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Genetic Diversity and Differentiation of Secale strictum Accessions Based on Phenotypic Traits and Seed Storage Protein Profiles

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Abstract

The genetic diversity of nine wild Secale strictum accessions was studied using seed storage protein profiles and phenotypic traits. Offsprings and phenotypically superior offsprings of the nine wild accessions were also evaluated and compared with their parental accessions to assess their genetic variability based on seed storage proteins and seed germination parameters. High genetic variation was observed for both seed storage protein profiles and phenotypic traits. The protein banding data were investigated in relation to phenotypic traits and indicated no influence of polymorphic bands on quantitative traits. Seeds of superior offsprings showed less genetic variability than both wild and offsprings of S. strictum accessions suggesting that more intensive breeding practices may have resulted in a further erosion of genetic variability. Neighbor-joining cluster analysis showed that wild populations, offspring and phenotypically superior offspring accessions were separated into three groups. This suggests that founder effects and subsequent selection have had more effect on the genetic differentiation among these accessions than geographical separation. The results demonstrated that the study of genetic diversity and differentiation between the parents and their offspring using seed storage protein profiles provides important information for the breeding and conservation of S. strictum germplasm.

Keywords: Secale strictum; Phenotypic traits; Differentiation; Genetic diversity; Seed storage proteins.

Introduction

Secale is a member of the Triticeae tribe in the grass family, Poaceae (syn. Gramineae). *Secale* is a small but important cereal genus that includes cultivated rye (*Secale cereale* L.) and several wild species [44]. *Secale strictum* C. Presl (mountain rye) is a wild perennial, diploid and outbreeding species. It is believed to have been the ancestor of annual cultivated ryes. *S. strictum* is found in dry mountainous areas, roadsides and

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cultivated field margins. Its range spans from southern Spain in the west to the Caspian Sea in the east. *S. strictum* is one of the important perennial grasses that naturally grows 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 overgrazed sub-steppic rangelands [34]. Because of its dense network of roots, *S. strictum* is recommended as a part of a seed mix for erosion control [3, 4]. A few studies have been conducted on *S. strictum* in different ecological conditions of Iran and revealed that there was considerable variation in herbage yield, seed yield and crude protein content [35]. Although *S. strictum* described as a palatable, leafy, short-lived, tufted perennial, which can provide winter grazing in subtropical areas with fair winter rainfall [35], it has some troublesome characteristics including small seed size, shattering and pre-harvest sprouting [16].

The wild and weedy rye species (*Secale* spp.) constitute a reserve of genetic diversity that has been underutilized for crop improvement. 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 [21, 37]. This is especially true for species like *S. strictum* which suffers severe inbreeding depression [44]. However, there is neither information on the genetic quality of wild *S. strictum* accessions nor the progeny may be used in breeding programs. Reports of studies based on different plant species provide conflicting results on the impact of domestication on the genetic diversity of populations [19, 29, 31, 49]. The impact of domestication on the genetic diversity of progeny populations is also poorly understood [19, 43]. Such studies on genetic diversity of initial selection materials are essential for successful breeding and creation of new cultivars.

Knowledge of genetic variability and relationships among traits are necessary for facilitating the transfer of useful genes and maximizing the use of available germplasm resources. The extent of genetic diversity in germplasm can be assessed through morphological characterization and genetic markers. The characterized material then helps the plant breeders to select the accessions to be utilized in hybridization program [15]. Variations in *S. strictum* has been studied morphologically [6, 19, 44, 49], cytologically [2, 22, 38, 41] and through isozymes [47, 48], and DNA based markers [9, 20, 36]. The genetic structure of the Iranian *S. strictum* accessions, however, still remains unclear despite its usefulness as a genetic resource.

Electrophoresis (SDS-PAGE) is widely used to describe seed storage protein diversity of crop germplasm [8]. This method can also be used as a promising tool for distinguishing cultivars of particular crop species. However, a few studies indicated that SDS-PAGE method was not efficient for cultivar identification [51]. To our knowledge, no studies have yet been made in Iran on the diversity of *S. strictum* germplasm based on seed storage protein electrophoresis, and its association with phenotypic traits. So the present study aimed to evaluate and compare the genetic structure of nine wild accessions of *S. strictum*, using neutral and potentially selective markers. It is generally much easier to characterize differences among accessions for molecular markers than for agronomical important traits. Because molecular markers are considered, a priori, neutral, while agronomic traits are most likely to be under selection even in natural populations, it is interesting to compare the inferences one can make from observations on these two kinds of traits.

In this study, we tried to i) estimate genetic variation in wild accessions of *S. strictum*; ii) study the pattern of differentiation among wild, offspring and phentypically superior offspring accessions based on total protein profiles; and iii) analyze seven quantitative traits in order to compare neutral and quantitative variation and thus evaluate the role of natural selection in the maintenance of morphological integrity in wild accessions.

