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Genetic Relationships Among Representatives of *Dasypyrum*, *Secale* and *Triticum* Species Revealed with RAPD and ISSR Markers

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Abstract

In this study the genetic similarity among *Dasypyrum*, *Secale* and *Triticum* species with RAPDs and ISSRs was analyzed. To show a level of similarity between the species, 12 populations of *Dasypyrum* (11 *D. villosum* and 1 *D. breviaristatum*), together with 12 accessions belonging to 3 *Secale* species and 12 accessions from 4 *Triticum* species were used. Genetic distances (GD) and bootstrap values were calculated and PCA analysis was conducted to present the relationships among the species. To estimate the genetic structure among and inside genera, as well as population differentiation, gene diversity (He), total genetic variation (Ht) and Wright's fixation index (Fst) were computed. The highest values were found in *Triticum,* within which Ht was equal to 0.332±0.023 and Fst was 0.42. It confirmed that the material studied was highly differentiated. Both systems found *Dasypyrum* more related with *Triticum*, as compared to *Secale*. With RAPDs, genetic distance (GD) between *Triticum* and *Dasypyrum* was 0.435, respectively 0.460 for *Secale* vs *Dasypyrum*. In the case of ISSRs these values were 0.374 and 0.407, respectively. Despite the fact that the difference between the two GD indices was insignificant, one should not exclude the possibility of successful hybridization of *Dasypyrum* and *Secale*, especially when using bridge species.

Keywords: diversity, molecular markers, similarity, *Triticeae*

Introduction

Dasypyrum L., *Triticum* L and *Secale* L. are three species from Triticeae tribe. The first taxon includes only two wild, allogamous species: *Dasypyrum villosum* L. 'Candargy' (diploid, haplome V) (*Dv*) and *D. breviaristatum* (Lindb. f.) 'Frederiksen' (diploid and autotetraploid, haplome V^b) (*Db*) (Baum *et al*., 2014; De Pace *et al*., 2011; Gradzielewska, 2006a). The other two genera include both wild and cultivated species.

Dasypyrum species, especially *Dasypyrum villosum,* are commonly known as relatives of bread wheat (*Triticum aestivum* L.) and they are widely used as donors due to their favorable traits to wheat (De Pace *et al*., 1990). *Dasypyrum villosum* (2*n*=2x=14, VV) possess many genes used in wheat breeding to improve the resistance; for example, resistance to powdery mildew, leaf and stem rusts, take-all, cereal eyespot, wheat streak mosaic virus (WSMV) and its vector wheat curl mite (De Pace *et al*., 2011; Gradzielewska, 2006b; Hyde, 1953; Liu *et al*., 1988; Murray *et al*., 1994; Yildirim *et al*., 1998; Zhang *et al*., 2005). Moreover, other desirable traits of *D. villosum* include salt and drought tolerance, winter hardiness and high protein content (Chen and Liu, 1982; Della Gatta *et al*., 1984; De Pace *et al,*. 2001; Liu *et al*., 1988). Until now, a lot of hybrids and amphiploids between *D. villosum* and *Triticum* species, substitution and translocation lines, have been developed successfully using resistance genes *Pm21* (powdery mildew), *YrV3* (yellow rust) and *Wss1* (wheat spindle streak mosaic virus) which have been transferred into wheat cultivars (Blanco *et al*., 1987; Chen *et al*., 1995, 2002; De Pace *et al*., 2011; Hou *et al*., 2013; Liu *et al*., 1988; Minelli *et al*., 2005; Zhang *et al*., 2005).

Secale cereale L. (rye) is considered an important crop in the colder parts of Northern and Eastern Europe and Russia. It is a diploid (2*n*= 2*x*= 14) cross-pollinated, annual cereal with an effective gametophytic self-incompatibility system (Geiger and Miedaner, 2009; Haffke *et al*., 2014). Rye is also considered as a hardy crop, which can grow in sandy soils of low fertility and is more tolerant to drought, cold and other adverse growing conditions than other cereal crops. Although there are two rye biotypes (spring and winter), most of the world supply is obtained from winter varieties (Haffke *et al*., 2014). Among the rye cultivation

areas Germany, Poland, Russia and Belarus have a significant contribution to rye production (FAOSTAT, 2013).

For a long time rye was found to be resistant to cereal diseases. Nevertheless, it has been increasingly infected by pathogens in recent times. There are several diseases which infect rye such as leaf and stem rust (*Puccinia recondita, P. graminis* f. sp. *secalis*), ergot (*Claviceps purpurea), Fusarium* diseases, leaf blotch (*Rhynosporium secalis*), pink snow mould (*Microdochium nivale*) or soil-borne viruses (Geiger and Miedaner, 2009; Pociecha *et al*., 2013). Due to this fact there is a need to find new resistance donors for rye, which will be used in breeding programs. A good resistance source would be *Dasypyrum villosum*. Both species, *Dasypyrum villosum* and *Secale cereal*, are diploids with the same number of chromosome in their genome, annual and cross-pollinated cereals. Therefore, it might be possible to transfer homologous genes between these two species, as it happened in the case of wheat, although interspecific hybridization reveals very little homology between wheat and *D. villosum* genomes (Hyde, 1953).

