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Application of the ISSR method to estimate the genetic similarity of *Dasypyrum villosum* (L.) P. Candargy Greek populations to *Triticum* and *Secale* species

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Abstract: In the study, the genetic similarity between *Dasypyrum villosum* L. (P.) Candargy and *Triticum* L. and *Secale* L. species was studied on the basis of ISSR markers. As a very polymorphic, effective and reproducible method, ISSR can be successfully employed to evaluate polymorphism between and inside different species. The polymorphic information content values (PIC) of ISSR method ranged from 0.57 to 0.87, with the mean value of 0.7. The genetic similarity of the forms analyzed ranged from 0.27 to 0.97, with the mean value of 0.47, indicating their high diversity. A higher similarity of *Dasypyrum villosum* to *Triticum* species, in comparison with *Secale* was found – the mean Dice genetic similarity index between genera was calculated at 0.40 for *Dasypyrum* and *Triticum*, and at 0.31 for *Dasypyrum* and *Secale*.

Key words: *Dasypyrum villosum*, *Secale*, *Triticum*, ISSR, molecular markers, genetic similarity

1. Introduction

Dasypyrum villosum (L.) P. Candargy, is a cross-pollinating, annual diploid grass (2n=2x=14, VV) from the tribe Triticeae, native to the north-eastern part of the Mediterranean region and the Caucasus areas (Frederiksen 1991). It possesses many traits useful for wheat breeding, such as resistance to powdery mildew, rusts, take-all fungus, cereal eyespot, wheat streak mosaic virus, barley yellow dwarf virus, wheat curl mite, salt and drought tolerance, as well as a low plant height, a good tiller ability and high protein content in kernels (De Pace *et al*. 1990, 2001; Zhang *et al*. 2005; Grądzielewska 2006b; Cao *et al*. 2009). Much progress has been made in transferring useful *D. villosum* genes to wheat through the development of addition, substitution and translocation lines. The resistance genes *Pm21*, *PchDv* and *Wss1* from *D. villosum* have been successfully transferred to common wheat. A T6VS·6AL translocation line with *Pm21* gene is extensively used in breeding programs (Yildirim *et al*. 1998; Zhang *et al*. 2005). On the other hand, the allelic diversity of *D. villosum* gliadin genes was shown to be beneficial for the quality improvement of wheat (De Pace *et al*. 2001).

Most researchers, on the basis of the similarity of morphology, place *Dasypyrum* near the *Triticum*/*Agropyron* complex and *Secale* (Sakamoto 1973; West *et al*. 1988). The major distinctive feature of the genus *Dasypyrum* is its two-keeled glume, a clearly autopomorphic feature that separates it from all other genera in the *Triticeae* (Galasso *et al*. 1997). Polymorphism of storage proteins and isozymes (Liu *et al*. 1995) and a comparatively high crossability with diploid, tetraploid and hexaploid wheats indicate a rather close relationship between *Dasypyrum* and *Triticum*, in spite of the low degree of chromosome pairing (Lucas & Jahier 1988). On the other hand, morphological, biochemical, cytological and hybridization experiments using DNA probes, suggest a closer relationship with *Secale* (Frederiksen 1991; Uslu *et al*. 1999; Grądzielewska 2006a).

ISSR is a simple technique, in which polymorphisms results from the differences in the length between inversely oriented and closely spaced microsatellites. The reproducibility and informativeness of this method is higher than in other marker systems using single arbitary primers. Moreover, ISSRs are inherited as dominant or rarely as codominant genetic markers and are random-type markers, so they are suitable for phylogenetic studies, evaluation of genetic diversity and identification of cultivar (Rakoczy-Trojanowska & Bolibok 2004). This study attempted to establish genetic relationships between *Dasypyrum villosum*, *Triticum* L. and *Secale* L. species on the basis of polymorphism of ISSR markers.

