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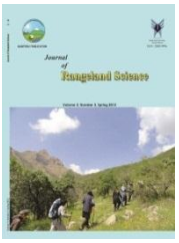

Full Length Article:

Effects of Selection on Genetic Parameters of *Secale montanum* Based on Seed Storage Protein Marker

Parvin Salehi Shanjani[A], Ali Ashraf Jafari[B], Roya Hoseinzadeh[C]

[A]Assistant Professor, Natural Resources Gene Bank, Research Institutes of Forests and Rangelands, Tehran, Iran. (Corresponding Author). E-mail: psalehi1@gmail.com
[B]Professor, Natural Resources Gene Bank, Research Institutes of Forests and Rangelands, Tehran, Iran.
[C]Azad University of Karadj, Karadj, Iran.

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Abstract. *Secale montanum* is one of the important perennial grasses growing naturally in arid to semiarid pastures and rangelands, with a typical Mediterranean climate, in northern and western Iran at altitudes of 800-2900 m. In this paper, seed storage protein profiles of nine wild populations of *S. montanum* from different regions of Iran and their phenotypically superior progenies as well as a multi-origin polycross (PLC) were studied. High levels of polymorphism were observed over all populations with the average number of bands and average heterozygosity. Superior progeny of different populations showed less genetic variability than wild parents in terms of band diversity, whereas PLC samples showed extremely high values of genetic parameters. Two locally common bands were observed in almost all wild parent populations, which are missing in superior progeny of different populations and PLC. These results provide highly support for the hypothesis that neutral genetic diversity has been reduced or inadvertently lost via artificial selection. Among wild parent populations and their superior progenies significant differences were observed in expected heterozygosity suggesting that more intensive breeding practices may have resulted in a further erosion of genetic variability. Neighbor-joining cluster analysis showed that wild populations and the phenotypically superior progeny of different populations were separated into two groups. This suggests that founder effects and subsequent selection have had more effect on the genetic differentiation between these accessions than geographical separation. This technology, seed storage protein profiling, has great potential for use in breeding programmes.

Key words: *Secale montanum*, Seed storage proteins, Superior progeny, Wild population

1. Introduction

Secale montanum Guss. (syn. *S. dalmaticum* Vis., common names: secale or mountain rye) is a diploid, outbreeding, perennial grass, widely distributed in central and southern Europe, northern Africa, Asia Minor, Transcaucasia, Iraq, Iran, and northern Pakistan (Davis, 1985). It normally inhabits dry, stony, or rocky hillsides, and as a weed in crops of *Triticum turgidum* Steud (Davis, 1985). *S. montanum* is one of the important perennial grasses naturally growing in arid to semiarid pastures and rangelands, with a typical Mediterranean climate, in northern and western Iran at altitudes of 800-2900 m. It is used for grazing and hay production as well as revegetating the overgrazed semi-steppe rangelands (Peymani-Fard, 1993). Because of its dense network of roots, *S. montanum* is recommended as a part of a seed mix for erosion control (Anderson and Brooks, 1975; Mclean and Clark, 1980). A few studies have been conducted on *S. montanum* in different ecological conditions of Iran and revealed that there was considerable variation in herbage yield, seed yield and crude protein content (Rahmani *et al.*, 2002). Although *S. montanum* is described as a palatable, leafy, short-lived, tufted perennial which can provide winter grazing in subtropical areas with fair winter rainfall (Rahmani *et al*., 2002), it has some troublesome characteristics including small seed size, shattering and pre-harvest sprouting (Gazanchian, 2006). Gordon-Werner and Dorffling (1988) have studied some of the mechanisms of drought tolerance in mountain rye. It is also used as a source of disease resistance genes (Andriyash, 1989) and cytoplasmic male sterility (Lapinski, 1991) in cereal rye breeding programmes in Eastern Europe. Attempts to breed a perennial grain type from hybrids between *S. cereale* and *S. montanum* were initially hindered by interchanges among three pairs of chromosomes, but eventually were successful (Reimann-Philipp, 1995).

