RESEARCH

Genetic Diversity in a Worldwide Collection of Sainfoin Using Morphological, Anatomical, and Molecular Markers

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ABSTRACT

Genetic information on sainfoin (*Onobrychis viciifolia* Scop.), an important forage species, is highly limited. In this study, genetic diversity and trait relationships of 80 sainfoin accessions were evaluated using morphological, anatomical, and inter-simple sequence repeats (ISSR) characteristics. Results of 2 yr of study indicated a wide range of variability in the germplasm using all three data sets. Based on morphological assessment, accessions were divided into three different groups that could be easily identified by traits such as palatability, resistance to powdery mildew, and percentage of plant shoot and leaf. On the other hand, great diversity was found for anatomical traits, especially vessel diameter, sieve diameter, width of phloem, and xylem/phloem ratio, possibly indicating different mechanisms of water and solute transport among populations. Results indicated that anatomical traits were less influenced by environmental constraints compared with agro-morphological traits. Accessions with high vessel diameter and large xylem diameter were may be palatable. Result of ISSR markers showed that high genetic variation among accessions can be closely related to "Isolation by distance" model, resulting in accessions falling into two main clusters (Iranian and the exotics), each having four subclusters. Most of the genetic variance was found among the accessions and less among the geographical groups. Results suggested that Asia and Eastern Europe may be the main center of diversity for this species.

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Abbreviations: CD, crown diameter; DF, days to flowering; DMY, dry matter yield per plant; FMY, fresh matter yield per plant; ISSR, inter-simple sequence repeats; LH, number of leaf hairs; LL, legume length; LW, legume width; NNS, number of nodes per shoot; P, palatability; PCV, phenotypic coefficient of variation; PDMY, percentage of dry matter yield; PH, plant height; PL, panicle length; POL, percentage of leaf; POS, percentage of shoot; RPM, reaction to powdery mildew; SD, sieve diameter; SL, seed length; SLW, seed length/width ratio; SPP, number of shoots per plant; VD, vessel diameter; WLC, width of lower cuticle; WOLC, width of leaf collenchymas; WP, width of phloem; WUC, width of upper cuticle; XPR, xylem/phloem ratio.

The genus *Onobrychis* (Fabaceae) contains nine sections with high potential for use as pasture plants (Rechinger, 1969). This genus includes nearly 130 species that are mainly distributed in the northern temperate regions Europe and Asia, especially Iran and Anatolia, making this area the main center of genetic diversity (Ranjbar et al., 2010; Zohary, 1987; Yildiz et al., 1999). Cultivated sainfoin (*Onobrychis viciifolia* Scop.) is an allotetraploid species ($2n = 4x = 28$), which is resistant to major alfalfa pests such as the alfalfa weevil [*Hypera postica* (Gyllenhal)], winter hardy, relatively drought tolerant, and also has a level of nitrogen fixation activity similar to other forage legumes (Emre et al., 2007; Prevost et al., 1987). Although advances in plant breeding have led to improved alfalfa (*Medicago sativa* L.) varieties, sainfoin production has continued to rely on old cultivars (Demdoum et al., 2012), and very little information is available about the

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genetic diversity of sainfoin accessions in its main center and other areas of the world.

Understanding genetic diversity in germplasm collections can greatly facilitate reliable classification of accessions and identification of subsets of core accessions with possible utility in breeding programs (Majidi et al., 2009). Different methods including morphological, anatomical, and molecular characteristics have been employed to investigate genetic diversity. Morphological traits are commonly used to analyze genetic diversity because they provide a simple way of quantifying genetic variation while assaying genotype performance under growing environments (Fufa et al., 2005). But they have number of limitations, such as low polymorphism, low heritability, and are influenced by stage of plant development (Zeid et al., 2003). Unlike morphological traits, anatomical characteristics seem to reflect diversity and phylogenetic relationships better because they are subjected to environmental constraints to a lesser degree. Vascular system is an anatomical characteristic that is responsible for the transport of water, ions, carbohydrates, and other nutrients (Chen et al., 2006). Kocsis et al. (2004) found considerable variation in leaf anatomical and morphological characters among 10 species of *Rondeletia* and suggested that the combination of anatomical and morphological leaf surface characters can be helpful in the systematics of these species. Hofreiter and Tillich (2002) demonstrated that a great diversity in root anatomy in Commelinaceae provides the possibility to distinguish all genera, most subgeneric groups, and sometimes even species.