2. Material and methods

Five Greek populations of *Dasypyrum villosum* and, respectively, five species and subspecies of *Secale* and *Triticum* were analysed (Table 1). All genotypes were kindly supplied by Dr Harold Bockelman, National Small Grains Collection U.S. Department of Agriculture, Agricultural Research Service, Aberdeen, Idaho, USA.

Total genomic DNAs were extracted from coleoptiles of several-day old seedlings in two replications, following the method of Milligan (1992).

ISSR analyses were conducted with 10 primers (Table 2) as described by Zietkiewicz *et al.* (1994) with modifications. The reaction was run in 20µl mixture containing: 1x PCR buffer (10 mM Tris pH 8.8; 50 mM KCl; 0.08% Nonidet P40) (Fermentas, Lithuania), 100 mM of each dNTP; 300 nM of primer; 2.5 mM $MgCl_{2;}$ 60 ng template DNA; 0,5 U *Taq* DNA Polymerase (Fermentas, Lithuania). The program of thermal cycling was as follows: initial denaturation at 95˚C for 7 min.; 38 cycles: denaturation at 95˚C for 30 s.; annealing lasted 45s but the temperature was changed: for the first 3 cycles, it was 54˚C, for the next 3- 53˚C, and for the remaining 32 cycles 52˚C, extension 72˚C for 2 min.; the last cycle was followed by incubation for 7 min. at 72˚C.

ISSR products were separated on a 1.5% agarose gel for 2h at 100V. The gel contained 0.01% bromium

Table 1. Characteristics of analysed *Dasypyrum villosum, Secale* and *Triticum* genotypes

Species/cultivar	Accession	Genome	Country of origin	Type	Life cycle
Dasypyrum villosum	W67282 W67283 W67284 W67285 W67286	V	Greece/ Central Macedonia	W	A
Secale cereale ssp. *afghanicum*	PI618662	R	Armenia	We	A
Secale cereale ssp. *ancestrale*	PI283971		Algieria	We	A
Secale cereale ssp. *cereale*	PI446017		Canada	C	A
Secale strictum	PI205222		Turkey	W	P
Secale vavilovii	PI573648		Russian Federation	W	A
Triticum aestivum ssp. *sphaerococcum*, Acarp	PI277142	ABD	India	C	A
Triticum monococcum ssp. *aegilopoides*	PI272519	A^m	Hungary	W	A
Triticum timopheevii ssp. *timopheevii*, Nigrum	PI282933	AG	Argentina	W	A
Triticum turgidum ssp. *dicoccoides*, Schweinfurthii	PI352328	AB	Germany	W	A
Triticum aestivum ssp. *aestivum*	PI572994	ABD	United States	C	A

Explanations: C – cultivated, W – wild, We – weedy, A – annual, P – perennial

Table. 2. Characteristic of polymorphism identified with ISSR markers

Primer sequence 5'-3'	All genotypes				*Dasypyrum villosum*				*Secale*				*Triticum*			
	No. of DNA fragments			PIC	No. of DNA fragments			PIC	No. of DNA fragments			PIC	No. of DNA fragments			PIC
	t	p	s		t	p	s		t	p	s		t	p	s	
Sr-01$(AG)_8$G	9	7	0	0.64	6	0	0	0.30	3	0	0	0.67	6	2	0	0.47
Sr-06$(GT)_8$C	13	11	1	0.67	6	0	0	0.50	10	4	1	0.49	11	8	3	0.68
Sr-22$(CA)_8$G	15	11	0	0.57	14	5	1	0.24	12	7	4	0.58	9	5	3	0.64
Sr-23$(CA)_8$GC	20	20	0	0.83	9	4	2	0.70	8	4	2	0.74	16	13	4	0.70
Sr-28$(TG)_8$G	15	13	0	0.64	10	3	0	0.46	9	4	1	0.54	10	5	0	0.54
Sr-31$(AG)_8$YC	28	28	6	0.79	13	1	0	0.54	16	6	3	0.61	16	15	6	0.84
Sr-32$(AG)_8$Y	25	23	2	0.70	14	3	2	0.54	13	6	3	0.66	19	12	3	0.54
Sr-36$(AC)_8$CG	13	12	1	0.64	5	0	0	0.61	9	4	3	0.56	13	6	2	0.36
Sr-37$(AC)_8$C	16	14	1	0.69	9	1	0	0.46	10	5	2	0.61	9	6	0	0.64
Sr-38$(CT)_8$G	20	20	1	0.87	9	4	2	0.70	13	10	5	0.73	14	12	8	0.81
Total	174	159	12	-	95	21	7	-	103	50	24	-	123	84	29	-
Average	17.4	15.9	1.2	0.70	9.5	2.1	0.7	0.50	10.3	5.0	2.4	0.62	12.3	8.4	2.9	0.62