The most frequent breeding methods applied to crop species involve different forms of mass selection, recurrent phenotypic selection and development of synthetic populations. Information about germplasm diversity and relationships among elite breeding materials is of fundamental importance in plant breeding (Hallauer and Miranda, 1988). This is especially true for species like *S. montanum* which suffers severe inbreeding depression (Geiger and Miedaner, 1999). However, there is neither information on the genetic quality of wild *S. montanum* populations nor information on the progeny to be used in breeding programmes. Reports of studies based on different plant species provide conflicting results on the impact of domestication on the genetic diversity of populations (Chaisurisri and El Kassaby, 1994; Rajora, 1999; Moran *et al.*, 2000; Godt *et al.*, 2001; Icgen *et al.*, 2006). Also the impact of domestication on the genetic diversity of progeny populations is poorly understood (Stoehr and El-Kassaby, 1997; Schmitdtling and Hiplins, 1998). Such studies on genetic diversity of initial selection materials are essential for successful breeding and creation of new cultivars.

S. montanum has been studied by morphological (Fredriksen and Petersen, 1998; Rahmani *et al*., 2002; Oram, 2013), cytological (Katsumasa *et al*., 1990; Petersen and Doebley, 1993, Petersen *et al*., 2004; Cuadrado and Jouve, 1997, 2002; Riley, 1955; Sheidai, 2008) isozymes (Vence *et al*., 1987a, b), restriction fragment length polymorphisms of plastid genome (cpDNA RFLPs) (Murai *et al*., 1989), restriction fragment length polymorphisms of mitochondrial DNA (mDNA RFLPs) (Skizai *et al*., 2007), rDNA spacer-lengths (Reddy *et al.*, 1990), ITS of the 18 s-5.8 s rDNA (De Bustos and Jove, 2002), Random

Amplified Polymorphic (RAPDs) (Del Pozo *et al.*, 1995), Amplified Fragment Length Polymorphisms (AFLPs) (Chikmawati *et al.*, 2005), ISSR and SCAR markers (Vaillancourt *et al.*, 2008) and microsatellite (Shang *et al.*, 2006; Jenabi *et al.*, 2011) analyses. The genetic structure of the Iranian *S. strictum* populations, however, still remains unclear despite its usefulness as a genetic resource.

Characterization of germplasm using biochemical fingerprinting has got special attention. The protein profiling of germplasm and use of genetic markers have been widely and effectively used to determine the taxonomic and evolutionary aspects of several crops (Murphy *et al.*, 1990; Khan, 1990; Das and Mukarjee, 1995; Ghafoor *et al*., 2002). Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is most economical, simple and extensively used biochemical technique for analysis of genetic structure of germplasm. Leaf total proteins have been used as genetic markers in breeding programs (Hirano, 1982; Vries, 1996; Kamel and Hassan, 2001; Reddy and Munirajappa, 2005; Mohamed *et al*., 2006), as well as in basic studies on population genetics (Torkpo *et al*., 2006) and reproductive biology (Cardeña *et al*., 1998). To our knowledge, no studies have yet been made in Iran on the diversity of *S. montanum* germplasm based on total protein electrophoresis. We therefore address two issues in the present study: (i) the structure of genetic diversity in different wild *S. montanum* populations, and (ii) the impact of selection on genetic diversity of progeny populations using seed storage proteins.

2. Materials and Methods

Seed material of nine wild populations of *Secale montanum* (accession No.: 467, 1275, 2283, 2292, 1549, 591, 2382, 1567 and 941) and their phenotypically superior progenies as well as the multi-origin polycross (PLC), provided from Iranian Natural Resources Gene Bank (INRGB), were used in the present study (a total of 190 entries).

For study of the extent of genetic variation based on SDS-PAGE markers, a total of 190 entries were selected from nine wild populations, their superior progenies and PLC (10 plants for each population). Preliminary experiments (data not shown) indicated that a larger sample (20 plants for each population) did not modify the results substantially regarding the amount or the structure of polymorphism. Seed storage proteins were extracted using 0.05M Tris-HCL pH=8, 0.2% SDS, 5M urea, 1% B-mercaptoethano l.