Materials and Methods

Seed Material Experiment Layout

Seed material of nine wild accessions of *Secale strictum* from different regions of Iran, provided from Iranian Natural Resources Gene Bank, Research Institute of Forests and Ranglands (RIFR), was used in the present study. The research was conducted on the experimental field at the RIFR. A total of 30 seedlings of each wild accession were grown in jiffy pots for forty days before transplanting into a field in October 2008. The field trial was arranged in a randomized complete block with three replications. Each plot included 36 spaced plants (0.40 x 0.40 m). Fertilizer application rates were 100 kg/h phosphorus (P) at sowing. The field was irrigated once a week during summer. No measurements were taken in the establishment year.

During the two-year investigation (2009 and 2010), seven phenotypic traits were observed in this research.

The data were collected and analyzed for the following seven phenotypic traits: harvesting index, stem number, grain yield (kgh^{-1}), dry matter yield (th^{-1}), plant height (cm), day to pollination and day to heading. The data presented here are average values over two years.

The seeds of 27 *S. strictum* accessions (the nine wild parent, nine offspring and nine phonotypically superior offspring accessions) were tested for germination characteristics. The normal ISTA (1993) laboratory germination test procedure was used with three replications. Seeds (150) of each accession were sterilized with 70% ethyl alcohol for five minutes, and then washed with distilled water. Three replicates (50 seeds per replicate) of sterilized seed were placed in Petri dishes on double Whatman papers (TP). For protection against moulds, the water used to moisten the seed samples and substrata contained 0.002% Benomyl fungicide. The samples were transferred into a germinator at (20±4°C) with 1000 lux light for 15 days. The percentage and speed of germination were recorded at 3, 6, 9, 12 and 15 days. The length of roots and shoots (mm) of 10 randomly-selected seedlings (15-day old) from each replicate were measured. After measuring shoot and root lengths, the caryopses were cut from the seedlings and fresh seedling weights of each replicate were recorded. The seedlings were then placed in an oven at 80°C for 24 hours, after which the dry weight of each replicate was recorded as a percentage of the fresh weight. Seed vigour index was calculated by multiplying germination (%) and seedling length [1].

Seed Storage Protein Analysis

In this study the extent of genetic variant was based on SDS-PAGE markers. A total of 560 *Secale strictum* genotypes were analysed: 180 seeds from nine wild *S. strictum* accessions (each accession 20 seeds); 180 seeds from offsprings of the nine wild accessions (each accession 20 seeds), and 180 seeds from superior offsprings of the nine wild accessions (each accession 20 seeds). Preliminary experiments (data not shown) indicated that a larger sample (30 plants for each accession) did not modify the results substantially regarding the amount or the structure of polymorphism. Seed storage proteins were extracted from seeds using protein extraction 0.05M Tris-HCL pH=8, 0.2% SDS, 5M urea, 1% B-mercaptoethanol. Electrophoresis was carried out in the discontinuous Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) system of Laemmli [23] using 12% (w/v) separating gel and 5% (w/v) stacking gel. 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.

Data Analysis

Analysis of variance was computed on collected data for each trait for phenotypic traits. The descriptive statistics and phenotypic correlation coefficients between traits were estimated using the SAS9.1 software. Seven classification variants had significant ($P \leq 0.01$) variation among accessions and were subsequently used for multivariate analysis. The Euclidean distances of accessions were computed on phenotypic traits and then used for the UPGMA cluster analysis method using NTSYS-PC software [39].

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. The indices of genetic diversity, such as the percentage of polymorphic loci (*PPL*) and expected heterozygosity (*He*), were calculated using POPGENE 32 software [55] on the basis of gene frequencies. At the same time, the genetic structure within and among accessions were detected using the software AMOVA-PREP1.01 [30] and WINAMOVA [10] in order to partition the genetic variation among local and exotic groups, among accessions within groups and among individuals within accessions. The significance of each variance component was tested with permutation tests [11]. Genetic distances were estimated according to Nei [32] and the resulting similarity matrix was subjected to principal component analysis (PCA), UPGMA algorithm using NTSYS-pc 2.01 [39], and neighbor-joining (NJ) analysis using MEGA4 software [45]. Wright's *Fst* was used to estimate three datasets differentiation [52, 53]. A 999 random permutation Mantel test [17] was used to assess the correlation between the calculated distance matrices (using phenotypic and total protein profile data). The Pearson correlation among the genetic index within accession, phenotypic traits and ecological factors was analyzed using the SPSS 11.0 software.

