Australian Journal of Crop Science AJCS

AJCS 7(7):1024-1031 (2013) ISSN:1835-2707

Genetic diversity analysis of agro-morphological and quality traits in populations of sainfoin (*Onobrychis sativa*)

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Abstract

In order to study the variations and relations among morphological and quality traits, 12 populations (accessions) of sainfoin (*Onobrychis sativa*), were assessed under sward growth condition using split plot design with two replications for three years. In this regard, each variety was cultivated in plot area directly and different traits were assessed when 50% of plants produced flowers. Results of combined analyses showed that there were significant differences among 3 years for all of traits except plant density. Based on these results, the average values of 6.47 and 10.31 ton ha^{-1} dry matter (DM) yield were obtained in the first and second year, respectively. Phenotypic correlation between DM yield with both dry matter digestibility (0.35*) and crude fiber (0.58*) were positive, whereas its relationship with crude protein (CP) was negative (-0.82**). Using principal component analysis, the first four independent components with values more than 1.0 accounted for 88% of total variation, namely plant density, forage quality, seed yield and dry matter yield component. Based on Ward cluster analysis method, 12 populations were divided into 3 groups. Ultimately, superior populations were identified to improve the breeding of sainfoin in terms of high quality and forage yield coupled with resistance to powdery mildew disease.

Keywords: Sainfoin (*Onobrychis sativa)*, forage yield, quality traits, morphological traits, correlation, PCA and cluster analysis.
Abbreviation: Dry matter digestibility (DMD); Crude fiber (CF); Crude protein (CP); Dry matter (DM) yield; Principal component analysis (PCA); Leaf and stem ratio (LSR); Acid detergent fibre (ADF); Water-soluble carbohydrates (WSC); Near infrared spectroscopy (NIR);Poly-cross (PC).

Introduction

Sainfoin (*Onobrychis sativa* Scop.) is one of the most important forage legumes which are appreciated by farmers due to high palatability and high nutritional value properties (Delgado et al., 2008). Sainfoin has been harvested for hundreds of years in many zones of the world, including Asia, Europe and North America (Frame et al., 1998). Sainfoin sometimes is also known as holy clover; a perennial forage legume with deep roots that are often grown in conjunction with forage grasses to reduce bloat hazard as well as to improve soil fertility due to its nitrogen fixing ability (Lu et al., 2000). Depending on the growth conditions, DM yield of sainfoin may range between 7 and 15 ton ha^{-1} (Frame et al., 1998). Traditional harvesting is normally practiced at the bud to mid-flowering stage for the first cut, approximately 70% producing of the total annual yield. Forage quality is the ability of pasture or harvested forage to produce a desired livestock response. Specific levels of forage quality are required for different classes of livestock, seasons and conditions. Forage quality varies considerably due to several factors such as species, leaf-to-stem ratio, stage of growth, soil agents, climate, harvesting, disease and pests (Arzani et al., 2001). Information on nutritive value of plant in each phonological stage could help range managers to choose suitable grazing time to achieve higher animal performance without detriment to vegetation (Arzani et al., 2004). Forage quality declines with advancing maturity which also influences forage consumption by animals (Ball et al., 2001). With increasing CF (crude fiber), the percentage of digestibility and plant energy will decrease (Hoffman et al., 2003). Problems related to seed production of forage crops in less intensive production regions have not been sufficiently studied. In reality, most attentions have been mainly focused on resolving problems about the harvest of major profitable crops in developed regions (Porqueddu et al., 2000). First researches of sainfoin in Canada showed that regrowth had more forage quality if a cut was taken at bud or early flowering stage, but yield was higher when the first harvest was done at a more mature stage (Goplen et al., 1991). Whereas, Mowrey and Matches (1991) stated that protein, lignification and fiber content do not have significant relation with early, medium and late bloom. Sainfoin regrowth is slow, and it is important to allow enough time to replenish macro and micro nutrient contents of root in order to maintain its persistence and longevity. Since the regrowth of sainfoin is slower than alfalfa, the second and third cuts may be harvested at intervals of about 7 weeks after the previous cut. The slow regrowth of sainfoin compared to alfalfa may be due to considerable differences in the root reserves. The final cut should probably be harvested when no further regrowth is likely to occur (Mowrey and Matches, 1991; Frame et al., 1998). The aims of this study were to determine the pattern of variation for forage dry matter (DM) yield, morphological and quality traits in 12 populations of sainfoin (*Onobrychis sativa*), and to identify different groups of

Table 1. Information and collection areas of sainfoin accessions.