Explanations: t – total, p – polymorphic, s – specific ethidine in TBE buffor (89 mM Tris-borate, 2.5 mM EDTA).

Polymorphism information content values (PIC) of ISSR method were calculated according to Nei (1973) on the basis of the results received. The assay efficiency index, AEI (mean number of polymorphic fragments), was also calculated (Pejic *et al*. 1998). For each genotype x marker combination, the presence (1) or absence (0) of an ISSR allele was treated as an independent character. The data matrix was then used to calculate the genetic similarity (GS) index between pairs of all the genotypes analyzed using Dice formula (Nei & Li 1979). Genetic relationships among *Dasypyrum, Secale* and *Triticum* accessions were estimated using the unweighted pair-group method with arithmetic mean (UPGMA) cluster analysis of the GS matrix, employing the NTSYS-pc 2.10q (Rohlf 2001).

3. Results

The primers used amplified 174 total products, out of which 159 were polymorphic (91.4%). The AEI index was calculated at 15.9. Individually, in *Triticum* 68.3% products were polymorphic, and in *Secale* and *Dasypyrum* respectively 48.5% and 22.1%. The AEI indices were calculated at 8.4 in *Triticum*, 5.0 in *Secale*, and only 2.1 in *D. villosum* (Table 2).

Calculated values of the polymorphic information content (PIC) of the ISSR method ranged from 0.57 to 0.87, on average 0.7. Individually for three genera, mean PIC values were 0.50, 0.62 and 0.62 for *Dasypyrum*, *Secale* and *Triticum*, respectively (Table 2).

It was possible to distinguish all analyzed *Triticum* accessions, just as *Secale cereale* ssp. *cereale* (PI446017), *Secale strictum* (PI205222) and *Secale vavilovii* (PI573648) from the other genotypes on the basis of DNA fragments characteristic only of one genotype. The remaining genotypes – *Dasypyrum* populations, *Secale cereale* ssp. *afghanicum* (PI618662) and *Secale cereale* ssp. *ancestrale* (PI283971) – could be differentiated by marker profiles.

The number of profiles generated with all primers was 106 (from 5 to 14 for individual primers, on average 10.6), out of which 82 were specific. All genotypes could be differentiated from each other, based on the profiles obtained with min. 2 primers. When the three genera were analysed individually, in *Dasypyrum villosum* 26 profiles were obtained, within which 17 was specific. In *Secale* and *Triticum* these values were 36 (29) and 44 (38), respectively. On average, in *Dasypyrum*, *Secale* and *Triticum* the primers amplified 2.6, 3.6 and 4.4 profiles, respectively.