Recently, introduction of alien genetic variability to rye seems to be necessary, because of diseases attack augmentation, especially rusts and powdery mildew. Hence, looking for germplasm donors inside closely related species from *Triticeae* seems to be essential. *Dasypyrum villosum* seems to be a valuable source of germplasm for rye, particularly as these species have been shown to be related, in some findings even more than *Dasypyrum* with *Triticum* (Baum, 1977, 1978a, b, 1983; De Pace *et al*., 2011; Linde-Laursen *et al*., 1992; Lucas and Jahier, 1988; Uslu *et al*., 1999; Vershinin and Heslop-Harrison, 1998). To conduct hybridization and gene transfer between species, information about their genetic similarity and relationship is often essential. In the light of many studies, the taxonomic position of *Dasypyrum* vis-à-vis *Triticum* and *Secale* is not clear. Many of the morphological, biochemical and cytological, as well as some molecular analyses, suggest a closer relationship of *Dasypyrum* with *Secale* (Baum, 1983; Lucas and Jahier, 1988; Uslu *et al*., 1999; Vershinin and Heslop-Harrison, 1998). On the other hand, *Dasypyrum* has been shown to be closely related to wheat as regards the level of the prolamin storage proteins (Montebove *et al*., 1987; Shewry *et al*., 1987), isozymes (Liu *et al*., 1995; Montebove *et al*., 1987) and RFLP analyses (Qi *et al*., 1998, 1999).

Molecular markers are useful in taxonomical *troubleshooting*, phylogenetic studies and evaluation of genetic diversity. From many techniques applied, random-type markers like RAPD and ISSR are still successfully used for these goals in plants (Bishoyi *et al*., 2014; Linos *et al*., 2014; Mucciarelli *et al*., 2014).

Random Amplified Polymorphic DNA (RAPD) is a technique using short (8-12 base pairs) arbitrary primers to amplify random segments of DNA along the whole genome. This method has been shown to have some disadvantages, like sensitivity to the changes of reaction conditions and dominant inheritance of markers. Nevertheless, it has a low cost, requires small amounts of DNA and does not need prior knowledge of the genome sequences. The data received in a short time and number of markers obtained is sufficient to discriminate species and genera. The source of RAPD polymorphism are mutations in sites complementary to the 3' end of primer, as well as deletions or insertions between primer binding sites and repetitive sequences (Kojima *et al*., 1998; Lynch and Milligan, 1994; MacPherson *et al*., 1993).

ISSR is also a simple technique using single arbitrary primers. However, reproducibility and informativeness of ISSRs are higher than in the case of RAPD. Moreover, ISSRs are inherited as dominant and rarely as codominant genetic markers. In this method, polymorphisms results from the differences in the length between inversely oriented and closely spaced microsatellites (Reddy *et al*., 2002; Ziętkiewicz *et al*., 1994). In rye (Bolibok *et al*., 2005; Matos *et al*., 2001) and in wheat (Nagaoka and Ogihara, 1997) ISSRs have been applied for polymorphism detection.

The aim of this study is to present relations between an inside *Dasypyrum*, *Secale* and *Triticum* species on the genetic level with ISSR and RAPD markers. New findings on similarity of the wild *Dasypyrum* accessions to wheat and rye may shed light on genetic divergence and indicate further directions in intergeneric transfer.

Material and methods

Plant material and DNA extractions

12 populations of *Dasypyrum*, twelve species and subspecies of *Secale* and *Triticum* were analyzed (Tab.1). All genotypes were kindly supplied by Dr Harold Bockelman, National Small Grains Collection. U.S. Department of Agriculture, Agriculture Research Service, Aberdeen, Idaho, USA. Total genomic DNA was extracted from 15-30 coleoptiles of several days-old seedlings following the CTAB procedure (Doyle and Doyle, 1987).

RAPD and ISSR amplification

Amplification was performed according to RAPD method described by Williams *et al*. (1990) and the ISSR method described by Ziętkiewicz *et al*. (1994) with modifications. Analyses were conducted in a T1 Biometra thermal cycler. 15 µl of RAPD mixture contained: 1 × PCR Buffer (10 mM Tris pH 8.8, 50 mM KCl, 0.08% Nonidet P40), 160 µM of each dNTP, 5.3 pM of primer, 1.2 mM $MgCl_2$, 0.5 U *Taq* DNA Polymerase and 60 ng of template. 14 RAPD primers listed in Tab. 2 were used in amplification.

The applied program of thermal cycling was: initial denaturation 95 °C for 2 minutes and next 45 cycles: denaturation 94 °C for 45 s, annealing 37 °C for 45 s, extension 72 °C for 45 s and last cycle was followed by incubation at 72 °C for 10 minutes. Amplification was conducted in two repeats for each genotype.