Taxon	Locality	Gene Bank Code	Altitude (M)	Coordinates	Accession
Onobrychis sativa	Shahrekord	18	2070	32°19`N50°51`E	Shahrkord-18
Onobrychis sativa	Golpaygan	181	1830	33°27`N50°17`E	Golpaygan-181
Onobrychis sativa	Esfahan	182	1590	32°38`N51°39`E	Esfahan-182
Onobrychis sativa	Hamedan	281	1850	34°48`N48°31`E	Hamedan-281
Onobrychis sativa	Bijar	624	2020	35°52`N47°36`E	Bijar-624
Onobrychis sativa	Kashan	962	1600	33°59`N51°28`E	Kashan-962
Onobrychis sativa	Gorgan	1586	1350	36°50`N54°26`E	Gorgan-1586
Onobrychis sativa	Gorgan	1601	1350	36°50`N54°26`E	Gorgan-1601
Onobrychis sativa	Orumieh	1763	1332	37°33`N45°04`E	Orumieh-1763
Onobrychis sativa	Karaj	3001	1312	35°50`N51°00`E	Karaj-3001
Onobrychis sativa	RIFR*	Early	-	-	PC-Early
Onobrychis sativa	RIFR	Late	-	-	PC-Late

* RIFR; Research Institute of Forests and Rangelands, PC; Ploycross

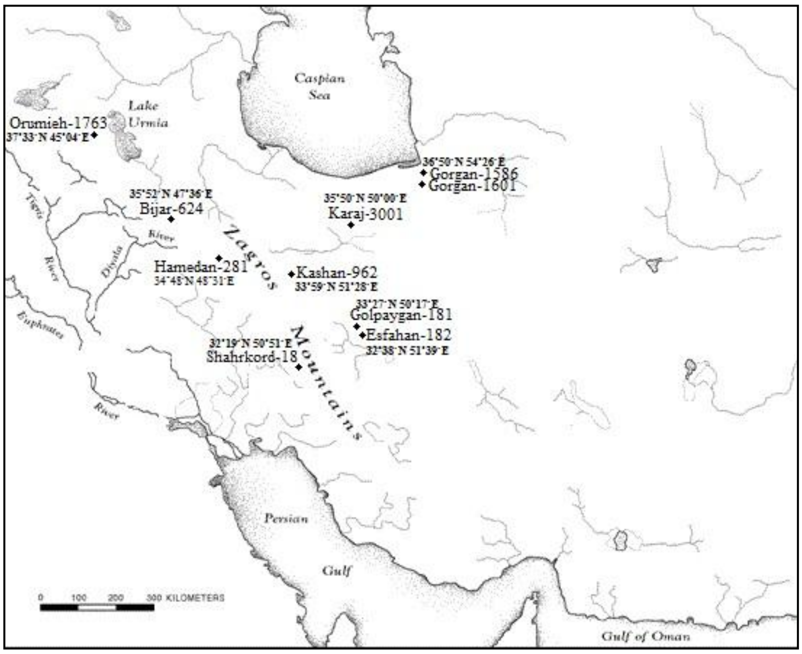

Fig 1. Collecting sites of sainfoin accessions in Iran.

accessions using a multivariate approach. We aim to assess and identify high quality and high-yielding hybrid rootstock lines that are resistant to powdery mildew disease, critical to any sainfoin breeders. The findings of the current study are also essential for future research, especially to study the correlation between genetic diversity of sanfoin with morphological and quality traits.

Results

Combined analysis and mean comparison

The results of combined analysis showed that harvesting year significantly affect all traits under study (P≤0.01), whereby the second year had the higher values for dry matter yield, morphological traits, dry matter digestibility, crude protein and ash. The effects of accessions were significant on DM yield, acid detergent fibre and plant height (P≤0.05). Similar results were obtained for crude protein, crude fiber, water-soluble carbohydrates, ash and flowering (P≤0.01).The interaction effect between year and genotype showed significant differences for ash, plant height and stem number (Table 2). Orumieh accession showed the highest values of DMD and CP at the second year with 74.27% and 27.31%, respectively. Karaj accession also showed remarkable DMD and CP results with 74.17% and 26.13%, respectively. CF% was the least for Gorgan-16, Orumieh and Poly Cross-Late in the second year (approximately 20%). Accessions of Poly Cross-Early and Golpaygan showed higher values of WSC (24.11%) and ADF (37.48%) in the first and third year. In the second year, the total ash value was more than two other years with approximately of 8% in all accessions (Fig. 2).