Electrophoresis was carried out in the discontinuous Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) system of Laemmli (1970) using 12% (w/v) separating gel and 5% (w/v) stacking gel (Fig. 1). The molecular weights of the dissociated protein were estimated by using molecular weight standard proteins "MW-SDS-70 Kit". Gels were gently shaken until the background of the gel became clear and polypeptide bands were clearly visible.

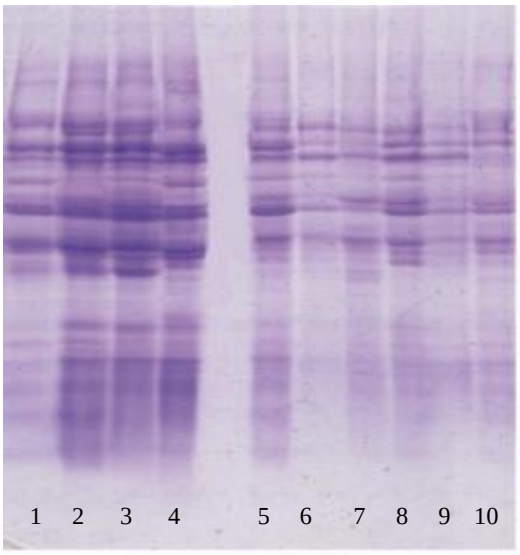

Fig. 1. Seed storage protein band profiles of the multi-origin polycross (PLC) samples (1-4) and the superior progenies (5-10) of *S. montanum*

2.1. Data analysis

For protein profile data, to avoid taxonomic weighting, the intensity of bands was not taken into consideration, only the presence of bands was taken as indicative. The scores were 1 for the presence and 0 for the absence of a band. Then, the indices of genetic diversity, such as the number of bands (Na), locally common bands with frequency ≤ 25% (Nalc), Percentage of Polymorphic Loci (PPL) and expected heterozigosity (He), were calculated using POPGENE 32 software (Yeh *et al*., 1999) on the basis of gene frequencies. At the same time, the genetic structure within and among populations were detected using the software AMOVA- PREP 1.01 (Miller, 1997) and WINAMOVA (Excoffier, 1995) in order to partition the genetic variation among local and exotic groups, among populations within groups and among individuals within populations. The significance of each variance component was tested with permutation tests (Excoffier *et al*., 1992). Genetic distances were estimated according to Nei (1978) and the resulting similarity matrix was subjected to Principal Component Analysis (PCA), UPGMA algorithm using NTSYS-pc 2.01 (Rohlf, 2004), and Neighbor- Joining (NJ) analysis using MEGA4 software (Tamura *et al*., 2007). Wright's Fst and Nm were used to estimate population differentiation and gene flow, respectively. The rate of gene flow was estimated indirectly from the proportion of total diversity that was found among populations (Wright, 1931, 1951). A 999 random permutation Mantel test (Gower 1966) was used to assess the correlation between the calculated distance matrices.

3. Results

On the basis of the relative mobility of total proteins on the gel, 32 polypeptide bands of different sizes ranging from 6.606 to 269.153 kDa, from nine wild parent populations, their superior progenies and PLC samples of *S. montanum*, were identified. Different populations showed quite different band frequency distributions among wild parent, progeny and PLC genotypes (Fig. 2). Among the nine wild populations, the mean PPL and He values were 62.50% and 0.253, respectively. Population p-Bojnurd (Na, PPL and He values: 31, 81.25% and 0.345, respectively) and p-Zanjan1 (Na, PPL and He values: 32, 87.50% and 0.332, respectively) had the highest level of variability, whereas population p-Zanjan3 had the lowest level of variability (Na, PPL and He values: 27, 18.75% and 0.079, respectively) (Table 1). Among the superior progenies, the mean *PPL* and He values were 56.45% and 0.221, respectively. Within the superior progenies, PPL value ranged from 37.50% (s-Karaj3) to 71.88% (s-Karaj1), He value ranged from 0.116 (s-Esfahan) to 0.71.88 (s-Zanjan) (Table 1). The PPL and He values of PLC (93.75% and 0.409, respectively) were higher than all the wild parent and superior populations. Almost in all wild populations (except p-Zanjan3) two locally common bands (with frequency ≤ 25%) were observed, missing in superior progeny of different populations and PLC. Comparison of different wild populations with their superior progenies showed significant decrease in the genetic parameters of superior progenies in the most populations.