Despite morphological and anatomical traits, molecular markers detect diversity at the DNA level, and largely overcome the problems that are associated with morphological-based classification (Awasthi et al., 2004). Among the many molecular methods, inter-simple sequence repeats (ISSR) markers have been successfully used to determine the level of genetic diversity within and among populations. Target microsatellite sequences are abundant throughout the genome and have high potential to determine intra- and intergenomic diversity (Zietkiewicz et al., 1994). Several researchers have used both morphological and molecular markers to estimate genomic diversity in many species including forage legumes, such as alfalfa (Tucak et al., 2008), falcata (*M. sativa* spp. *falcata* L.; Li et al., 2009), and red clover (*Trifolium pratense* L.; Paplauskiene and Dabkeviciene, 2008). In sainfoin, Delgado et al. (2008) found high levels of variation in 44 Spanish sainfoin accessions using 12 morphological traits. Demdoum et al. (2012) reported diversity in 23 sainfoin accessions based on six simple sequence repeat primers and suggested that the pattern of genetic diversity was related to the geographic distribution of the accessions. Carbonero (2011) showed that there was strong correlation between genetic diversity of agro-morphological traits and geographical origins of sainfoin accessions.

Reliable characterization of native plant germplasm and its comparison with exotic accessions is an essential step toward better use of genetic resources in plant improvement programs. Little information is available on genetic diversity of sainfoin, especially using molecular and anatomical markers. The objectives of the present study were: (i) to determine the level of genetic diversity accessions of sainfoin from different geographical regions of Iran and some areas of the world using morphological, anatomical, and molecular (ISSR) markers; (ii) to assess the genetic relationships of Iranian and exotic populations with a focus on their genetic structure; and (iii) to estimate phenotypic and genotypic relationships of morphological traits.

MATERIAL AND METHODS

Plant Materials

Eighty accessions of sainfoin, including 46 Iranian and 34 exotic, were used across three separate studies (56 for morphological, 44 for anatomical, and 75 for molecular study) (Table 1). Iranian accessions were collected from different geographical regions nationwide. Foreign accessions were provided by the gene bank of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) and the United States Department of Agriculture (USDA). All accessions were germinated and grown in a greenhouse in January 2010 before transfer to the field.

Established seedlings of accessions were space-planted in the field according to a randomized complete block design with three replications. Each plot contained 20 seedlings that were planted on 1 Mar. 2010. Finally, 56 accessions, including 46 Iranian and 10 exotic, were established in the field and evaluated during 2010 and 2011. The experiment was conducted on a Typic Haplargid, silty clay loam soil at Isfahan University of Technology Research Farm (32°30′ N, 51°20′ E), Isfahan, Iran. The soil was calcareous, containing 390 g kg^{-1} calcium carbonate equivalent, 5.0 g kg^{-1} organic C, and 0.77 g kg^{-1} total N, with pH 8.3. The soil was nonsaline and nonsodic. The mean annual temperature and precipitation were 14.5°C and 140 mm, respectively. Irrigation supply was nonlimiting and done when 50% of the total available water was depleted from the root zone during the growing season.