ISSR analyses were conducted with 17 primers (Tab. 2). The reaction was run in 15 µl mixture, which contained: 1 × PCR Buffer (75 mM Tris pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20), 160 µM of each dNTP, 4.7 pM of primer, 1.5 mM $MgCl_2$, 0.4 mM of spermidine, 0.5 U *Taq* DNA Polymerase and 60 ng of template. Amplification was carried out in a T1 Biometra thermal cycler with the

Tab. 1. Characteristics of analyzed material (*Dasypyrum, Secale, Triticum*)

No.	Species	Acc.no/Acc.name	Ploidy/ genomic composition	Growth habit	Country
1	*Dasypyrum villosum*	PI 251477	2x/VV	O, A, -, W	Turkey
2	*Dasypyrum villosum*	PI 251478	2x/VV	O, A, -, W	Turkey
3	*Dasypyrum villosum*	PI 368884	2x/VV	O, A, -, W	Turkey
4	*Dasypyrum villosum*	PI 368885	2x/VV	O, A, -, W	Turkey
5	*Dasypyrum villosum*	PI 368886	2x/VV	O, A, -, W	Turkey
6	*Dasypyrum villosum*	PI 470279	2x/VV	O, A, -, W	Turkey
7	*Dasypyrum villosum*	PI 491576	2x/VV	O, A, -, W	Italy
8	*Dasypyrum villosum*	PI 598390	2x/VV	O, A, -, W	Former Soviet Union
9	*Dasypyrum villosum*	PI 598391	2x/VV	O, A, -, W	Greece
10	*Dasypyrum villosum*	PI 636505	2x/VV	O, A, -, W	Greece
11	*Dasypyrum villosum*	PI 639750	2x/VV	O, A, -, W	Greece
12	*Dasypyrum breviaristatum*	PI 516547	$4x/V_bV_b$	O, P, -, W	Marocco
13	*S. cereale* ssp. *afghanicum*	PI 618662	2x/RR	O, A, Wi, Uc	Armenia
14	*S. cereale* ssp. *ancestrale*	CIse 107	2x/RR	O, A, Sp, Uc	Japan
15	S. cereale ssp. ancestrale	PI 445975	2x/RR	O, A, Sp, Uc	USSR
16	*S. cereale* ssp. *cereale*	CIse 174/UC-90	2x/RR	O, A, Sp, B	USA
17	*S. cereale* ssp. *cereale*	PI 446004/Brittle Rye-2	2x/RR	O, A, Wi/Sp, G	Canada
18	*S. cereale* ssp. *cereale*	PI 590948 /Ac Rifle	2x/RR	O, A, Wi, Cv	Canada
19	*S. cereale* ssp. *segetale*	CIse 105	2x/RR	O, A, Wi/Sp, Uc	Italy
20	*S. cereale* ssp. *segetale*	PI 283982	2x/RR	O, A, Wi/Sp, Uc	Former Soviet Union
21	*S. strictum*	PI 240286	2x/RR	O, P, Sp, W	Turkey
22	*S. strictum*	PI 383757/Yabani caudar	2x/RR	O, P, Wi, W	Turkey
23	*S. vavilovii*	PI 618682	2x/RR	S, A, Wi/Sp, W	Poland
24	*S. vavilovii*	PI 573649	2x/RR	S, A, Sp, W	Afghanistan
25	*T. aestivum* ssp. *compactum*	PI 129528/Sandomierka	6x/AABBDD	S, A, Wi, L	Poland
26	*T. aestivum* ssp. *sphaerococcum*	PI 352499/Cawnpore	6x/AABBDD	S, A, Sp, L	India
27	*T. aestivum* ssp. *aestivum*	CItr 14108/Chinese S	6x/AABBDD	S, A, Sp, G	USA
28	*T. aestivum* ssp. *aestivum*	PI 572966; alloplasmic population (cytoplasm from *D. villosum*)	6x/AABBDD	S, A, Wi, B	USA
29	*T. monococcum* ssp. *aegilopoides*	PI 272520	2x/AmAm	S, A, Wi, W	Hungary
30	*T. monococcum* ssp. *aegilopoides*	PI 401412	2x/AmAm	S, A, Wi, W	Iran
31	*T. monococcum* ssp. *monococcum*	CItr 13963	2x/AmAm	S, A, Sp, Uc	USA
32	*T. timopheevii* ssp. *armeniacum*	PI 427394	4x/AAGG	S, A, Wi, W	Iraq
33	*T. timopheevii* ssp. *timopheevii*	PI 282932/I-1022	4x/AAGG	S, A, Sp, W	Argentina
34	*T. turgidum* ssp. *dicoccoides*	PI352328/Spontaneo villosum	4x/AABB	S, A, Sp, W	Germany
35	*T. turgidum* ssp. *dicoccon*	CItr 12213/Garden	4x/AABB	S, A, Sp, Uc	India
36	*T. turgidum* ssp. *durum*	CItr 11477/Golden Ball	4x/AABB	S, A, Sp, Cv	USA

Note: O – open pollinated, S – self-pollinated, A – annual, P – perennial, W – wild, L – landrace, Cv – cultivar, Uc – uncertain improvement status, B – breeding material, G – genetic material, Wi – winter, Sp – spring

program of thermal cycling as follows: initial denaturation 95 °C for 7 min, 38 cycles with denaturation step at 95 °C for 30 s, different annealing temperatures (the first three cycles 54 °C – 45 s, the next three cycles 53 °C – 45 s and 32 cycles 52 °C for 45 s) and extension 72 °C – 2 min for each step. The last cycle was followed by incubation at 72 °C for 7 minutes. Amplification was conducted in two repeats for each genotype.