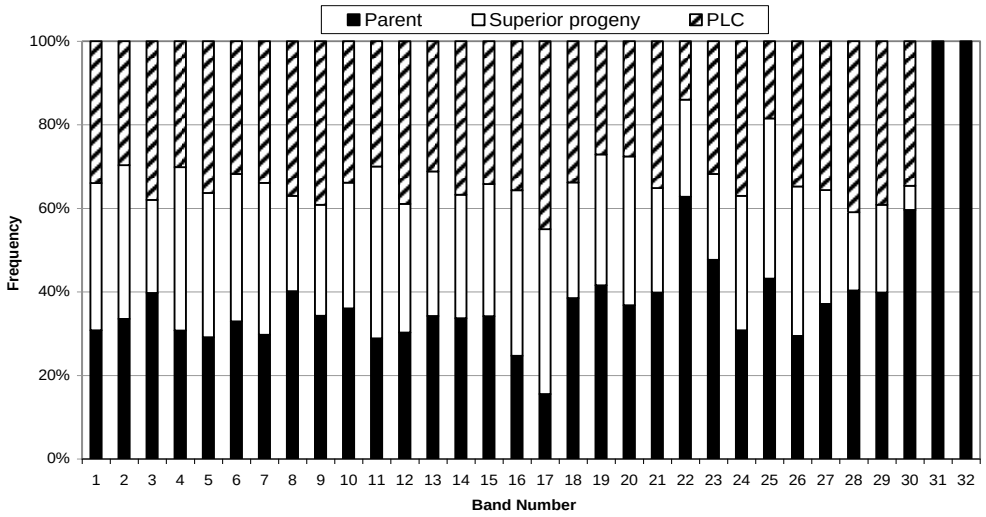

Fig. 2. Seed storage protein band frequencies of the wild parent, the superior progenies and multi-origin polycross (PLC) samples of *S. montanum*

Table 1. Genetic diversity parameters of the wild parent populations, the superior progenies of different populations and PLC of *S. montanum*

	Na		Nalc		PPL		He	
	Parent	Superior Progeny	Parent	Superior Progeny	Parent	Superior Progeny	Parent	Superior Progeny
467	32	25	2	0	87.50	68.75	0.332	0.311
1275	32	29	2	0	59.38	68.75	0.256	0.274
2283	27	25	0	0	18.75	50.00	0.079	0.217
2292	32	29	2	0	71.88	68.75	0.319	0.285
1549	31	26	2	0	81.25	46.88	0.345	0.190
591	31	27	2	0	62.50	37.50	0.250	0.163
2382	31	29	2	0	78.13	71.88	0.293	0.265
1567	32	22	2	0	46.88	43.75	0.181	0.116
941	32	27	2	0	56.25	50.00	0.222	0.166
Mean					62.50	56.45	0.253	0.221
PLC		30		0		93.75%		0.409

Na = observed number of bands; Nalc = Number of locally Common Bands (frequency <= 50%); He = Nei's gene diversity; PPL = Percentage of polymorphic loci

The coefficient of genetic differentiation (Fst) among wild parent populations was 0.3 (31% of total genetic variation resided among, and 69% within populations). Among the superior progenies of different populations, Fst was 0.360. The level of gene flow (Nm) between wild parent populations was 1.373. Fst between wild parent populations and their superior progenies was 0.363; indicating only about 20% genetic variation resided among the three groups (wild parents, superior progenies and PLC). The level of gene flow (Nm) between superior progenies and their wild progenitors was 0.714 individuals per generation. AMOVA analysis showed that the variation among the wild parent populations and within the populations accounted for 31% and 69% of the total variation, respectively ($P < 0.01$) (Table 2). In the progeny data set, these values were 38% and 62% ($P<0.01$), respectively. AMOVA analysis also revealed a highly significant genetic differentiation ($P < 0.01$) among the wild parent populations and their progenies. Of the total genetic diversity, 20% was attributable to between-group diversity and the rest (80%) to differences within groups (Table 2).