To compare the DM yield of 12 accessions, the populations of Shahrkord and Golpaygan showed higher forage yield, with DM yield of 14.3 and 12.09 ton ha^{-1} in the second year. Similarly, Akbarzadeh and Salariin (1995) reported that the highest forage yield was produced for a period of three years at the second year. It became clear that population of PC-Early was tall (85.6 cm) in the second year, and two populations of Kashan and Esfahan were short, with average plant height of 49.5 cm and 49.4 cm in the first year, respectively. Accessions of Shahrkord and Esfahan had the most crowded density with 38.5 stem per m^2 in the second year. In terms of flowering date, populations of PC-Early and Late took the least number of days to appearance of 50% flowering, with 32 and 31.17 days in the first year, respectively (Fig. 3).

Analysis of phenotypic correlations

The analysis on phenotypic correlations between traits is shown based on average of years (Table 3). The correlation

Table 2. Summary of combined analysis and the level of significant mean squares.

S.O.V	df	DM Yield	DMD %	CP %	CF %	WSC %	ADF %	ASH %	Plant Height	Flowering Date	Stem No.
Accession (a)	11	3.4*	12.5	2.3**	10.4**	3.74**	8.7*	0.21**	70*	19.1**	22
Replication	1	128**	0.2	1.3	2.7	5.74*	14	0.18*	9.3	0.6	282**
Error 1	11	3.2	7.1	0.56	1.8	0.96	3	0	23	1.6	19.6
Year (b)	2	202**	282**	441**	496**	299**	113**	50**	4233**	14676**	30
a*b	22	4.1	15.6	3.7	8.2	1.76	10	0.18*	71**	16.5**	18.1
Error 2	24	6.4	8.3	5.7	5.6	1.33	6.84	0.08	14.9	1.99	16.6
CV%		25	4	11	8	6	9	4	6	2	12

*significant at 0.05 probability level, ** significant at 0.01 probability level.