RAPDs and ISSR products were separated on agarose gels, respectively in 1.5% and 2.5%, which contained 0.1% ethidium bromide in 1 x TBE buffer (89 mM Tris-borate, 2.5 mM EDTA). Separation was carried out for 1.5 h at 120 V. DNA marker GeneRulerTM 100 bp Plus DNA Ladder was used as a size ruler.

Data analysis

The amplified RAPD and ISSR fragments were scored as absent (0) or present (1), then formed into a binary matrix. Results of RAPD and ISSR allowed to calculate polymorphism information, which include PIC (polymorphism information content) according to Riek De *et al*. (2001) and Assay Efficiency Index (AEI, mean number of polymorphic fragments) (Pejic *et al*., 1998). Genetic Distance (GD) indexes between all the analyzed genotypes were calculated using Nei's formula (Nei, 1972). GD and bootstrap values were calculated for the relationships between the species of *Secale*, *Dasypyrum* and *Triticum* using PHYLIP (Felsenstein, 1989) and visualised as dendrograms constructed using Treeview software (Page,

Tab. 2. RAPD and ISSR primers and characteristics of the polymorphism identified

Marker	Detected polymorphism					
	Primer sequence 5'-3'	No. of polymorphic fragments	Total PIC	PIC *Dasypyrum*	PIC *Triticum*	PIC *Secale*
RAPD	A-07 GAA AAG GGT G	25	0,26	0,35	0,35	0,30
	A-20 GTT GCG ATC C	17	0,39	0,25	0,41	0,40
	G-04 GGA GTA CTG G	19	0,27	0,30	0,26	0,34
	G-10 CCG ATA TCC C	17	0,38	0,29	0,39	0,39
	J-05 CTC CAT GGG G	13	0,38	0,32	0,39	0,35
	J-10 AAG CCC GAG G	34	0,30	0,31	0,40	0,30
	J-13 CCA CAC TAC C	16	0,34	0,37	0,34	0,30
	J-19 GGA CAC CAC T	19	0,31	0,33	0,41	0,34
	T-02B CTA CAC AGG C	53	0,20	0,34	0,35	0,35
	T-03 GTC CTT AGC G	22	0,22	0,20	0,34	0,27
	U-225 CGA CTC ACA G	15	0,42	0,40	0,38	0,35
	U-295 CGC GTT CCT G	14	0,39	0,29	0,40	0,40
	U-534 CAC CCC CTG C	13	0,35	0,40	0,37	0,27
	U-535 CCA CCA ACA G	10	0,40	0,39	0,42	0,36
	Total	287	-	-	-	-
	Average	20,5	0,33	0,32	0,37	0,34
ISSR	SR-01 $(AG)_8G$	35	0,24	0,31	0,36	0,22
	SR-06 $(GT)_8C$	25	0,29	0,29	0,32	0,34
	SR-11 $(AC)_8G$	30	0,33	0,31	0,34	0,30
	SR-14 $(GA)_7YG$	34	0,30	0,32	0,35	0,32
	SR-16 $(GA)_8C$	29	0,33	0,35	0,39	0,38
	SR-17 $(GA)_8YC$	49	0,24	0,30	0,36	0,31
	SR-22 $(CA)_8G$	18	0,29	0,38	0,39	0,40
	SR-23 $(CA)_8GC$	63	0,22	0,27	0,35	0,33
	SR-27 $(TC)_8G$	43	0,22	0,27	0,37	0,32
	SR-31 $(AG)_8YC$	29	0,30	0,26	0,34	0,24
	SR-32 $(AG)_8YT$	35	0,27	0,33	0,35	0,33
	SR-33 $(AG)_8T$	36	0,30	0,33	0,28	0,29
	SR-36 $(AC)_8CG$	40	0,24	0,25	0,33	0,31
	SR-37 $(AC)_8C$	49	0,24	0,33	0,35	0,23
	SR-38 $(CT)_8G$	33	0,29	0,29	0,38	0,33
	SR-39 $(GA)_8GG$	39	0,27	0,32	0,30	0,34
	SR-40 $(AC)_8T$	34	0,36	0,34	0,39	0,38
	Total	621	-	-	-	-
	Average	36,5	0,28	0,31	0,35	0,31

1996) by UPGMA (unweighted pair-group method with arithmetic averages). To estimate the genetic structure among and inside the tribe, gene diversity - He (Lynch and Milligan, 1994) was calculated with AFLPsurv (Vekemans *et al*., 2002). Variation between species within genus (Hw) and variation between genera (Hb) were established as components of total genetic variation (Ht). Wright's fixation index (Fst), which is a measure of population differentiation and genetic distance was computed based on genetic polymorphism data scored by measuring the genetic correlation between pairs of genes sampled within a population relative to pairs of genes sampled within the overall set of populations (also interpreted as the proportion of the total gene diversity that occurs among as opposed to within populations, Lynch and Milligan, 1994). PCoA based on matrix of Pearson correlation coefficients was performed in XlStat v.7.5.2.