Table 2. Analysis of Molecular Variance (AMOVA) of the wild parent populations, the superior progenies of different populations and PLC of *S. montanum* based on total protein profile

Source	Degrees of Freedom	Sum of Squares	Mean of Squares	Est. Var.	Variation %	P
Among groups (parents, superior progenies and PLC)	2	158.354	79.177	1.412	20%	
Within groups	187	1044.567	5.586	5.586	80%	0.001
Among parent populations	8	180.578	22.572	1.851	31%	
Within parent populations	81	329.000	4.062	4.062	69%	0.010
Among superior progeny populations	8	199.289	24.911	2.140	38%	
Within superior progeny populations	81	284.100	3.507	3.507	62%	0.010

The genetic distance of the populations using Nei's genetic distances is shown in (Table 3). The data ranged from 0.041 (P-1275 and P-2292) to 0.703 (P-1567 and S-1567), with an average of 0.279 (Table 3). To elucidate the genetic relationships among wild parent populations, superior progeny populations and PLC samples, an UPGMA dendrogram was produced using Nei's genetic distances (Fig. 3). The nine parent populations and their superior populations were grouped into three clusters, cluster I having 11 populations including PLC, the superior progenies of almost all populations and two wild parent populations, cluster II consisted of wild parent populations, and cluster III, having only one population (S-1567).

The total protein data were also used for conducting Principal Coordinate Analysis (PCoA) to study further the genetic diversity among the nine wild parent *S. montanum* populations and their progenies (Fig. 4). The results of the PCA showed that most of the wild populations were clearly separated from their progenies (Fig. 4). The first three principal coordinates accounted for 80% of the total variation among the populations or progenies.

Correlation coefficients among pairwise genetic distance matrices of wild parent samples and their superior progenies were calculated using mantel's test. Regression and correlation analysis between genetic distances showed very low correlation, but significant ($p < 0.04$) (Fig. 5).

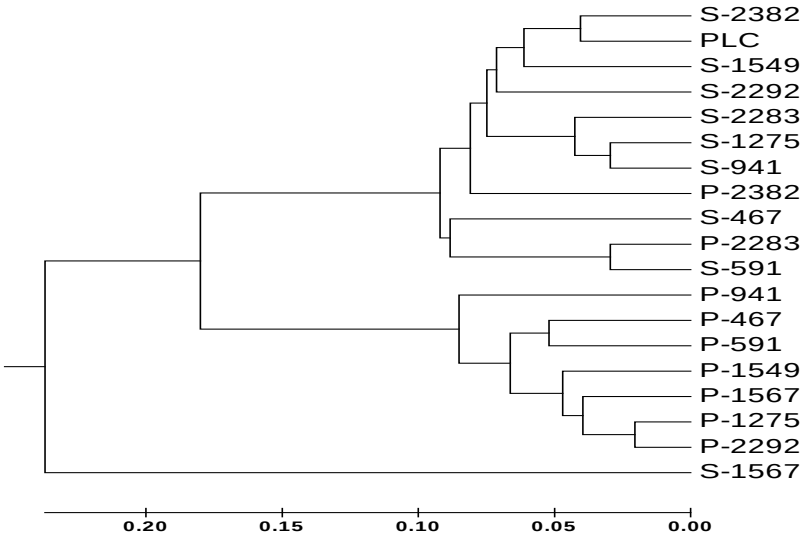

Fig. 3. Phenograms of the wild parent populations (with P prefix), the superior progenies of different populations (with S prefix) and multi-origin polycross (PLC) of *S. montanum* based on seed storage protein profiles

Table 3. Pairwise values for Nei's genetic distances of the wild parent populations (with P prefix), the superior progenies of different populations (with S prefix) and PLC of *S. montanum*