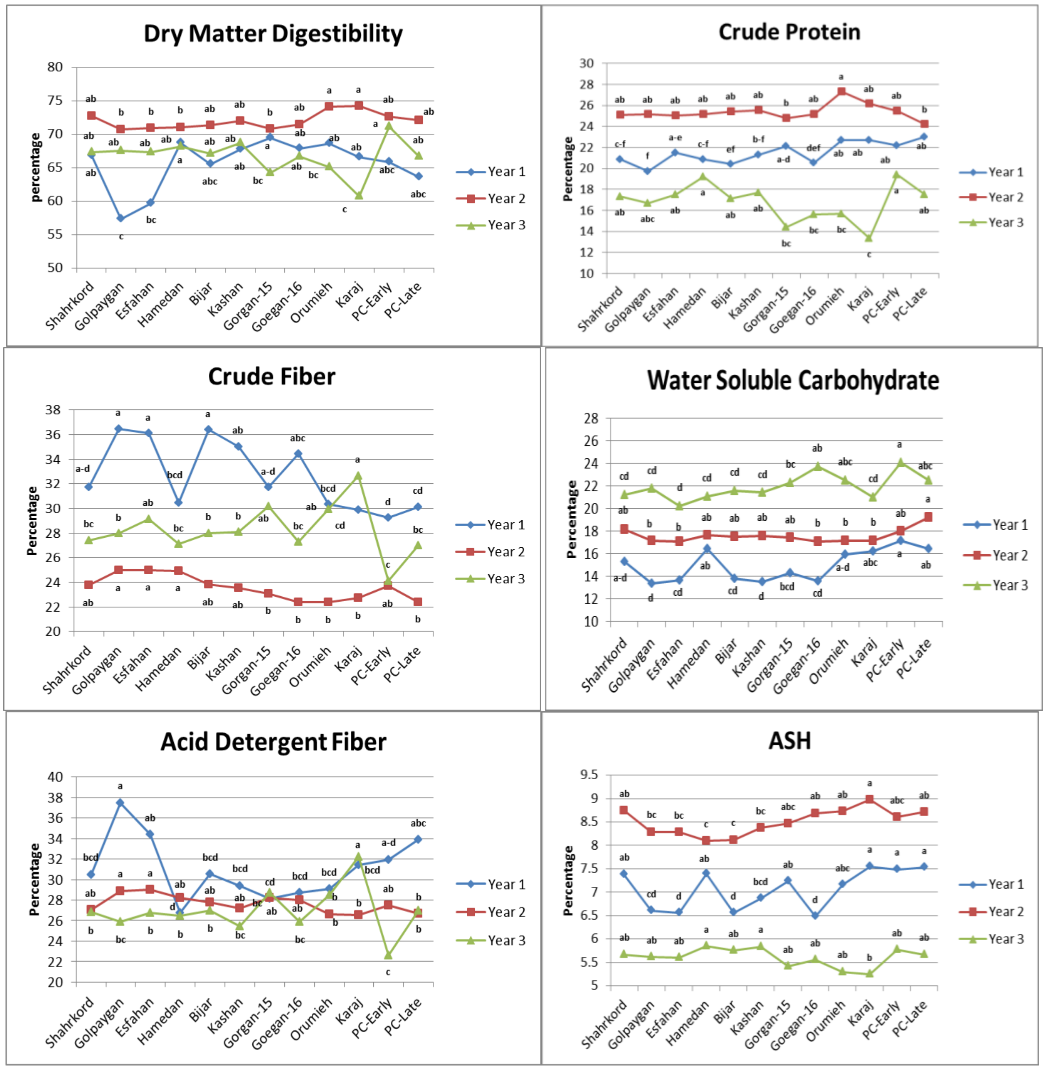

Fig 2. Mean comparison among accessions of *Onobrychis sativa* for quality traits in three years. The means of the populations with same letters are not significantly different based on DMRT (P<0.05).

coefficient between dry matter yield and plant density with dry matter digestibility and crude fiber were significantly positive and also negatively correlated with CP and WSC. However, their associations with the acid detergent fibre and ash traits were found to be non-significant. Plant height revealed significantly positive association with DMD and ash while its relationship was extremely negative with ADF. Flowering date was positively and negatively correlated with CF and WSC (Table 3).

Principal component analysis (PCA)

In PCA analysis, the particular values (variance) resulting from 1 to 4 axes were more than 1 accounted for 37, 26, 17 and 8 percent of variation, respectively. The first four components of the PCA explained 85% of the observed variability. For the first component; plant density, vigour and total ash with positive coefficient and CP with negative coefficient yield were the most important traits for classifying populations into clusters. The second component was strongly correlated with traits of DMD, ADF, LSR and inflorescence seed and this component was regarded as quality components. Traits of flowering date, CF, WSC, seed yield, 1000-gw and inflorescence length had significant correlation with third component and was termed as seed yield.

Table 3. Correlation analysis between morphological and quality components in 12 populations of sainfoin.

Traits	Plant Height	Flowering Date	Stem No.	Plant Density	DM Yield	DMD %	CP %	CF %	WSC %	ADF %	ASH %	Mildew Disease
Flowering Date	-0.1											
Stem NO.	0.36*	0.46**										
Plant Density	0.2	0.41*	0.55**									
DM Yield	0.21	0.43*	0.54**	0.76**								
DMD %	0.52**	0.06	0.38*	0.43*	0.35*							
CP %	0.03	-0.33	-0.47**	-0.82**	-0.82**	-0.42*						
CF %	-0.07	0.57**	0.60**	0.66**	0.58*	- 0.25	-0.71**					
WSC %	0.13	-0.65**	-0.46**	-0.53**	-0.58**	0.11	0.52**	-0.77**				
ADF %	-0.49**	-0.01	-0.33	-0.39*	-0.31	-0.97**	0.41*	-0.25	-0.15			
ASH %	0.44*	0.01	0.3	0.40*	0.32	0.80**	-0.39*	0.19	-0.02	-0.72**		
Disease	0.17	-0.39*	-0.54**	-0.69**	-0.61**	-0.22	0.80**	-0.80**	0.73**	0.22	-0.29	
LSR	-0.64**	0.13	-0.37*	-0.22	-0.1	-0.69**	0.09	-0.08	-0.17	0.71**	-0.52**	0.07

*significant at the 0.05 probability level, ** significant at the 0.01 probability level.