Results and discussion

In this study the genetic similarity among *Dasypyrum*, *Secale* and *Triticum* species with RAPDs and ISSRs was estimated. To show the level of *Dasypyrum* similarity vs. *Secale* and *Triticum*, 12 populations of *Dasypyrum* (11 *D. villosum* and 1 *D. breviaristatum*), together with 12 accessions belonging to 3 *Secale* species and 12 accessions from 4 *Triticum* species were analyzed (Tab. 1). 14 RAPD and 17 ISSR primers (Tab. 2) showing high level of polymorphism and reproducibility were selected, respectively from 80 and 60 previously screened.

As expected, ISSRs were more informative than RAPDs. In *Dasypyrum* 170 RAPD bands (from a total of 287) were amplified and only 4 fragments were monomorphic (polymorphic ones comprised 97.65%, AEI-11.9). Among 662 total ISSR markers, 451 were shown in *Dasypyrum*, out of them 96.45% were polymorphic (AEI-25.6). For *Secale* 154 total RAPD bands were scored, out of them 130 were polymorphic (84.42%) and from 340 ISSR bands, 299 were polymorphic (87.94%). AEI index was calculated to 9.29 and 17.59, respectively. For *Triticum* species out of 177 RAPD fragments, 170 were polymorphic (96.04%) and in the case of ISSRs from a total of 423 bands, 417 were polymorphic (98.58%). AEI were 12.14 for RAPD and 24.53 for ISSR. Summing up, ISSRs amplified more polymorphic bands, as was also obtained by other researchers (Kojma *et al*., 1998; Matos *et al*., 2001), but oddly, the percentage of polymorphic bands detected with both methods was nearly on the same level inside the

species. This may result from the fact that only highly polymorphic primers were selected to analyses.

The highest percentage of all (RAPD together with ISSR) polymorphic loci was found in *Dasypyrum*, followed by *Triticum* and *Secale* (Tab. 3). Gene diversity (He) was the highest in *Triticum*, followed by *Dasypyrum* and *Secale* (Tab. 3). Total genetic variation (Ht), which was 0.332±0.023 (Ht±S.E, standard error), was composed of variation between species within genus (Hw=0.192±0.00) and variation between genera (Hb=0.140±0.09). A similar level of Ht (0.34 0.02) was found in cultivated rye before and was higher comparing with that of weedy rye (Ht=0.27 ± 0.03) (Chikmawati *et al*., 2012). It was reflected in the more diverse distribution area of cultivated rye group. In the presented experiment more variation was observable within species and Wright's fixation index (Fst), reflecting the proportion of the total gene diversity that occurs among as opposed to within genus, was 0.42. It confirmed that the studied biological material was highly differentiated. Chikmawati *et al*. (2012) observed lower genetic differentiation of the cultivated ryes vs. weedy ryes (Fst=0.16 and 0.56, respectively), indicating a high genetic similarity among the accessions of cultivated rye. In heterotic groups of Chinese northern wheat cultivars and lines, based on DArT markers, Zhang *et al*. (2011) found the Fst value equal to 0.28, indicating that this germplasm is highly differentiated.

High informativeness of the chosen primers was also confirmed by the PIC index, which is the same as diversity index -DI (Botstein *et al*., 1980). PIC reflects informativeness of marker system employed and shows the distribution of identified polymorphisms. For dominant markers its values range from 0 (monomorphic) to 0.5 (highly discriminative) (Riek De *et al*., 2001). PIC has been widely used to compare diallelic and multiallelic molecular markers (Powell *et al*., 1996). In this study, the mean PIC indices of RAPD for the studied species were highly similar (Tab. 2), with values between 0.31-0.37 (Tab. 2). Powell *et al*. (1996) hypothesised that the numerical value of PIC index changes with the species analyzed, but the relative level of marker systems remains constant if the mechanisms generating DNA polymorphisms are similar across these species. The high PIC values found in the presented experiment indicates a high level of genetic diversity existing in the three species studied. Similarly, high values of PICs in the case of ISSR markers were found in rye (Bolibok *et al*., 2005). In rice, Sarla *et al*. (2005) also obtained high PICs for ISSR, as they examined highly diverse material containing varieties, landraces, ancestral landraces and wild accessions. High PICs were also detected for RAPD, for example in an orchid *Dendrobium nobile* Lindl. (Bhattacharyya and Kumaria, 2014) or an Indian medicinal plant *Solanum trilobatum* L. (Shilpha *et al*., 2013).

Tab. 3. Distribution of the genetic variation among and within *Dasypyrum*, *Secale* and *Triticum* (Lynch and Milligan, 1994)

Genus	Polymorphic loci		He ± S.E	Nei's genetic distance	
	Number	Proportion [%]		*Dasypyrum*	*Secale*
Dasypyrum	554	61.1	0.207±0.007	–	–
Secale	377	41.6	0.146±0.006	0.228	–
Triticum	523	57.7	0.223±0.007	0.150	0.188