	P-467	P-1275	P-2283	P-2292	P-1549	P-591	P-2382	P-1567	P-941	S-467	S-1275	S-2283	S-2292	S-1549	S-591	S-2382	S-1567	S-941
P-467	0.000																	
P-1275	0.175	0.000																
P-2283	0.398	0.334	0.000															
P-2292	0.126	0.041	0.302	0.000														
P-1549	0.096	0.077	0.364	0.097	0.000													
P-591	0.104	0.087	0.370	0.074	0.104	0.000												
P-2382	0.275	0.287	0.213	0.257	0.251	0.280	0.000											
P-1567	0.224	0.066	0.329	0.092	0.108	0.173	0.320	0.000										
P-941	0.186	0.156	0.321	0.153	0.142	0.186	0.266	0.197	0.000									
S-467	0.197	0.073	0.307	0.101	0.126	0.157	0.341	0.122	0.143	0.000								
S-1275	0.179	0.108	0.305	0.104	0.139	0.157	0.268	0.133	0.144	0.089	0.000							
S-2283	0.318	0.334	0.405	0.388	0.316	0.392	0.347	0.343	0.274	0.219	0.239	0.000						
S-2292	0.331	0.210	0.277	0.243	0.253	0.306	0.303	0.242	0.198	0.137	0.076	0.125	0.000					
S-1549	0.314	0.133	0.203	0.118	0.236	0.227	0.288	0.131	0.156	0.156	0.115	0.383	0.162	0.000				
S-591	0.240	0.122	0.279	0.136	0.204	0.192	0.352	0.146	0.197	0.043	0.098	0.193	0.135	0.157	0.000			
S-2382	0.373	0.260	0.353	0.276	0.339	0.356	0.284	0.331	0.264	0.171	0.127	0.195	0.077	0.197	0.190	0.000		
S-1567	0.224	0.163	0.275	0.168	0.201	0.230	0.295	0.189	0.117	0.122	0.130	0.173	0.118	0.118	0.129	0.132	0.000	
S-941	0.287	0.239	0.419	0.227	0.304	0.272	0.300	0.303	0.232	0.211	0.097	0.252	0.141	0.293	0.159	0.212	0.251	0.000
PLC	0.222	0.246	0.148	0.243	0.217	0.254	0.095	0.282	0.224	0.204	0.078	0.130	0.100	0.091	0.179	0.081	0.361	0.142

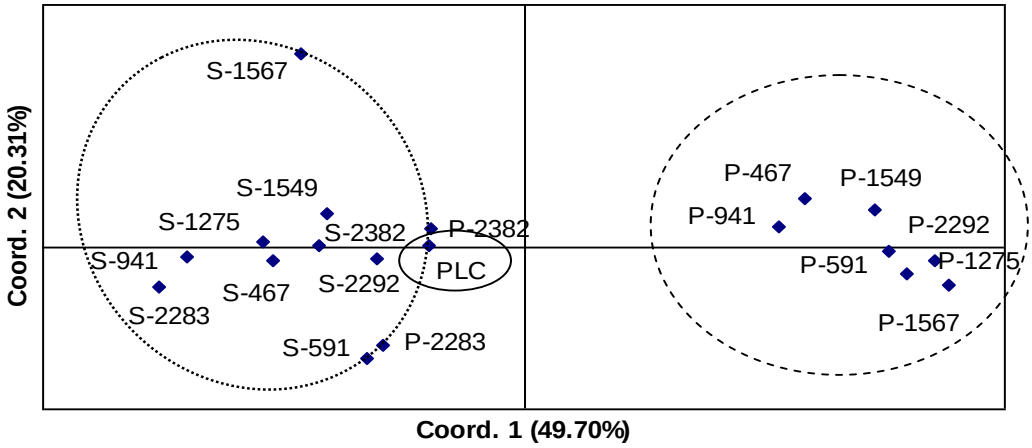

Fig. 4. Two-dimensional graph based on the ordination scores of the principal coordinate analysis of the wild parent populations (with P prefix) and the superior progenies of different populations (with S prefix) and PLC of *S. montanum*