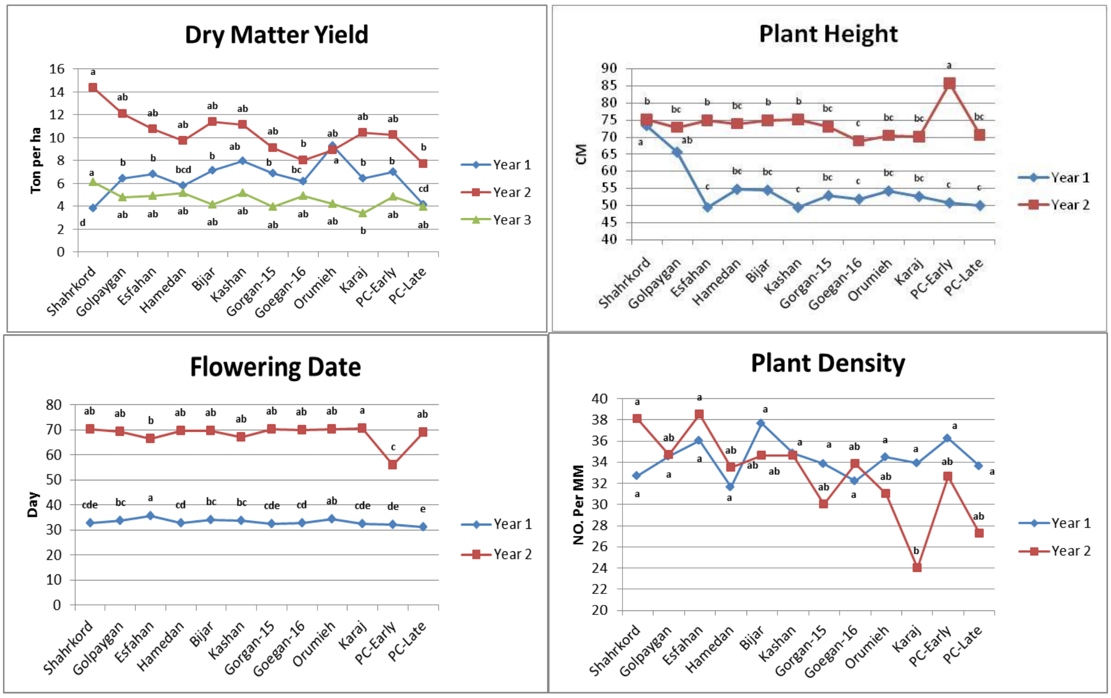

Fig 3. Mean comparison among accessions of *Onobrychis sativa* for dry matter yield and morphological traits. Effect of accessions was significant in these traits. The means of the populations with same letters are not significantly different based on DMRT (P<0.05).

The final factor was termed as forage DM yield because it had significant relations with plant height, disease and DM yield (Table 4).

Cluster analysis

In the cluster analysis, all 17 traits of 12 accessions were used and grouped into 3 different categories with dendrogram slice in 8.1 of Euclidean distance (Fig. 4). The mean comparison among clusters is illustrated in Table 5. Accessionsin cluster1 had high DM yield, low CP, higher vigour, crowded density of stems, sensitive to diseases and average values of DMD. Cluster 2 showed accessions with lower DM yield, higher CP, poor growth condition, high LSR and average vales for DMD. PC-Early was the sole genotype showed in cluster 3 and it had average DM yield and CP, high DMD, crowded density of stems and resistance to diseases. This cluster was superior for forage quality. Dispersion of 12 accessions (in 3 clusters) based on the first and second components are shown in Figure 5. The first axis (X) was formed by plant density and ash (positive coefficient) and CP (negative coefficient). Accessions of the first cluster were upper right and correspond to plant density, ash, number of stems and low CP. In contrary, the scattered accessions of the second cluster were on the left of X axis and had the highest value of protein. The second axis (Y) was associated with DMD (positive coefficient) and ADF, LSR (negative coefficient).
The third cluster was on top of the Y axis with high DMD percentage (Fig. 5).