The Dice similarity indices ranged from 0.27 between PI 205222 (*Secale strictum*) and W6 7284 (*D. villosum*) to 0.97 between W6 7283 (*D. villosum*) and

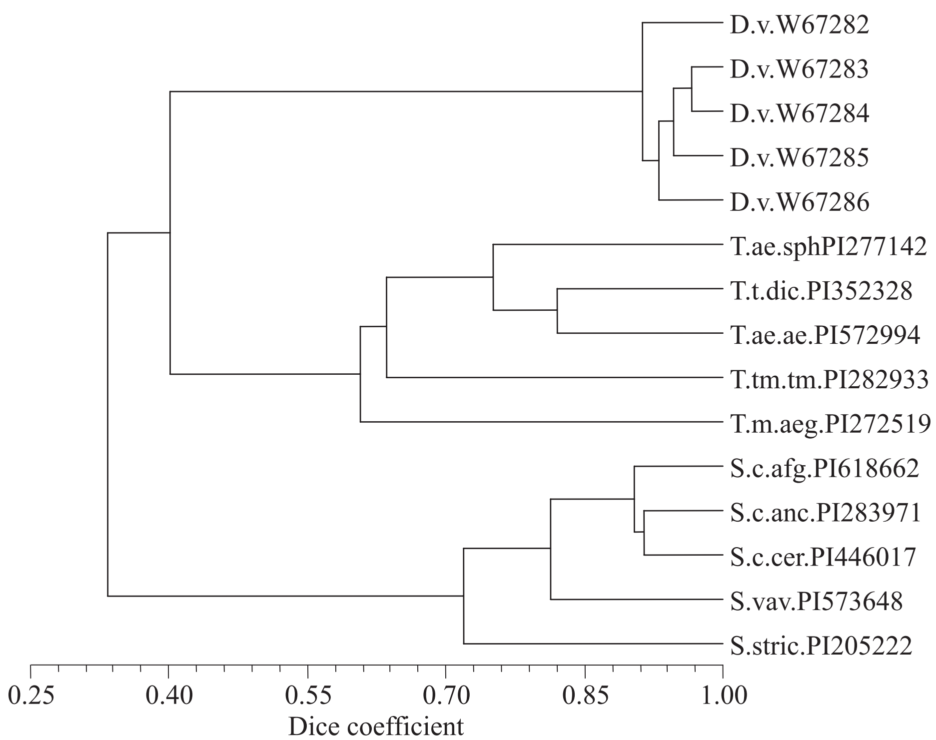

Fig. 1. The dendrogram of *Dasypyrum villosum, Secale* and *Triticum* genetic similarities constructed with UPGMA method based on the polymorphism of ISSR markers

W6 7284 (*D. villosum*). The mean genetic similarity was calculated at 0.47. PI 205222 had the least affinity with the others (0.56). In the three genera analyzed separately, the mean genetic similarity index in *D. villosum* was 0.93, in *Secale* – 0.80 and in *Triticum* 0.67. In *Secale*, the greatest affinity (0.91) was between *S. cereale* ssp. *cereale* and *S. cereale* ssp. *ancestrale* (PI 446017 and PI 283971), and the smallest (0.70) between *S. cereale* ssp. *afghanicum* and *S. strictum* (PI 618662 and PI 205222). In wheats, *Triticum turgidum* ssp. *dicoccoides* (PI 352328) and *Triticum aestivum* ssp. *aestivum* (PI 572994) were the most similar (0.82) and *Triticum monococcum* ssp. *aegilopoides* (PI 272519) and *Triticum timopheevii* ssp. *timopheevii* (PI 282933), the most different (0.56). The mean genetic similarity of *Dasypyrum villosum* to *Secale* and *Triticum* species was calculated as 0.31 and 0.40.

On the dendrogram constructed on the basis of genetic similarities matrix, *Dasypyrum villosum* populations, *Secale* and *Triticum* species formed three main clusters (Fig. 1). In the *Triticum* cluster, two of the *T. aestivum* subspecies (ssp. *sphaerococcum* and ssp. *aestivum*) together with *Triticum turgidum* ssp. *dicoccoides*, formed a subcluster. Three subspecies of *S. cereale* (ssp. *afghanicum*, ssp. *ancestrale* and ssp. *cereale*) formed a subcluster in the main *Secale* cluster (Fig. 1).