Tab. 4. Number of specific RAPD and ISSR markers identified

Specific for		Genome	RAPD	ISSR
Genus	*Dasypyrum*	V+Vb	0	3
	Dasypyrum + *Secale*	V+Vb+R	0	3
Genome	*D. villosum* + *Secale*	V+R	0	1
Species	*D. villosum*	V	1	4
	S. cereal	R	1	0
	S. strictum	R	4	3
	S. vavilovii	R	0	0
	S. cereale +*S. vavilovii*	R	1	7
	T. timopeevii	AG	3	4
	T. monococcum	Am	6	4
	T. turgidum	AB	0	3
	T. aestivum	ABD	4	3
	T. monococcum + *T. turgidum*	Am, AB	0	1
	T. timopeevii + *T. turgidum*	AG, AB	2	0
	T. aestivum + *T. turgidum*	ABD, AB	2	1
	T. aestivum + *T. timopeevii* + *T. turgidum*	ABD, AG, AB	3	0
Accession	*D. villosum*		8	30
	D. breviaristatum		5	24
	Secale		2	11
	Triticum		5	20
Total			47	122

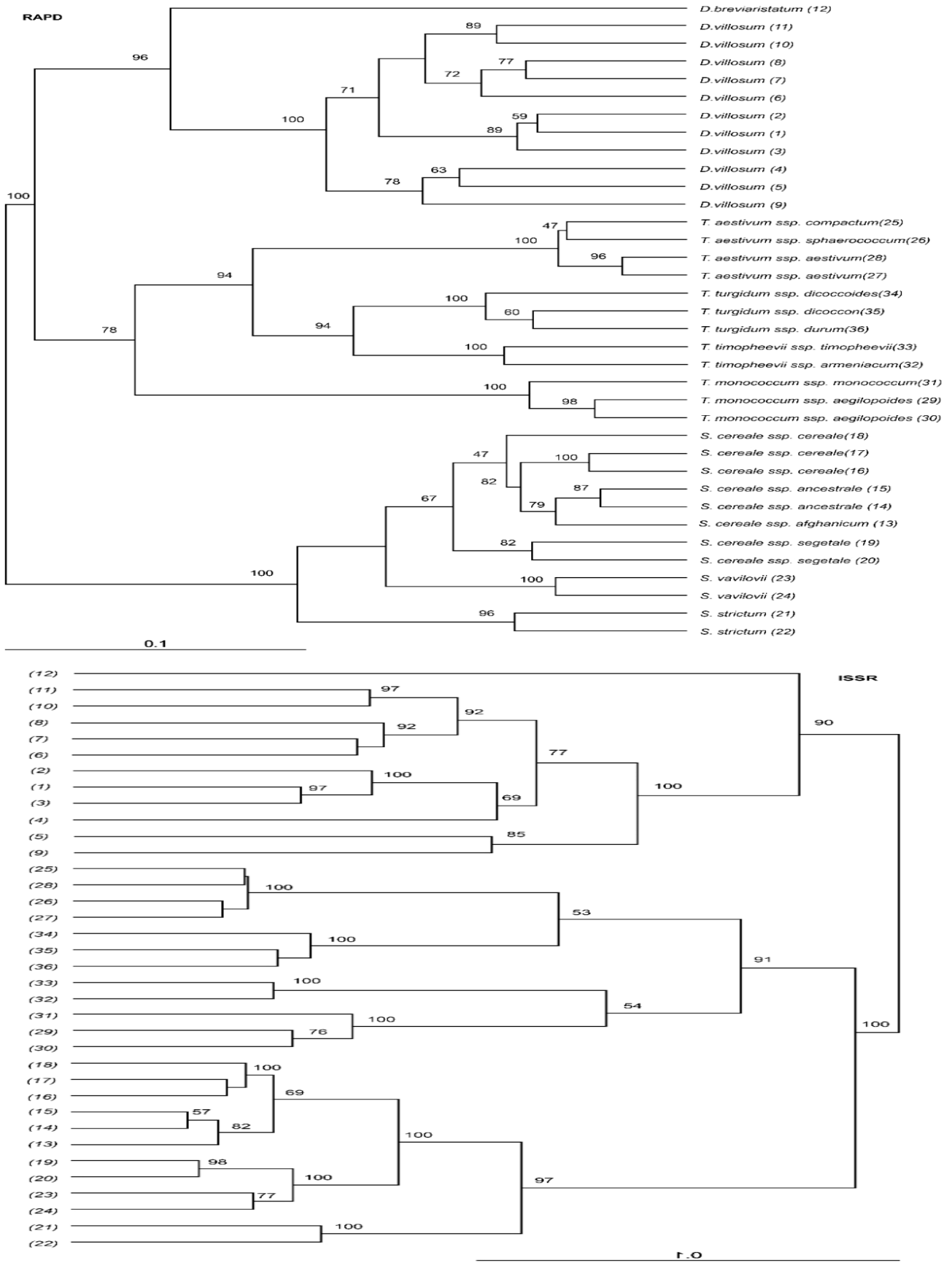

Fig. 1. Cluster analysis dendrogram estimated among the *Dasypyrum*, *Secale* and *Triticum* species based on RAPD and ISSR markers using the unweighted pair group method of arithmetic average (UPGMA) and Nei's distances. Bootstrap values are given for nodes

In this experiment some fragments specific for genus, genome, species or genotype were found (Tab. 4). Many of these fragments were amplified with ISSR. Only three ISSR bands and none of RAPD were characteristic for *Dasypyrum* genus, while 3 other were shared with *Secale*. One RAPD and 4 ISSR markers of V genom of *Dv* were identified. For respective species of *Secale*, six RAPD fragments and 10 ISSRs were specific. The largest number of 20 RAPD and 16 of ISSR specific bands was identified for *Triticum* species. A significant number of bands, many more of ISSRs, specific to one of the analyzed accessions, were detected (Tab. 4). Some of these markers could be converted to SCARs suitable to trace introgression events, identification of genus, species or accessions.