Discussion

Comparison among the three different years showed that the second year had the highest forage yield which is common in perennial forage crops. According to Jafari et al., (2003), development of forage plants are made in the first year and its growth is less to some extent. In the combined analysis, since interaction of year and accessions showed different variations, hybrids will need to be evaluated in several environments in forage crops breeding. Effect of environment in all ecosystems and especially in agro ecosystems has been realized widely on crop traits (Collins, 2001). In this regard,

Table 4. Matrix of coefficients vectors and variance proportion from the first four principal component axes in 12 accessions of sainfoin.

Variable	PC1	PC2	PC3	PC4
Plant Height	0.16	0.32	-0.13	*-0.35*
Flowering	0.17	-0.1	*0.38*	-0.36
Stem NO.	*0.36*	0.03	0.04	-0.1
Plant Density	*0.32*	0.04	0.05	-0.02
DM Yield	0.25	-0.03	0.12	*-0.52*
DMD %	0.24	*0.34*	-0.08	0.03
CP %	*-0.31*	0.05	-0.16	-0.28
CF %	0.27	-0.12	*0.31*	0.14
WSC %	-0.2	0.26	*-0.34*	0.05
ADF %	-0.22	*-0.35*	0.08	-0.09
ASH %	*0.25*	0.27	-0.08	0.21
Inflo. length	0.13	-0.22	*-0.42*	-0.09
Seed Yield	0.23	-0.26	*-0.31*	-0.05
1000 GW	0.22	-0.17	*-0.29*	0.22
Inflo. Seed	0.07	*-0.39*	-0.22	0.11
Disease	-0.28	0.17	-0.17	*-0.37*
LSR	-0.21	*-0.32*	0.21	-0.05
Eigenvalue	6.66	4.66	3.03	1.48
Proportion	0.37	0.26	0.17	0.08
Cumulative	0.37	0.63	0.8	0.88

*The bold and underline coefficients have significant correlation with the relevant axes.

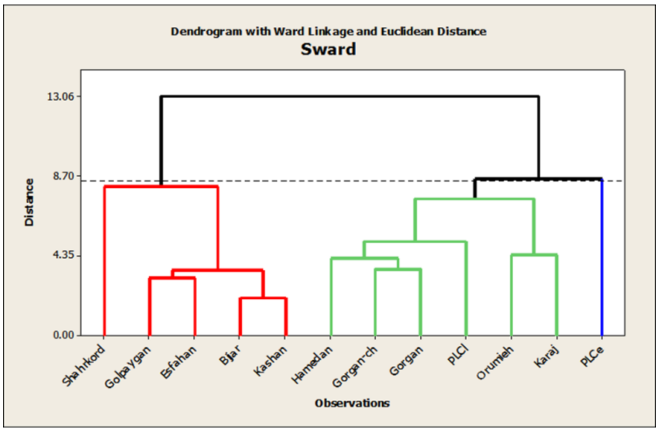

Fig 4. Cluster analysis with Ward Linkage. 17 traits of 12 accessions were used and grouped into 3 different categories with dendrogram slice in 8.1 of Euclidean distance.

the species diversity of natural ecosystems is extremely variable (Edwards and Hilbeck, 2001). In the present investigation, comparative studies on sanfoin populations also showed high levels of genetic variation. In general, the association of genetic variation with environmental conditions has been noted due to natural selection and local gene dispersal (Hamrick and Godt, 1996). The genetic closeness between the two geographically distant but ecologically similar populations of Gorgan-1586 and Gorgan-1601 (both distributed in regions with Elevation 1350 m and moderate and humid climate) reflect ecological impact, rather than geography. Quality increment is one of the most important goals in sainfoin breeding programs. Data from animal nutrition studies revealed the need for improvement in the nutritive value of new recommended sanfoin varieties. Increase of DMD, water soluble carbohydrates (WSC), and CP% coupled with low fibre content had higher priority in terms of forage quality for life weight gain and dairy production (Wilkins and Humphreys, 2003). Although the relationships between DM yield and CP was negative, however, the higher values of DMD and CP were obtained in the second year when the accessions had the highest forage quality. The findings depicted in the current study were parallel to the results obtained by Jafari and Godarzi (2006) in their works on alfalfa. It should be noted that for forage breeding projects, production of early population is important for early spring grazing, while late population can be used for delayed grazing in summer time in cold regions. So, accessions of Poly Cross-Early and Poly Cross-Late, Esfahan and Kashan are recommended for spring grazing or as the first harvesting crop. The consistently negative relationship between crude fiber and digestibility suggests that reduction of ADF might be an effective way to improve the quality of forage (sanfoin). In this intensive system, the number of stems per m^2 was high in the early maturity accessions without any significant difference from flowering and decreased in the late accessions. The low stems density in the late accessions observed before summer was probably due to the higher temperature of that period (Kallenbach et al., 1996). Consequently, the correlations between both LSR and Mildew disease with DMD were significantly negative, but plant density was shown to have positive correlation with DMD. These linkages showed that sensitive accessions with high LSR had poor forage quality. Nakhjavan et al. (2011) stated that reduction in plant height results in lower DM in addition to the production of more leaves and enhanced