Results

Genetic Diversity among Wild Accessions

ANOVA suggested significant differences among nine wild accessions of *S. strictum* for all the seven phenotypic traits. High CV values were obtained for grain yield and harvesting index (Table 1). Pearson correlation showed a positive relationship between day to pollination with day to flowering and stem number; and a negative correlation with plant height, grain yield

Table 1. Evaluation of data on seven phenotypic traits and genetic parameters in nine wild parent populations of *S. strictum*

	Phenotypic traits							Genetic parameters		
	Day to pollination	Day to heading	Plant height (cm)	Stem number	Grain yield ($kg.h^{-1}$)	Dry matter yield ($t.h^{-1}$)	Harvesting index	*Na*	*PPL*	*He*
Zanjan1	73.27ab	61.3a	55.53e	44.31b	250.67b	7.14cde	4.16bc	32	87.50	0.332
Zanjan2	75.58a	63.19a	62.47d	55.10a	265.00b	8.19b	3.73c	32	59.38	0.256
Zanjan3	70.07b	57.933b	64.09cd	58.41a	187.33bc	7.58cb	3.00cd	27	18.75	0.079
Zanjan4	76.43a	64.09a	54.03e	53.89a	120.67c	7.40bcd	1.81d	32	71.88	0.319
Bojnurd	63.04c	51.508c	72.98a	56.40a	547.00a	10.42a	5.74b	31	81.25	0.345
Karaj3	60.18c	48.405d	69.02b	45.39b	617.00a	7.98b	8.75a	31	62.50	0.250
Karaj1	52.91d	35.15e	66.15bc	35.25c	581.00a	6.14f	9.17a	31	78.13	0.293
Karaj2	53.00d	34.517e	67.46b	31.22c	252.17b	6.72def	4.71bc	32	46.88	0.181
Esfehan	70.61b	53.318c	66.41bc	51.17ab	249.83b	6.55ef	4.21bc	32	56.25	0.222
CV	4.11	4.74	3.83	11.70	26.01	8.08	29.13			

Table 2. Pearson correlation analysis for the relationships between phenotypic parameters of nine wild parent populations of *S. strictum*

	Day to pollinatin	Day to heading	Plant height	Stem number	Grain yield	Dry matter yield
Day to heading	0.977					
p value	0.0001					
Plant height	-0.661	-0.606				
p value	0.052	0.084				
Stem number	0.758	0.811	-0.112			
p value	0.018	0.008	0.775			
Grain yield	-0.652	-0.538	0.687	-0.276		
p value	0.057	0.135	0.041	0.473		
Dry matter yield	0.166	0.317	0.381	0.591	0.294	
p value	0.67	0.406	0.312	0.094	0.442	
Harvesting index	-0.759	-0.689	0.593	-0.524	0.937	-0.047
p value	0.018	0.04	0.093	0.148	0.0001	0.905

and harvesting index. Day to heading positively correlated with stem number, while a negative value was obtained between day to heading and harvesting index. Grain yield positively correlated with both plant height and harvesting index (Table 2).

Genetic distance among nine wild *S. strictum* entries was also estimated using data on seven phenotypic traits using Euclidean distances, which ranged from 1.306 (between Karaj1 and Karaj3) to 7.60 (between Karaj2 and Bojnurd) with an average value of 3.58 (Table 3). The Euclidean distances matrix was subjected to agglomerative hierarchical clustering utilizing UPGMA method to construct a dendrogram (Fig. 1a). Nine entries of *S. strictum* were classified into two groups (Fig. 2a), suggesting no relationship between phenotypic traits and the origin of these *S. strictum* accessions.

On the basis of the relative mobility of seed storage proteins on the gel, 32 polypeptide bands of different sizes ranging from 6.606 to 269.153 kDa, from nine accessions of *S. strictum*, were identified. The percentages of polymorphic bands over the total bands detected ranged from 18.75% (Zanjan3) to 87.50% (Zanjan1) with an average of 62.50% (Table 4). Assuming Hardy-Weinberg equilibrium, the value of Nei's genetic diversity (*He*) ranged from 0.079 (Zanjan3) to 0.345 (Bojnurd) (Table 1). High polymorphism was found within accessions and the probability that two randomly sampled polypeptides in a given accessions are different was 25.3% (*He* = 0.253). The pairwise values for Nei's genetic distances between the analysed accessions ranged from 0.041 (between Zanjan2 and Zanjan4) to 0.398 (between Zanjan1 and Zanjan3), with an average of 0.201 (Table 3). To elucidate the genetic relationships among *S. strictum* accessions, an emphasized UPGMA dendrogram was

Table 3. Pair-wise values for squared Euclidean distances (below diagonal) and Nei's genetic distances (above diagonal) of nine wild parent populations of *S. strictum*