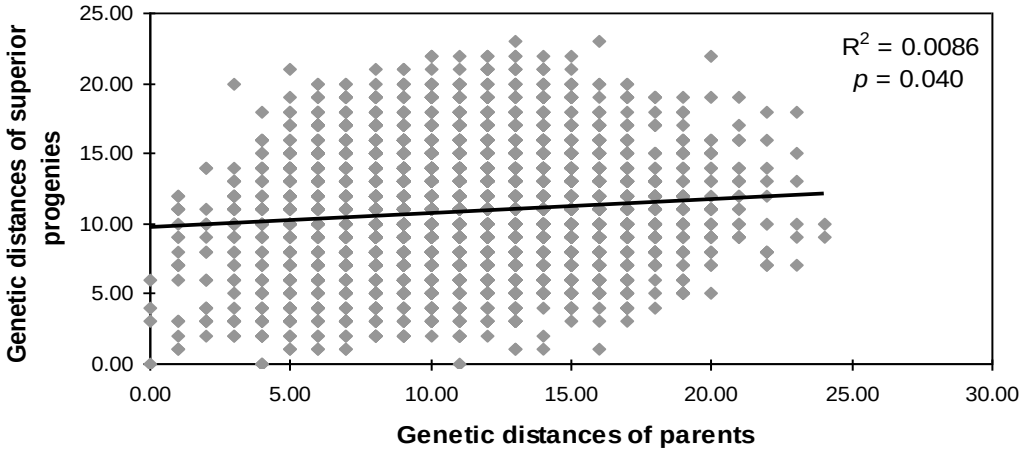

Fig. 5. Correlations between genetic distances among the wild parent populations and the superior progenies of different populations of *S. montanum*

4. Discussion

Loss of genetic diversity and increased population differentiation from source populations are common problems associated with breeding programmes established from a small number of founders. Like many other studied plant species where cultivars have lower genetic diversity than their wild relatives (Doebley, 1989; Gepts, 2004; Zhou *et al*., 2005; Miller and Schaal, 2006; Wu *et al*., 2006; Salehi Shanjani *et al*., 2013), the superior progenies of different *S. montanum* populations maintain lower levels of genetic diversity as their parents (mean He value for wild parent populations was significantly higher than that of their progenies). The heterozygosity of PLC samples was higher compared to all wild parent or superior progeny populations. Despite the retention of genetic diversity in PLC, a detectable shift in gene frequency was revealed by the distribution of band frequencies. These results demonstrates that artificial breeding practices result in a decrease in genetic variability in terms of band diversity but which is not necessarily detectable from levels of heterozygosity.

Selective breeding often produces an improvement in phenotype. Artificial selection can separate adult individuals from a parent generation into two groups, those selected and those to be discarded, based on the characteristics that are determined by the changes in the gene frequency (Li *et al*., 2010). This has been

confirmed in many species, such as Bluebunch Wheatgrass (Larson *et al*., 2000), maze (Labate *et al*., 1997; Zou *et al*., 2010), Cassava (Oslen and Schall, 1999; Manu-Aduening *et al*., 2013) and rice (Virk *et al*., 2003). In the present study, significant genetic differentiation among the wild parent populations and their superior progenies is also due to band frequency alterations. The most striking change in band frequencies is the loss of low frequency bands, which is proved to be a common phenomenon in cultivars as a consequence of small population size, genetic drift, and selection (Gosling, 1982; Dillon and Manzi, 1987; Launey *et al*., 2001; Zhang *et al*., 2004). The major reason for the genetic differentiation between the wild parent populations and their progenies in this study appears to be artificial selection as the superior progenies have been extensively selected. This investigation further demonstrates that gene frequency change is the genetic basis of character improvement in selective breeding.

Genetic diversity is always changing, but the report on the state of the worlds plant genetic resources (FAO, 1996), summarizing country reports, suggests that "recent losses of diversity have been large, and that the process of erosion continues." It points out that while loss of genes is of particular concern, loss of gene complexes and unique combinations of genes (as in different landraces) can also have important consequences. Genetic erosion may thus be defined as a permanent reduction in richness or evenness of common localized alleles or the loss of combination of alleles over time in a defined area. This definition recognizes that diversity has two distinct components in (i) the number of different entities and (ii) their relative frequencies. It also suggests that it is specifically loss of locally adapted alleles that is most significant. Two locally common bands were detected in almost all wild parent populations, which is very important part of genetic diversity, are missing in superior progenies and PLC. This process considered as genetic diversity erosion. Genetic erosion will be detrimental to the short-term viability of individuals and populations, the evolutionary potential of populations and species, and the direct use of genetic resources (Brown *et al*., 1997). Recent genetic erosion and/or the risk of imminent genetic erosion are key factors in determining the priority given to different areas for conservation interventions whether ex situ, in situ or a combination of both.