Table 5. Comparisons between means of 3 clusters for the traits used in classification.

Traits	F probability	Cluster 1 n=5	Cluster 2 n=6	Cluster 3 n=1
DM Yield	*	7.8 a	6.5 b	7.35 a
Plant Height	**	74.4 b	71.3 c	84.6 a
Flowering Date	ns	33.9 a	32.6 a	32.1 a
Plant Density	**	36.1 a	29.9 a	32.6 a
DMD %	*	67.6 ab	65.3 b	71.1 a
CP %	ns	20.7 a	21.9 a	22.2 a
CF %	**	35.1 a	31.1 b	29.2 b
WSC %	**	17.7 b	18.4 b	19.7 a
ADF %	*	26.3 ab	28.1 a	22.6 b
ASH %	ns	5.7 a	5.5 a	5.8 a
Inflo. length	ns	9.1 a	8.7 a	8.5 a
Seed Yield	*	400 a	288 b	266 b
1000 GW	ns	21.6 a	20.7 a	20.1 a
Inflo. Seed	ns	8.7 a	7.8 ab	4.2 b
Mildew Disease	**	2.8 b	3.4 ab	4.3 a
LSR	*	0.36 ab	0.42 a	0.25 b

*significant at the 0.05 probability level, ** significant at the 0.01 probability level. The means of the clusters (rows) with same letters are not significantly different based on DMRT P<0.05.

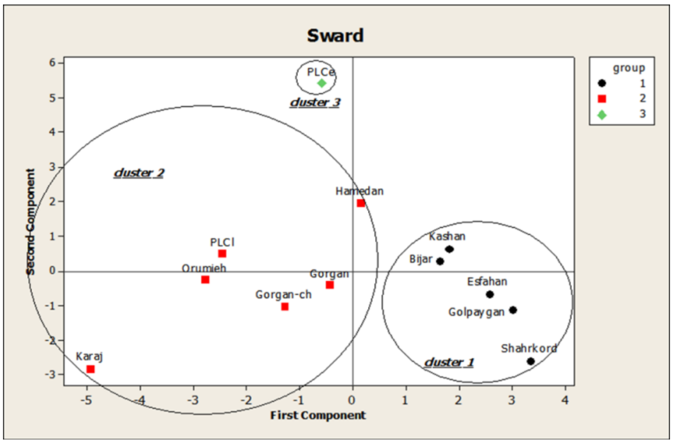

Fig 5. Factor analysis of traits based on 3 clusters of 12 accessions of *Onobrychis sativa.* The distribution of populations on the first two component scores was in agreement with cluster analysis

forage quality. High leaf to stem ratio (LSR) is usually desirable because leaves are more palatable and retain higher digestibility over time than stems. Turk and Celik (2006) observed a positive and significant correlation between DM yield and stem number and plant height and there was also a positive and significant correlation between these two traits. In PCA analysis, the normal distributions observed in morphological traits which show only a few accessions were characterised by extreme values almost similar to the agronomical traits. These traits might be of interest for breeding purposes. Distinction of cluster analysis could be explained by climatic factors as the accessions were from north of Iran, generally characterised by a temperate climate, and tend to have an earlier flowering date. A synthetic variety is developed by inter crossing a number of genotypes and accessions of known high combining quality and yield that are known to give superior hybrid performance. Seeds of synthetic variety are used for some generation. It is possible to produce these populations by crossing accessions that are more genetically diverse (Hosainianejad et al., 2011). Therefore, synthetic variety is more likely to be achieved by crossing the accessions from first and third clusters. By crossing these two accession groups and selection via a progeny test, breeding of sainfoin varieties could be improved, especially in terms of improving quality and producing higher forage yield coupled with resistance to powdery mildew disease. Accessions of the second cluster can be used as a valuable genetic resource in transgenic programs to increase the percentage of CP in high yield populations. In conclusion, the distribution of populations of the first two component scores was in agreement with cluster analysis.