	Zanjan1	**Zanjan2**	**Zanjan3**	**Zanjan4**	**Bojnurd**	**Karaj3**	**Karaj1**	**Karaj2**	**Esfehan**
Zanjan1	0	0.175	0.398	0.126	0.096	0.104	0.275	0.224	0.186
Zanjan2	3.743	0	0.334	0.041	0.077	0.087	0.287	0.066	0.156
Zanjan3	3.916	2.242	0	0.302	0.364	0.370	0.213	0.329	0.321
Zanjan4	2.286	2.439	2.836	0	0.097	0.074	0.257	0.092	0.153
Bojnurd	4.768	1.983	3.547	4.006	0	0.104	0.251	0.108	0.142
Karaj3	3.644	3.145	3.644	1.789	4.518	0	0.280	0.173	0.186
Karaj1	4.505	2.072	3.047	2.69	2.231	1.306	0	0.320	0.266
Karaj2	4.948	6.162	6.095	4.172	7.596	3.286	2.164	0	0.197
Esfehan	2.959	4.802	4.48	2.511	6.211	2.758	3.449	3.073	0

produced using Nei's genetic distances (Fig. 1b). The nine wild accessions were grouped into two clusters. This clustering pattern, made on the basis of SDS-PAGE, grouped the accessions differently and gave no clear indication of phenotypic performance or origin/source (Fig. 1b). In agreement with these results, AMOVA showed that most of the genetic variation was found within accessions (69%).

Correlation coefficients among pairwise genetic and phenotypic distance matrices were calculated using Mantel's test. Regression and correlation analysis between genetic and phenotypic distances showed no

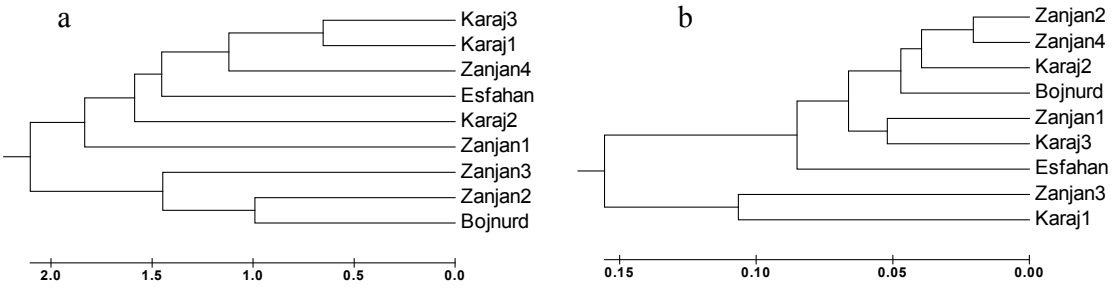

Figure 1. Phenogram of nine wild populations of *S. strictum* based on phenotypic traits (a) and total protein profiles (b), produced by the UPGMA clustering method.

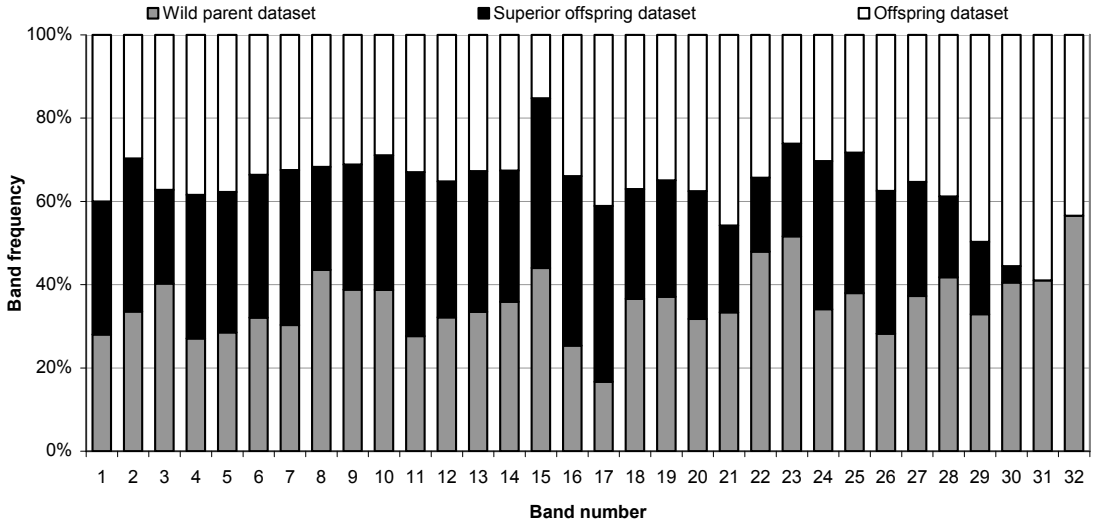

Figure 2. Seed storage protein band frequencies of the wild parent, offspring and superior offspring samples of *S. strictum*.

significant correlation ($p > 0.05$).

Genetic Differentiation between Wild Accessions and Their Offsprings

ANOVA suggested significant differences among seeds of 27 *S. strictum* accessions (the nine wild parents, offspring and superior offspring accessions, which were pooled as three distinct groups) for all the 10 germination parameters (Table 4). The mean values for the parental, offsprings and superior offspring accessions showed a wide variation for almost all the traits (Table 4). Apart from five (out of nine accessions) superior offspring accessions that did not germinate, almost all the germination parameters had higher mean values for the wild parent accessions.