In conclusion, this study demonstrates the high levels of polymorphism detectable with seed storage proteins even within superior progenies of *S. montanum*. This technology has the potential to be of great use in monitoring levels of genetic variation within wild populations as well as for parentage and relatedness purposes. Between wild parent populations and their progenies significant differences were observed in expected heterozygosity suggesting that more intensive breeding practices may have resulted in a further erosion of genetic variability. These results also show that allelic diversity is a more sensitive measure of differences in genetic variation between wild and progeny populations than overall heterozygosity. This is likely to be a result of the loss of low frequency alleles when new populations are created from larger founder ones. These results provide highly support for the hypothesis that neutral genetic diversity has been reduced or inadvertently lost via artificial selection. Neighbor-joining cluster analysis showed that wild populations and the phenotypically superior progeny of different populations were separated into two groups. This suggests that founder effects and subsequent selection have had more effect on the genetic

differentiation between these accessions than geographical separation. Differences in genetic variation observed among superior progenies may be a result of geographical separation of their parent populations. This technology has great potential for use in breeding programmes.

5. Acknowledgement

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اثر انتخاب بر پارامترهای ژنتیکی ***Secale montanum*** بوسیله مارکر پروتئین‌های ذخیره‌ای بذر

پروین صالحی شانجانی، استادیار، بانک ژن منابع طبیعی ایران، موسسه تحقیقات جنگلها و مراتع کشور، تهران، ایران (نویسنده مسئول)
علی اشرف جعفری، استاد، بانک ژن منابع طبیعی ایران، موسسه تحقیقات جنگلها و مراتع کشور، تهران، ایران
رویا حسین‌زاده، دانش‌آموخته کارشناسی ارشد، دانشگاه آزاد اسلامی واحد کرج، ایران

چکیده

Secale montanum یکی از گیاهان علوفه‌ای چندساله است که به صورت طبیعی در مراتع خشک و نیمه خشک ارتفاعات ۸۰۰-۲۹۰۰ متری شمال و غرب ایران رویش دارد. در این مقاله الگو پروتئین‌های ذخیره‌ای بذر نه جمعیت وحشی ***S. montanum*** به همراه نتاج برتر و نمونه پلی‌کراس آنها مطالعه شدند. نتایج نشان دادند که جمعیت‌های ***S. montanum*** دارای میانگین تعداد باند، هتروزیگوسیتی و پلی‌مورفیسم بالایی می‌باشند. گوناگونی باندهای نتاج برتر جمعیت‌های مختلف کمتر از والدهای وحشی‌شان بود، درحالیکه پارامترهای ژنتیکی نمونه پلی‌کراس بسیار بیشتر از والدهای وحشی می‌باشد. دو باند عمومی مختص به محل در الگو پروتئین‌های ذخیره‌ای بذر تقریباً تمام جمعیت‌های وحشی مشاهده گردید که در نتاج برتر جمعیت‌های مختلف و نمونه پلی‌کراس وجود نداشت. این نتایج از فرضیه اثر انتخاب مصنوعی در کاهش تنوع ژنتیکی حمایت می‌کند. وجود اختلاف معنی‌دار در هتروزیگوسیتی مورد انتظار بین جمعیت‌های وحشی و نتاج برترشان نشان می‌دهد که هرچه عملیات اصلاحی وسیعتر باشد فرسایش تنوع ژنتیکی نیز بیشتر می‌شود. دندروگرام حاصل از تجزیه کلاستر Neighbor-Joining نشان داد که جمعیت‌های وحشی و نتایج برترشان در دو گروه جداگانه قرار می‌گیرند. این نتایج نشان می‌دهد که اثر تعداد محدود والد و انتخاب‌های بعدی بیش از جدایی جغرافیایی در تمایز ژنتیکی نقش دارد. نتایج این پژوهش نشان داد که مارکر پروتئین‌های ذخیره‌ای بذر پتانسیل بالایی برای استفاده در برنامه‌های اصلاحی دارد.

کلمات کلیدی: ***Secale montanum***، پروتئین‌های ذخیره‌ای بذر، جمعیت‌های وحشی، نتاج برتر