The genetic relationships among *Dasypyrum*, *Secale* and *Triticum* were pictured as dendrograms constructed based on ISSRs and RAPDs (Fig. 1). On both dendrograms three genera formed 3 main clusters. Inside *Dasypyrum* cluster, *Db* was an outgroup, and accessions of *Dv* were clustered together. Turkish *Dv* accessions were demonstrated to be differential as they clustered into three different subclusters. One of them formed a group with accessions from USSR and Italy, two other on RAPD based dendrogram and one based on ISSR polymorphism clustered with one accession from Greece, and three formed their own subclaster (Fig. 1).

Cluster analysis gives no clear answer as to the position of *Triticum* species vs. *Secale* and *Dasypyrum*. Results of RAPD analysis suggest closer relation between *Triticum* and *Dasypyrum*, which is further not confirmed by ISSR analysis where *Secale* and *Triticum* are better clustered (Fig. 2). This problem may be further resolved with PCA analysis (Fig. 2). Distribution of genotypes on the first two principal components accounting for 37.3% of overall genetic variation indicates that *Triticum* is located between *Dasypyrum* and *Secale*. Moreover, the accession of *D. breviaristatum* is grouped with *Triticum*. This finally supports that *Dasypyrum* is more closely related to wheat when compared with rye. Additionally, taking into account genetic distances, gene transfer from *Dasypyrum* to rye may be more difficult than to wheat. These observations are further supported by values on Nei's genetic distance, which is the lowest for combination of *Triticum* and *Dasypyrum* (0.150) and the highest for *Secale* vs *Dasypyrum* (0.228).

The high level of polymorphism found inside *Dasypyrum* and topography of *Dasypyrum* clade was expected, as the situation within the genus is yet to be untangled. The V^b genome of *D. breviaristatum* was repeatedly shown to be highly different from V^v of diploid *D. villosum*, due to defficiency of chromosomes pairing, their different morphology and composition (Blanco *et al*., 1996; Linde-Laursen and Frederiksen, 1991; Pignone *et al*., 2000; Sakamoto, 1986; Uslu *et al*., 1999; Vershinin *et al*., 1996). Moreover, Uslu *et al*. (1999) suggested even higher distance of *Db* to *Dv* than of the latter to *Th. bessarabicum* and *S. cereale*. Yang *et al*. (2006) also showed by RAPD a closer relationship of *Db* with *Th. intermedium* and *T. aestivum* than with *Dv*. The results presented here with RAPDs as well as with ISSRs showed that in cluster analysis *Db* constitute an outgroup, but belonging to *Dasypyrum* (Fig. 1). Similarly, Fan *et al*. (2013),

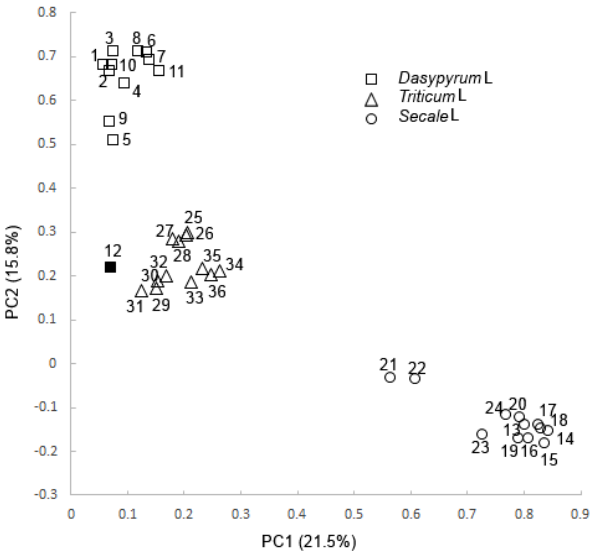

Fig. 2. Distribution of representatives of *Dasypyrum*, *Secale* and *Triticum* on the two first components of variation

basing on molecular evolution of a single-copy gene encoding plastid acetyl-CoA carboxylase (*Acc1*), showed *Dv* in the same clade as *Db*; however, *Dv* was placed closer to one of *Elymus repens* accessions than to *Db*. On the other hand, Baum *et al*. (2014) through analyses of 5S nrRNA gene sequences found that the *Db* 4x cytotype is an allotetraploid containing haplomes of *Dv* and diploid cytotype of *Db* (VVV^bV^b). Close relationship between *Dv* and *Db* was also supported earlier with Southern-blot with repeated genome sequences and plastidal DNA probes, RFLPs, isosymes and in situ with gliadin (Blanco *et al*., 1996), rDNA and repetitive probes (Galasso *et al*., 1997). However, the results of PCA analysis confirm previous reports that *Db* may be grouped together with *Triticum* and Nei's distances to *Secale*, *Triticum* and *Dasypyrum villosum* are respectively 0.424, 0.458 and 0.344 for RAPDs and 0.399, 0.379 and 0.343 for ISSRs. In *Triticeae* a high level of synteny exists at the genes or single-copy markers, but in repetitive DNA the significant differences, crucial for evolution and speciation, was found (Galasso *et al*., 1997). These explain similarities as well as differences found among species and genera.

