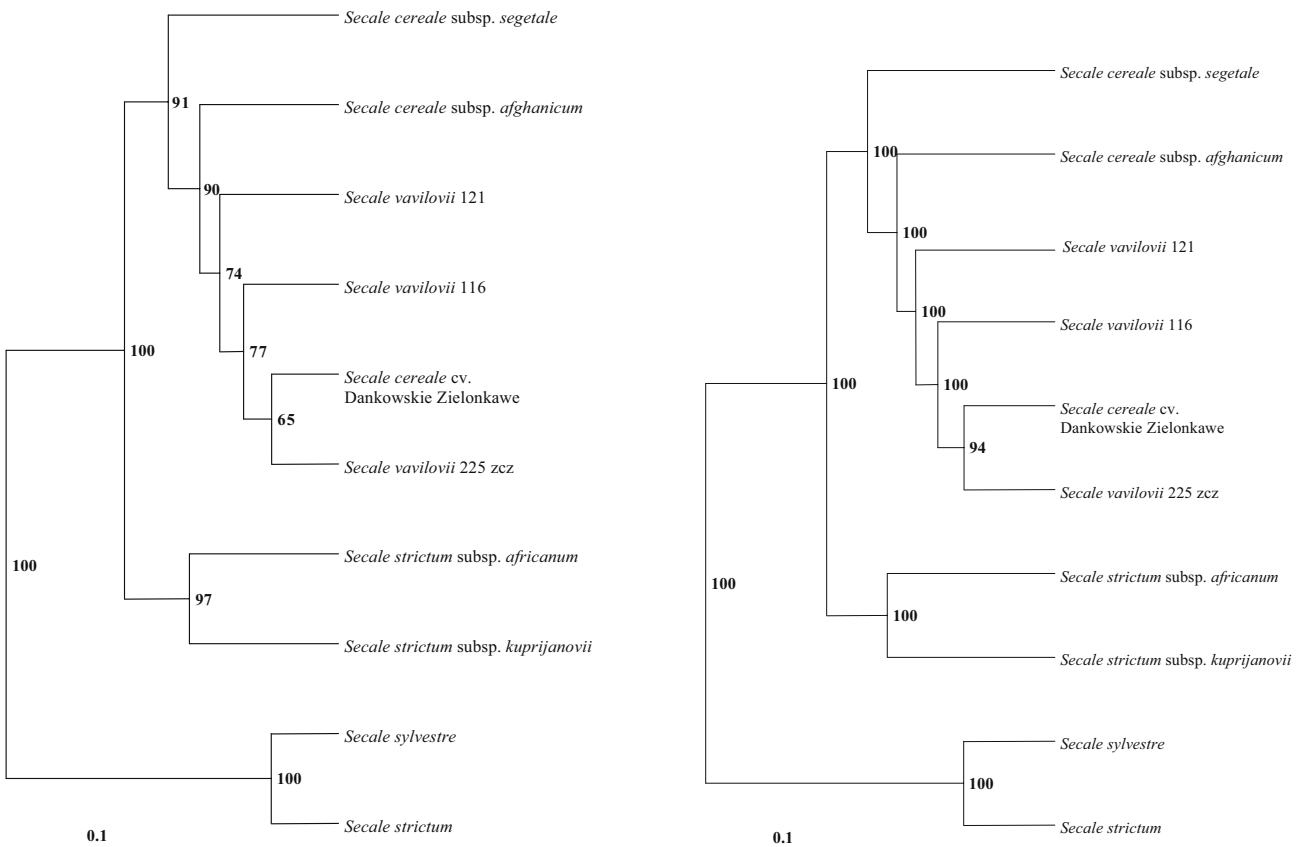

Figure 4. Bootstrap (A) and jackknife (B) resampling analysis of the cumulative dendrogram robustness.