Seed storage proteins profiling in the 27 *S. strictum* accessions (the nine wild parents, offspring and superior offspring accessions) were indicated 32 polypeptide bands of different sizes ranging from 6.606 to 269.153 kDa. Results showed quite different band frequency distributions among wild parent, offspring and superior offspring genotypes (Fig. 2). A summary of genetic variability measures for the analyzed accessions is shown in Table 4. The *PPL* and *He* ranged from 18.75% and 0.07, respectively, (for parental accession from Zanjan3, P-Zanjan3) to 100% and 0.439, respectively, (for offspring accession from Zanjan2, O-Zanjan2). In seven out of nine source regions, wild parent accessions showed higher levels of genetic diversity than superior offspring accessions with respect to *PPL* and *He*. However, most of the parent accessions (six out of nine) recorded lower values with respect both *PPL* and *He* compared to their respective offspring accessions. The observed number of bands (*Na*) in all superior offspring accessions ranging from 22 to 29 were lower than all parental (ranging from 27 to 32) and offspring

Table 4. Germination traits and genetic diversity parameters of wild parent (with P prefix), offspring (with O prefix) and superior offspring (with So prefix) accessions of *S. strictum* (as separate datasets)

pop	Germination traits										Genetic parameters		
	Germination %	Growth speed	Root length (mm)	Shoot length (mm)	Seedling length (mm)	Root /shoot length	Vigor Index	Seedling fresh weight (g)	Seedling dry weight (g)	Dry /fresh weight	*Na*	*PPL*	*He*
Parent													
P-	98.00	21.50	60.50	69.00	129.50	0.75	32.00	0.34	0.04	0.13	32	87.50	**0.332**
P-	98.00	24.50	143.50	109.50	253.00	1.25	61.90	1.30	0.09	0.07	32	59.38	0.256
P-	98.00	24.50	109.50	143.00	252.50	0.75	61.90	1.33	0.11	0.08	27	18.75	0.079
P-	100.00	24.25	112.00	100.00	212.00	1.10	53.00	1.50	0.09	0.06	32	71.88	**0.319**
P-	96.00	24.00	130.50	119.00	249.50	1.20	59.70	1.41	0.09	0.07	31	81.25	**0.345**
P-Karaj3	98.00	24.13	134.00	121.00	255.00	1.05	62.45	1.45	0.10	0.07	31	62.50	0.250
P-Karaj1	84.00	19.00	161.50	113.00	282.50	1.60	61.85	1.52	0.11	0.07	31	78.13	**0.293**
P-Karaj2	96.00	23.25	109.00	106.50	215.50	0.95	52.30	1.40	0.10	0.07	32	46.88	0.181
P-	88.00	20.13	73.00	109.00	182.00	0.60	40.05	0.83	0.06	0.07	32	56.25	0.222
Mean	95.11a	22.81a	114.83a	110.00a	225.72a	1.03a	53.91a	1.23a	0.09a	0.08b	31.11	62.5	0.243
Superior													
So-	-	-	-	-	-	-	-	-	-	-	25	68.75	0.311
So-	-	-	-	-	-	-	-	-	-	-	29	68.75	**0.274**
So-	-	-	-	-	-	-	-	-	-	-	25	50.00	**0.217**
So-	62.00	15.13	43.00	70.00	113.00	0.55	17.60	0.21	0.03	0.08	29	68.75	0.285
So-	62.00	15.13	68.00	87.50	155.50	0.55	31.80	0.92	0.07	0.07	26	46.88	0.190
So-	88.00	21.63	104.50	135.00	239.50	0.81	52.85	1.65	0.11	0.07	27	37.50	0.163
So-	-	-	-	-	-	-	-	-	-	-	29	71.88	0.265
So-	76.00	18.25	116.00	112.00	228.00	1.00	43.25	1.07	0.11	0.10	22	43.75	0.116
So-	-	-	-	-	-	-	-	-	-	-	27	50.00	0.166
Mean	72.00b	16.1b	93b	93.50b	162.25b	0.63b	30.10b	0.82b	0.07b	0.08b	26.55	56.25	0.221
Offspring													
O-	8.00	6.50	9.00	27.50	36.50	0.25	2.60	0.14	0.02	0.14	31	62.50	0.279
O-	84.00	20.63	65.50	66.50	132.00	0.95	27.65	0.40	0.05	0.11	32	100.00	**0.439**
O-	40.00	10.00	20.50	37.50	58.00	0.55	6.25	0.33	0.02	0.04	32	65.63	**0.198**
O-	36.00	9.00	17.50	38.50	56.00	0.40	5.25	0.18	0.03	0.14	30	93.75	**0.406**
O-	78.00	19.13	29.50	51.50	81.00	0.50	15.80	0.34	0.05	0.15	31	37.50	0.171
O-Karaj3	82.00	20.13	56.00	70.00	126.00	0.75	24.20	0.31	0.04	0.12	31	63.50	**0.277**
O-Karaj1	72.00	17.63	37.50	47.50	85.00	0.78	15.20	0.22	0.03	0.14	31	50.00	0.176
O-Karaj2	48.00	10.88	41.00	70.50	111.50	0.55	13.35	0.42	0.06	0.13	32	68.75	**0.298**
O-	76.00	19.00	28.00	61.50	89.50	0.40	16.90	0.40	0.05	0.12	32	71.88	**0.264**
Mean	58.22c	14.76b	33.83c	52.33c	86.17c	0.57b	14.13c	0.30c	0.04c	0.12a	31.33	68.06	0.276

The seed sources are abbreviated as follows: *P* parent, wild populations; *So* superior offspring of wild populations; and *O* offspring of wild populations. *: The seed sources were not germinated.