4. Discussion

Dasypyrum villosum is commonly considered as a wild relative of wheat (Yuan & Tomita 2009). Many hybrids and lines between *Dasypyrum villosum* and different *Triticum* species were produced, and some resistance genes were transferred (Yildirim *et al.* 1998; Zhang *et al.* 2005). Common wheat – *Triticum aestivum* L. – is economically a very important bread cereal in the world, but rye – *Secale cereale* ssp. *sereale* belonging to *Secale* genus – is also important, especially for eastern and northern Europe. Many researchers found *D. villosum* phylogenically closer to *S. cereale* then to many other species of the *Triticeae*, including wheat (Lucas & Jahier 1988; Uslu *et al.* 1999; Hodge *et al.* 2010).

In this study, the genetic similarity between *Dasypyrum villosum* and *Triticum* and *Secale* species was examined for the first time using ISSR method. As expected, the detected polymorphism was very high (91.4%). The material studied was very different; furthermore, ISSRs are known as identifying a high level of polymorphism. Similarly, high polymorphism level of ISSRs was determined in rye (82%) (Fernández *et al.* 2002) and barley (83%) (Tams *et al.* 2004). In this study, polymorphism identified separately in the three genera was high in *Triticum* and *Secale* (68.3% and 48.5% respectively) and relatively small in *D. villosum* (22.1%). The obtained findings agreed with expectations, as different species and subspecies of *Triticum* and *Secale* were examined, and only one *Dasypyrum* species – *D. villosum*. Moreover, all *D. villosum* populations were native to Central Macedonia region of Greece.

The distribution of identified polymorphism is also reflected in PIC values. The mean value of this index for ISSR method was 0.7. In *Triticum* and *Secale* PIC was identical (0.62), and lower in *D. villosum* (0.50). In triticale (Shang *et al.* 2006) and in rice (Sarla *et al.* 2005) similar mean values of PIC were calculated with SSRs. In rice, Sarla *et al.* (2005) found even higher (in comparison with the results obtained in this study) mean PIC value with ISSRs – 0.82. However, they examined highly diverse material containing varieties, landraces, ancestral landraces and wild accessions. Within these groups, the PIC values were lower, and similar to those calculated in this study.

The results obtained were also reflected in Dice genetic similarity indices: mean values were at 0.93 in *D. villosum*, 0.80 in *Secale* and 0.67 in *Triticum*. On the dendrogram, the three genera studied were placed in individual main clusters. In the *Secale* cluster, the species grouped in accordance with common taxonomy (De Bustos & Jouve 2002). In the *Triticum* group, only *Triticum turgidum* ssp. d*icoccoides* did not cluster as expected. This tetraploid PI352328 (AB) was shown to be more similar to *Triticum aestivum* ssp. *aestivum* PI572994 (ABD), than two hexaploid species, *Triticum aestivum* ssp. *aestivum* (PI572994) and *Triticum aestivum* ssp. *sphaerococcum* (PI277142), to each other. *S. strictum* appeared as the least similar to the others with GS at 0.56. *Dasypyrum villosum* was shown to be more similar to *Triticum* than to *Secale* with the mean genetic similarity at 0.40 and 0.31, respectively. Thus, on the level of the sequences amplified between SSRs, *D. villosum* seems to have higher affinity to *Triticum* species, as has been shown so far with crossability, polymorphism of storage proteins and isozymes (Liu *et al.* 1995; Hodge *et al.* 2010). To confirm the findings obtained in this study, investigations including more *Triticum*, *Secale* and *Dasypyrum* species are conducted with the ISSR, RAPD, SSR and SRAP methods.

5. Conclusions

1. The ISSR technique is very polymorphic and informative and can be applied to the evaluation of genetic similarity between *Dasypyrum*, *Secale* and *Triticum*.
2. The genetic similarity of Greek populations of *D. villosum* from Central Macedonia analyzed was high.

3. Generally, in *Secale* and *Triticum* the species clustered in accordance with common taxonomy.
4. Dice genetic similarity indices calculated and topology of dendrogram constructed show a higher genetic similarity of *D. villosum* to *Triticum* than to *Secale* species.

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