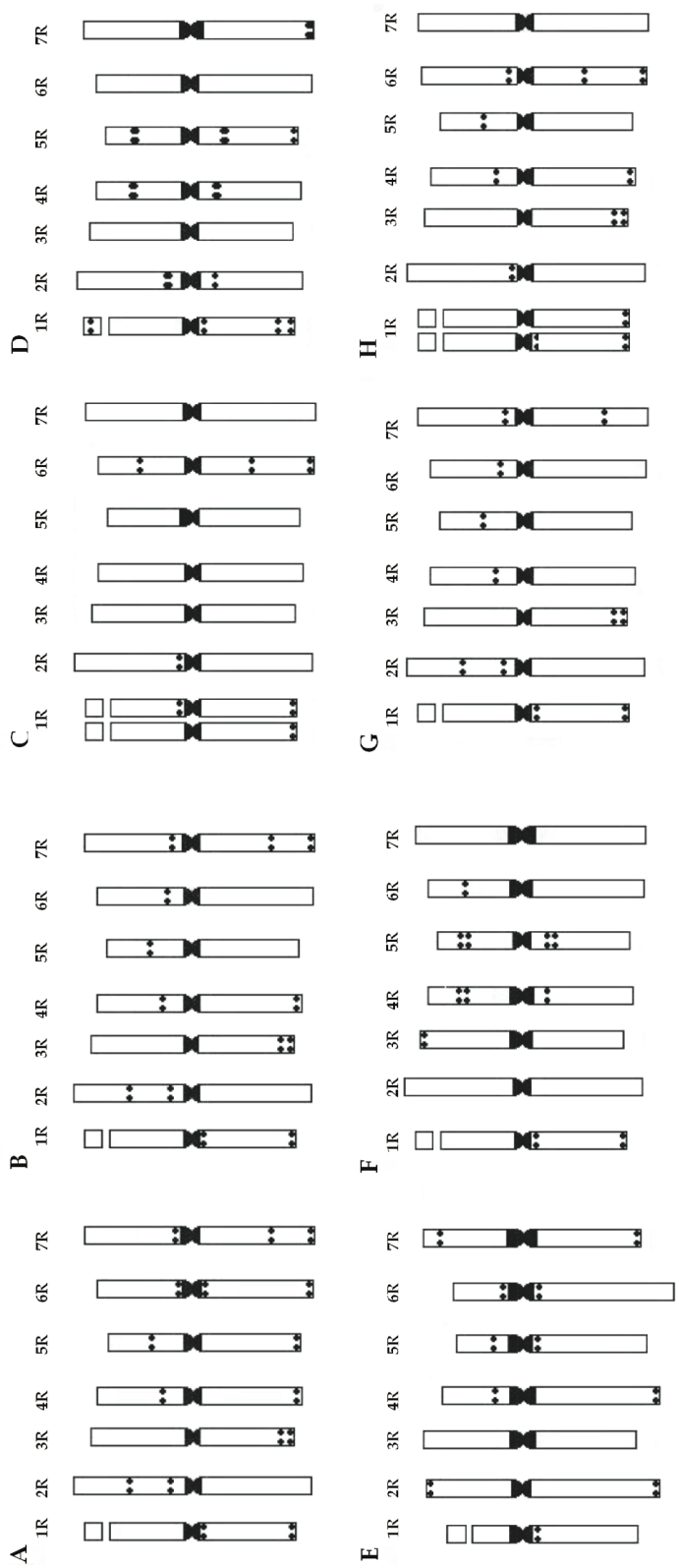

Figure 5. Schematic drawing of AAC distribution on rye chromosomes: *Secale cereale* and *Secale cereale* subsp. *segetale* (A), *Secale vavilovii* inbred line 225 żcz (B), *Secale cereale* subsp. *afghanicum* (C), *Secale sylvestre* (D), *Secale strictum* and *Secale strictum* subsp. *kuprijanovii* (E), *Secale strictum* subsp. *africanum* (F), *Secale vavilovii* inbred line 121 (G), *Secale vavilovii* inbred line 116 (H).