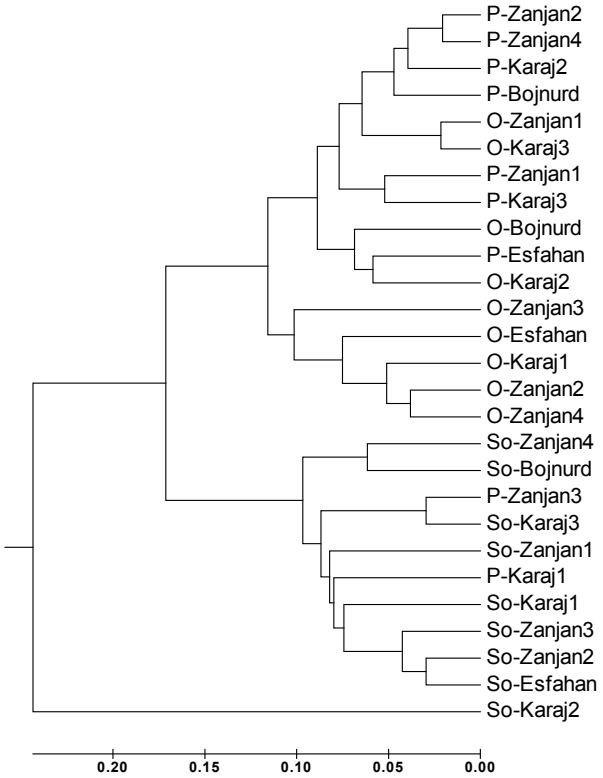

Figure 3. Dendrogram of wild parent (with P prefix), offspring (with O prefix) and superior offspring (with So prefix) accessions of *S. strictum* based on seed storage protein profiles, produced by the UPGMA clustering method.

accessions (ranging from 30 to 32). In all wild accessions and their offsprings, two locally common bands (with frequency ≤ 25%) were observed, which were absent in superior offsprings. We further compared the mean genetic parameters of three *S. strictum* datasets. The highest values were found in the offsprings, whereas the superior offsprings showed the least values (Table 4). Comparing three *S. strictum* datasets, the coefficient of genetic differentiation (*Fst*) between superior offspring genotypes with both wild parent and offspring genotypes was considerable (0.222 and 0.233, respectively), whereas, the value between wild parent and offspring genotypes was much lower (*Fst*=0.119).

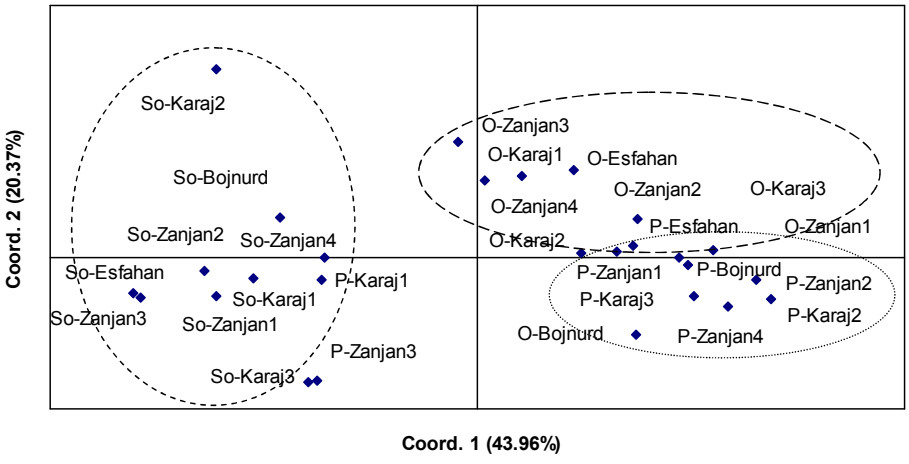

Figure 4. Two-dimensional graph based on the ordination scores of the principal coordinate analysis of wild parent (with P prefix), offspring (with O prefix) and superior offspring (with So prefix) accessions of *S. strictum* based on seed storage protein profiles.