Materials and methods

Plant materials

Ten accessions (Shahrkord-18, Golpaygan-181, Esfahan-182, Hamedan-281, Bijar-624, Kashan-962, Gorgan-1586, Gorgan-1601, Orumieh-1763 and Karaj-3001) of sainfoin (*Onobrychis sativa*) plus two poly-cross populations (Early and Late) were provided by natural resources gene bank, Research Institute of Forests and Rangelands, Tehran, Iran (Table 1, Fig. 1).

Experimental design

The present work was carried out at Alborz Research Centre for three years annually, in Karaj, Iran (35˚42' N 51˚31' E) at altitude 1291-m above sea level. Subsequently, different traits were analysed and evaluated at Institute of Biological Sciences, University of Malaya, Malaysia. This research was assessed under sward growth conditions using split plot design with two replications. The plot area was 1 x 2 m comprising of four rows with 25 cm spacing. The distance between lines of plots was 50 cm. The first irrigation was given7 days after cultivation while next irrigations were made according to the plant requirement. In the long run of the experiment, agricultural cares including weed control and fertilization schedule were made based on scientific advices and recommendations. In this respect, each plot was tilled, disk was applied and also fertilized with 200 to 250 kg ha-1 ammonium phosphate and 100 to 150 kg ha-1 urea. For weed control, either mechanical control with leveller or early harvesting of the first cut showed a good effect.

Traits measurement

Dry matter (DM) yield and quality traits were measured for three years separately while morphological traits and seed traits were assessed for two and one year, respectively. Plant density (No m^{-2}) and stem height (cm), flowering period (day) after 50% flower emergence, inflorescence length (cm), seed yield (kg ha^{-1}) and 1000-grain weight (g) were recorded based on the methods described by Mohajer et al., (2011). Ten plants of each plot were selected, then their seed numbers were measured and averaged (depicted as inflorescence seeds). Plant density was evaluated based on the number of plants in each plot. In this way, powdery mildew disease and plant density traits were evaluated qualitatively (1: sensitive and weak, 5: showing resistance and strong). After the first cut (50% of flower emergence), samples were taken from each plot. The samples were dried at 70°C for 48 hours. The leaf to stem ratio (LSR) on weight basis was measured after separation and weighing of leaf and stem. The separated leaves and stems were mixed and ground with 1 mm mill. The six quality traits studied in the current investigation were dry matter digestibility (DMD), water soluble carbohydrates (WSC), crude protein (CP), crude fibre (CF), acid detergent fibre (ADF) and total Ash (measured using near infrared spectroscopy, NIR). Details of the methodology and calibrations used are as described by Jafari et al., (2003).

Statistical analysis

Data was combined and analysed using split plot design under Randomised Complete Block (RCB). Duncan multiple range test (DMRT) method was used for determination of superior populations. Phenotypic correlations among characteristics were estimated via pair-wise combinations (Kwon and Torrie, 1964). All variables were used in principal components and cluster analysis, whereby the variables were standardized for cluster analysis (Ward method) using Minitab 16 and SAS9 softwares.

Conclusions

The three year assessment of the agronomic characteristics of sainfoin collections revealed a wide range of variations in all observed traits. The major part of the genetic variation was attributed to the differences between individual plants, causing any grouping based on overall genetic distances to be rather changeable. Nevertheless, the genetic differentiation between populations and geographical regions exists and is real, but is more or less unclear by the vast genetic variations within the accessions. Remarkable levels of DM and forage quality production were obtained from the second harvesting year whereas effect of year was significant in most traits. Principal component analysis helped to integrate all data and eventually to select several varieties of interest, both commercial accessions and landraces, on agronomic criteria. Based on this conclusion, the best accessions were selected for further evaluation in larger plots for their persistence and other agronomic parameters. Increasing the area for cultivation of Sainfoin depends greatly on improvements in seed production, which is still the main cause of the slow development of forage crop production.

Acknowledgements

The authors would like to thank the Research Institute of Forests and Rangelands, Iran for the facilities and University of Malaya, Malaysia for financial support provided (IPPP Grant No. PV025/2011B and UMRG grant RP025/2012A).

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