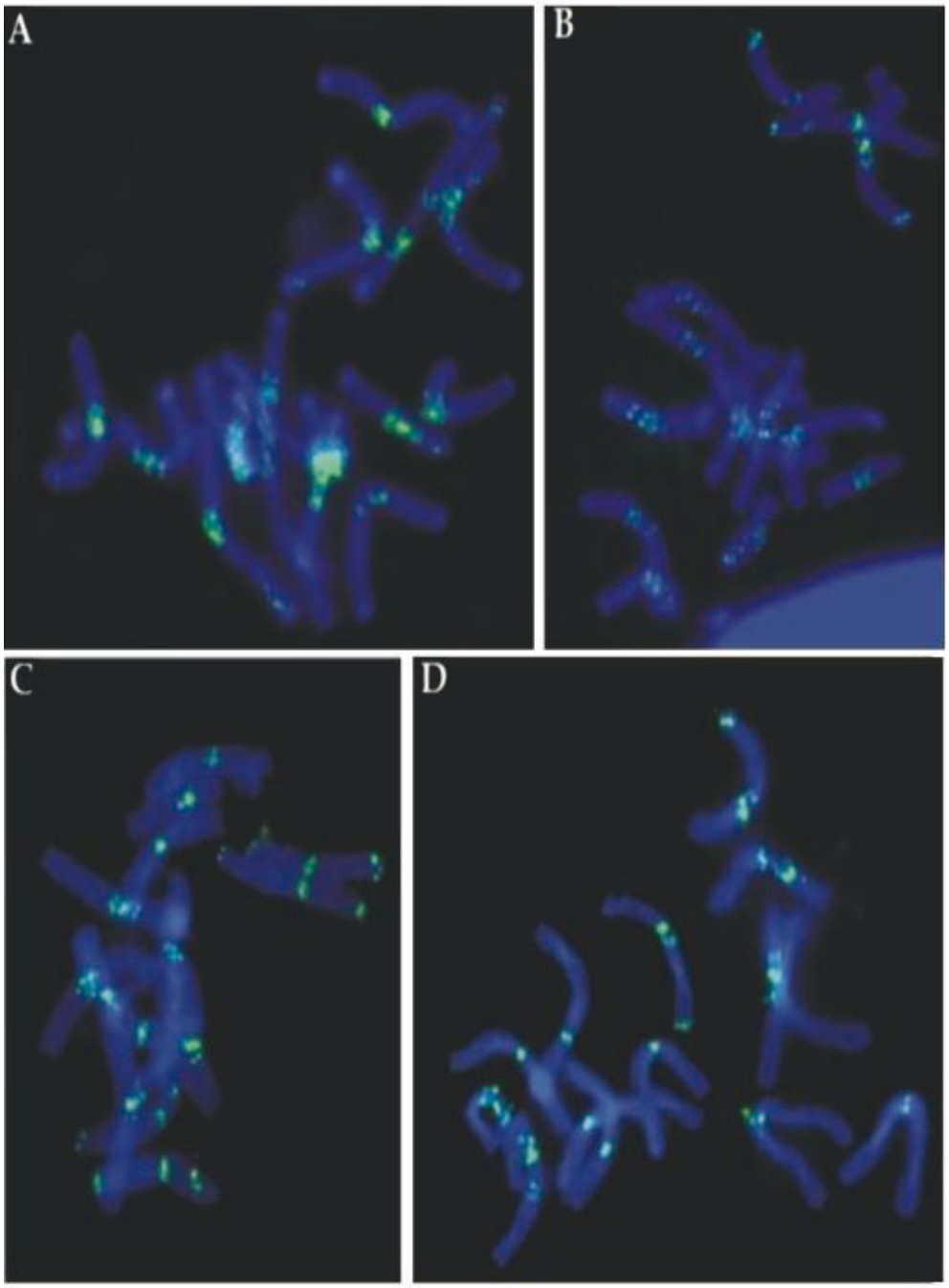

Figure 6. Exemplary images presenting the distribution of AAC sequence clusters in chromosomes of *Secale*: *Secale cereale* and *Secale cereale* subsp. *segetale* (A), *Secale vavilovii* inbred line 225 żcz (B), *Secale strictum* and *Secale strictum* subsp. *kuprijanovii* (C), *Secale strictum* subsp. *africanum* (D).

cluster on the long arm of 1R was found, whereas in others there was a lack of the hybridization signal in this region. In comparison to the other 2 lines, the line 116 showed no intercalary signals on 2RS, 7RS, and 7RL (Figure 5), while the distribution of the AAC motif in the 6R chromosome was the same as in *Secale cereale* (Figure 5).

4. Discussion

The presence and distribution of repetitive sequences can reveal evolutionary changes across groups of plants. The variability of microsatellites and retrotransposons, which are types of repetitive sequences, was used to assess genetic relationships among and within selected species of

the genus *Secale*. A high degree of similarity was revealed between *Secale strictum* and *Secale sylvestre*. Ren et al. (2011) also found these 2 species to be very similar. Stutz (1972) suggested that *Secale sylvestre* evolved from *Secale strictum* and that these 2 species share a common ancestor. The results presented here show that *Secale sylvestre* differs considerably in AAC cluster distribution either from *Secale strictum* and other species. Significant distinctions in patterns of AAC distribution between *Secale sylvestre* and *Secale strictum* suggest an early separation of these 2 species. The differences should be considered in 2 ways: first, the amplification of microsatellites could appear at different chromosomal sites in 2 diverging species, and, second, events of differential deletions in evolving species could follow microsatellite amplifications in a common ancestor (Cuadrado & Jouve, 2002; Achrem & Rogalska, 2006). The distinctness of *Secale sylvestre* was also confirmed by molecular studies of chloroplast DNA (Murai et al., 1989; Petersen & Doebley, 1993), repetitive sequences (Cuadrado and Jouve, 2002; Shang et al., 2006; Zhou et al., 2010), 18S-5.8S-26S rDNA (De Bustos & Jouve, 2002), and AFLP-based analysis of genetic diversity (Chikmawati et al., 2005).