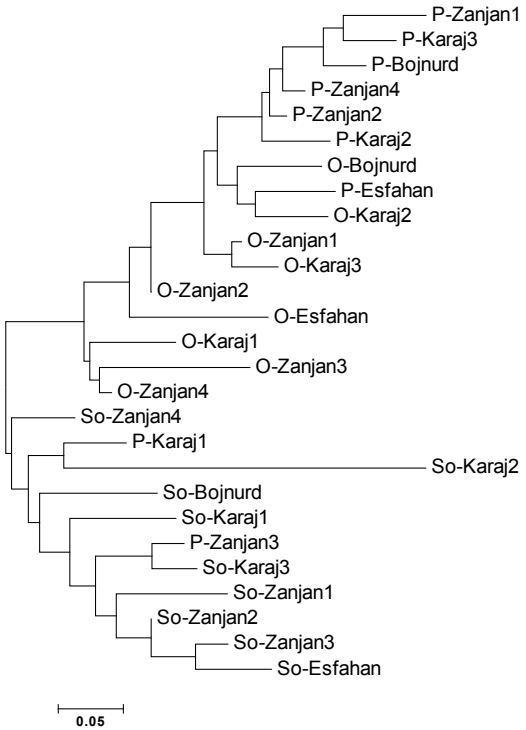

Figure 5. Dendrogram of wild parent (with P prefix), offspring (with O prefix) and superior offspring (with So prefix) accessions of *S. strictum* based on seed storage protein profiles, produced by the NJ clustering method.

To elucidate the genetic relationships among three *S. strictum* datasets (the wild parents, offsprings and superior offsprings) an UPGMA dendrogram was produced using Nei's genetic distances (Fig. 3). The 27 accessions from three *S. strictom* datasets were relatively separated from each other. The seed storage protein data were also used for conducting principal component analysis (PCA) to further study the genetic diversity among the 27 *S. strictom* accessions (Fig. 4). The results of the PCA showed that the three accessions of *S. strictom* datasets are clearly separated from each other (Fig. 4). The first three principal coordinates accounted for 80% of the total variation among the accessions or datasets. The first principal coordinate, which accounted for 44% of the total variation, clearly separated the parental and offspring accessions from the superior offspring accessions. The parental accessions were separated from the offspring accessions along the second principal coordinate, which explained 20% of the total variation (Fig. 4). The only exception among offspring accessions was O-Bojnurd, which clustered with parental accessions. Overall patterns of genetic differentiation were also examined using NJ analysis (Fig. 5). The resulting tree had long terminal branches, which suggested that the accessions and datasets were well differentiated.

AMOVA using seed storage proteins revealed that variation among datasets accounted for 17% of the total variance, among accessions within dataset and within accessions for 27% and 58% of the total variation, respectively (Table 5). An important observation in this research is that selection of phenotypically superior offsprings has resulted in higher between-accession variation among superior offspring accessions compared

Table 5. Analysis of molecular variance (AMOVA) for wild parent, offspring and superior offspring (with So prefix) accessions of *S. strictum* (as separate datasets).

Source	df	SS	MS	Est. Var.	% Total	Prob
Among datasets	2	250.974	125.487	1.115	17%	0.010
Among Pops/datasets	25	538.930	21.557	1.762	27%	0.010
Within Pops	252	992.300	3.938	3.938	56%	0.010
Total	279	1782.204	150.982	6.815		

Table 6. Analysis of molecular variance (AMOVA) for wild parent, offspring and superior offspring accessions of *S. strictum*.

Dataset	Among population (%)	Within population (%)	*p*
parent	31	69	0.01
Superior offspring	38	62	0.01
Offspring	26	74	0.01

to parental and offspring accessions implying greater differentiation among them (different bands lost) following selective retention of phenotypically superior offsprings (Table 6).

Discussion

The present survey examined nine wild accessions of *S. strictum* from Iran. High genetic variation was observed in total protein profiles and phenotypic traits. Parallel to our findings, significant variation was observed with respect to morphological, phonological, biological and molecular properties among populations in previous studies [9, 13, 20, 22, 33, 35, 38, 41, 42]. The reason for this variation detected within accessions may be related to genetic structure, which is probably due to the heterozygosity of cross-pollination of *S. strictum* [27]. The cross-pollination mechanism, sexual reproduction, high seed ratio and incompatibility to produce offspring of the *Secale* species could have resulted in accumulation of abundant genetic variation during the long evolutionary history [5]. This indicated that improvement through simple selection for these traits is possible. However, broadening the genetic base from diverse sources is recommended to include most of the genetic determinants of these traits [15].

Seed storage protein profiles variation revealed that wild accessions of *S. strictum* held more genetic variation within rather than among accessions (69%, 31%, respectively). According to Hamrick and Godt [18], reproductive biology is the most important factor in determining the genetic structure of plant populations. They showed that out-crossing plant species tend to exhibit between 10% and 20% genetic variation among populations, while self-pollination species exhibit on average 50% variation among populations. Therefore, the 31% genetic variation among studied accessions can be explained by partial inbreeding. Although studies on the biology of flowering and pollination indicate it as an out-crosser, *S. strictum* shows partial self-compatibility [46].