Inside *Triticum* clade the phylogenetic relationships showed identical arrangement on both dendrograms. The accessions studied were separated into species according to present classification and genomic composition. The first outgroup consisted of three diploid species of *T. monococcum* (Tab. 1, 29-31). Next, five tetraploid wheat species clustered together and a group consisting of *T. aestivum* hexaploid was separated. These results do not reveal new taxonomic relationships, but may serve as a good background for establishing relationships with more distantly related taxa, such as *Dasypyrum* and *Secale*.

The evolutionary events in *Secale* showed on dendrograms were generally the same as those presented earlier (Bolibok-Brągoszewska *et al*., 2014; Couadrado and Jouve, 2002; De Bustos and Jouve, 2002). In the case of *Secale* the RAPD and

ISSR clades were very similar, with two exceptions (Fig. 1). On the RAPD dendrogram one accession of *S. cereale* ssp. *cereale* was not grouped with the two other, but in the case of ISSRs, all three accessions were put together. On the other hand, *S. cereale* ssp. *segetale* were clustered with other *Secale* subspecies with RAPDs, but based on ISSRs they were shown to be closer to *S. vavilovii*. The first was in agreement with Bolibok-Brągoszewska *et al*. (2014) and the last with the dendrogram of Chikmawati *et al*. (2005) constructed basing on AFLPs.

The relationships of *Triticum*, *Secale* and *Dasypyrum* were estimated several times. Generally, it can be noticed that morphologically *Dasypyrum* resembles *Secale* species and was put as a sister group of *Secale* within the same clade (Baum, 1978a, 1983; Frederiksen and Seberg, 1992; Kellogg, 1989; Seberg and Frederiksen, 2001). Similar relationship was found on the level of non-coding sequences as in heterochromatic regions (Linde-Laursen *et al*., 1992; Vershinin and Heslop-Harrison, 1998) and species specific repetitive sequences- *pHv62* and *pSc119.2* (Schubert *et al*., 1990; Uslu *et al*., 1999). On the other hand, at the level of coding sequences as storage proteins (Montebove *et al*., 1987; Shewry *et al*., 1987) and isozymes (Liu *et al*., 1995; Montebove *et al*., 1987), *Dasypyrum* was grouped with *Triticum*.

In the presented paper, the phylogenetic relationships among the three species were shown to be slightly different, as was visualised on the dendrograms. RAPDs assembled *Triticum* and *Dasypyrum* together, and Nei's average distance between these the two genera (0.435) was lower for *Triticum* vs *Secale* (0.447) and *Secale* vs *Dasypyrum* (0.461). ISSRs put *Triticum* together with *Secale*, indicating them to be more related (0.370) than each of them with *Dasypyrum* (0.374 and 0.407, respectively). Both marker systems analyzed together showed *Dasypyrum* to be more similar to *Triticum* than to *Secale*, as was calculated with GD indices (Tab. 3). Nevertheless, differential between the two GD indices was slight (0.078), so it could not exclude the possibility of successful hybridization among *Dasypyrum* and *Secale*, especially using bridge species, which was conducted earlier. The first fertile hybrid of *Secale* × *Dv* (*S. fragile* × *Dv*) was produced by Sando (1935). Kostoff (1937) obtained trigeneric hybrid with *T. dicoccum* as a bridge species. The hybrid of *S. cereale* with *Dv* reported by Nakajima (1951), had more success in crossing *Secale* with *Dasypyrum* by using tetraploid wheats as bridge species (Nakajima, 1969, 1970). Other trigeneric hybrids were produced by Jiang *et al*. (1989), Fu *et al*. (1997) and Knobloch (1968) and tetrageneric ones by Jahier *et al*. (1988) and Yuan *et al*. (1997). Tetraploidal amphidiploid *S. cereale* × *H. villosa* was produced by Łapiński and Gruszecka (1997). As was presented, hybridization of *Secale* and *Dasypyrum* was performed only several times, but little is known about useful *Dv* genes' influence on hybrids.

Conclusions

The current study demonstrates usefulness of RAPD and ISSR markers for estimation of genetic relationships among *Triticeae*, between representatives of three analyzed species: *Dasypyrum*, *Secale* and *Triticum*. The findings showed that the choice of random markers techniques is still appropriate and attractive due to a low cost of analysis and quick acquisition of data. The results obtained in the study enrich the earlier data achieved by morphological, storage proteins, isozymes and cytological analyses. Polymorphisms resulting from mutations in sites complementary to the 3' end of primer and from deletions or insertions between primer binding sites and repetitive sequences (RAPD), just as those found in the length between inversely oriented and closely spaced microsatellites (ISSR) showed *Dasypyrum* to be more related to *Triticum* species, comparing to *Secale*. Nevertheless, the slight differential between the two GD indices could not exclude the potential use of *Dasypyrum* germplasm to enrich a genetic pool of rye, which is a very important bread crop in *north-eastern* Europe and Russia.

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