According to Hammer (1990), *Secale strictum* should be considered to be the most ancient species of *Secale*. Subspecies of *Secale strictum*, i.e. *Secale strictum* subsp. *kuprijanovii* and *Secale strictum* subsp. *africanum*, showed high similarity to each other. Most surprising, however, was the low degree of similarity (approximately 42%–53%) between *Secale strictum* subspecies and *Secale strictum* species. These results support the hypothesis that *S. strictum* stopped evolving a long time ago, whereas its subspecies are still evolving. Another piece of evidence that *Secale strictum*, *Secale strictum* subsp. *kuprijanovii*, and *Secale strictum* subsp. *africanum* evolved independently (Khush & Stebbins, 1961) was provided by a comparative sequence analysis of the internal transcribed spacer (ITS) of the rDNA performed by Bustos and Jouve (2002). This conclusion is supported by the notable differences in the pattern of AAC sequence distribution among *Secale strictum* species and subspecies. *Secale strictum* and *Secale strictum* subsp. *kuprijanovii* chromosomes showed the same pattern of AAC cluster distribution, while the pattern observed in *Secale strictum* subsp. *africanum* chromosomes was found to deviate from those of all taxa studied. The distinctive distribution pattern of the AAC repeat in *Secale strictum* subsp. *africanum* chromosomes can be explained by the migration of this subspecies to South Africa during the Early Pleistocene, where it evolved separately from the rest of the genus. This polymorphism could be due to deletion and/or amplification from the preexisting sequences.

On the other hand, *Secale cereale* showed high similarity to its subspecies, i.e. *Secale cereale* subsp. *segetale* and *Secale cereale* subsp. *afghanicum*, providing support for the classification proposed by Hammer (1990), who recognized these 2 taxa as subspecies. Very similar conclusions can be drawn from SSR-based studies (Shang et al., 2006), ISSR-based studies (Ren et al., 2011), and the analysis of ITS rDNA regions (Bustos & Jouve, 2002). The physical localization of AAC clusters in *Secale cereale* was identical to *Secale cereale* subsp. *segetale*, while the differences in *Secale cereale* subsp. *afghanicum* could originate from distinct deletions or rearrangements of sequences that occurred during taxa differentiation.

ISSR and IRAP results individually and collectively revealed high similarity between *Secale cereale* and *Secale vavilovii* inbred lines. Interestingly, the degree of similarity appeared to be higher between *Secale cereale* and *Secale vavilovii* than between *Secale cereale* and its subspecies. The similarity of AAC repeat distribution between *Secale cereale* and *Secale vavilovii* also confirms that these 2 taxa share a recent common ancestor. Generally, the pattern of AAC distribution is rather conservative in the majority of *Secale* taxa; the differences could be explained by moderate polymorphism between and within species. Cuadrado and Jouve (2002) observed more variable patterns of AAG repeat distribution in chromosomes of *Secale* taxa. The results of their study confirmed a close relationship between *Secale cereale* and *Secale vavilovii* and suggested their common origin and early separation from *Secale strictum*. This hypothesis is also supported by the finding that *Secale vavilovii* and *Secale cereale* share the same translocation that distinguishes these taxa from *Secale strictum* (Singh & Röbbelen, 1977), as well as the presence of polymorphic locus 5S rDNA in arm 3RS of *Secale vavilovii* and *Secale cereale*. Such a high similarity inclines scientists towards classifying *Secale vavilovii* as a subspecies of *Secale cereale* (Frederiksen & Petersen, 1998; Ren et al., 2011).

The ISSR, IRAP, and SSR marker systems used in this study have proven to be useful in assessment of genetic diversity of *Secale*, and they may provide clues useful for the reconstruction of phylogenetic relationships. High values of MI indicate the usefulness of IRAP (MI = 4.376) and ISSR (MI = 5.097) markers. We found that *Secale sylvestre* and *Secale strictum* were clustered separately and were obviously divergent from other species. The similarity between these 2 species was over 0.85. The second cluster consisted of *Secale strictum* subsp. *africanum* and *Secale strictum* subsp. *kuprijanovii*, between which the similarity was 0.75. The remaining taxa were included in a large cluster, in which *Secale cereale* and the *Secale vavilovii* inbred line 225 żcz exhibited the greatest genetic similarity (0.80).

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