AMOVA analysis showed that, although the majority of the genetic variation resided within wild accessions (69.0%), a relatively high degree of genetic variation resulted from differentiation among accessions (31.0%). Jenabi et al. [20] confirmed the presence of a much more pronounced and significant differentiation among 19 *S. strictum* populations from Iran using nuclear SSRs (based on AMOVA, differences among populations account for 62% of the total nSSR variance). In agreement with other studies in *Secale* species [9, 20, 36, 42] the results of this work implied that the genetic diversity of *S. strictum* was not the result of the joint effects of one or several ecological factors, i.e., the ecological factors do not play an important role in influencing the protein profiles polymorphism of *S. strictum*. This study provides evidence that seed storage protein marker polymorphisms are an informative and suitable approach to evaluate the polygenic relationships in wild accessions of *S. strictum*.

Comparison of seed storage protein profiles revealed considerable differences between wild parent accessions of *S. strictom* and their offspring. Unfortunately, our understanding of the relationship between level of genetic diversity in parental populations and their offspring among *Secale* species is limited. In our study, interestingly, progenies of wild populations were genetically more diverse on account of higher values of percent polymorphic loci as well as heterozygosity compared to their parent populations. This may suggest the influence of out crossing from larger distances in these samples, as produces offspring by the fusion of gametes, resulting in offspring genetically different from the parent or parents.

Loss of genetic diversity and increased population differentiation from source populations are common problems associated with breeding programs established from a small number of founders. Like many other studied plant species where cultivars have lower genetic diversity than their wild relatives [14, 29, 40, 54, 57], the superior offsprings of different *S. strictum* accessions maintain lower levels of genetic diversity as their parents (mean *He* value for wild parent accessions was significantly higher than their superior offsprings). The heterozygosity of offspring samples was higher than all wild parent or superior offspring accessions. Despite the retention of genetic diversity in offsprings, 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, 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 [26]. This has been confirmed in many species, such as Bluebunch Wheatgrass [24], maze [58], Cassava [28] and rice [50]. In the present study significant genetic differentiation among the wild parent accessions and their superior offsprings 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 [25, 56]. The major reason for the genetic differentiation between the wild parent accessions and their superior offsprings in this study appears to be artificial selection as the superior offsprings 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 [12], 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, a very important part of genetic diversity which have been missed in superior progenies. 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 [6]. 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.

This study demonstrates the high levels of polymorphism detectable with seed storage proteins even within superior offsprings of *S. strictum*. A relatively high degree of genetic differentiation among wild populations (31%) indicates that comprehensive germplasm collection in major geographic regions is required to broaden the genetic base and sample the full extent of the available variation. The results demonstrate that the divergence of microenvironments have no obvious effect on the genetic diversity and genetic structure of *S. strictum*. Consequently, major attention should be paid to the sustainable conservation of the wild populations of *S. strictum* at different populations, when strategies for breeding and germplasm conservation are being implemented in future programs. Breeding strategies need to exploit the existing variation within the wild *S. strictum* germplasm. The study confirmed that genetic and morphological diversity work in different ways to determine the relationships among populations. To effectively exploit germplasms, we should utilize both methods in breeding work. Further studies are required to reveal whether there are other factors that cause genetic variation in *S. strictum*. Although *S. strictum* had not been listed as a species of conservation concern for Iran, it is an important economic species endemic to Iran. Therefore, the conservation and further reasonable utilization of the germplasm resources of this species is an urgent task.

This technology (SDS-PAGE) 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 accessions and their superior offspring 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. 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, offspring and phenotypically superior offspring accessions were separated into three groups. This suggests that founder effects and subsequent selection have had more effect on the genetic differentiation among 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 programs.

Conclusion

From the present study it can be concluded that there was a high genetic variation among *S. strictum* wild populations for both seed storage protein profiles and phenotypic traits. The results demonstrate that the divergence of microenvironments have no obvious effect on the genetic diversity and genetic structure of alfalfa. Consequently, major attention should be paid to the sustainable conservation of the wild populations of alfalfa at different populations, when strategies for breeding and germplasm conservation are being implemented in future programs.

The results showed the existence of genetic variability for each progeny and wild population, demonstrating differences in the progeny performance across locations. Besides, seeds of superior offsprings showed less genetic variability than both wild and offsprings of *S. strictum* accessions suggesting that more intensive breeding practices may have resulted in a further erosion of genetic variability. Separation of wild populations, offspring and phenotypically superior offspring accessions into three groups, suggests that founder effects and subsequent selection have had more effect on the genetic differentiation among these accessions than geographical separation. The results demonstrated that the study of genetic diversity and differentiation between the parents and their offspring using seed storage protein profiles provides important information for the breeding and conservation of germplasm. However, this study represents a first step in studying the impact of domestication on the genetic diversity of *S. strictum* accessions in Iran, and still needs to be supported with additional work at different markers.

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