Morphological Analysis

Thirteen morphological traits were measured for each accession during the 2 yr of study in three harvests (1 June, 1 July, and 1 August). Traits measured included days to flowering (DF), number of shoots per plant (SPP), number of nodes per shoot (NNS), plant height (PH), panicle length (PL), fresh matter yield per plant (FMY), dry matter yield per plant (DMY), percentage of dry matter yield (PDMY), palatability (P), percentage of leaf (POL), percentage of shoot (POS), and crown diameter (CD). The P, POL, and POS were calculated as ratio of leaf dry matter to stem dry matter, ratio of leaf dry matter to dry matter yield, and ratio of shoot dry matter to dry matter yield, respectively. Reaction to powdery mildew (RPM) was scored on a nominal scale of 1 for sensitive to 5 for resistant (Table 2). The measurements were taken on all of the plants in each plot and the mean of plot was used for analysis.

Table 1. Information for *Onobrychis viciifolia* germplasm investigated in this study.

No.	Geographical location	Code	Longitude (°E)	Latitude (°N)	Experiment†	Morphological cluster	Anatomical cluster	Molecular cluster
1	Iran (Marand)	VICAZmS1	45°06′	38°25′	$E_1E_2E_3$	A	A	A_I
2	Iran (Oshnavyeh)	VICAZaS2	45°06′	37°02′	$E_1E_2E_3$	C	A	A_I
3	Iran (Oshnavyeh 2)	VICAZaS25	45°06′	37°02′	E_1E_3	B	–	A_I
4	Iran (Urmia)	VICAZoS24	45°06′	37°32′	E_1E_3	B	–	A_{II}
5	Iran (Kermanshah)	VICKES3	47°03′	34°18′	$E_1E_2E_3$	A	A	A_{II}
6	Iran (Baft)	VICKEbS33	56°35′	29°13′	E_1E_3	A	–	A_{III}
7	Iran (Asadabad)	VICHAazS5	48°06′	34°47′	$E_1E_2E_3$	A	A	A_{II}
8	Iran (Khomayn)	VICMAkS7	50°04′	33°38′	$E_1E_2E_3$	A	A	A_{IV}
9	Iran (Khomayn 2)	VICMAkS38	50°04′	33°38′	E_1E_3	A	–	A_{III}
10	Iran (Arak)	VICARS17	49°42′	34°03′	E_1E_3	A	–	A_{IV}
11	Iran (Arak 2)	VICARS35	49°42′	34°03′	E_1E_3	A	–	A_{III}
12	Iran (Damavand)	VICTEdS6	52°03′	35°43′	$E_1E_2E_3$	A	A	A_{II}
13	Iran (Aligudarz)	VICLOaS4	49°41′	33°24′	$E_1E_2E_3$	A	A	A_{II}
14	Iran (Aligudarz 2)	VICLOaS11	49°41′	33°24′	E_1E_3	A	–	A_{IV}
15	Iran (Khorramabad)	VICLOkS31	48°21′	33°27′	E_1E_3	A	–	A_{III}
16	Iran (Shahrkian)	VICCHsS12	50°53′	32°16′	E_1	B	–	–
17	Iran (Borujen)	VICCHS15	51°17′	31°58′	E_1E_3	A	–	A_{IV}
18	Iran (Shhrekord)	VICCHsS19	50°51′	32°20′	E_1E_3	A	–	A_{IV}
19	Iran (Gandoman)	VICCHgS21	50°20′	32°06′	E_1E_3	A	–	A_{IV}
20	Iran (Gishnizjan)	VICCHgS22	51°05′	31°31′	E_1E_3	B	–	A_{IV}
21	Iran (Faradonbeh)	VICESfS45	51°12′	32°00′	E_1E_3	A	–	A_{III}
22	Iran (Fereydunshahr)	VICESfS9	50°07′	32°57′	$E_1E_2E_3$	A	A	A_{IV}
23	Iran (Zavareh)	VICESrS16	52°41′	33°34′	E_1E_3	A	–	A_{IV}
24	Iran (Khonsar)	VICESKS10	50°19′	33°13′	$E_1E_2E_3$	A	A	A_{IV}
25	Iran (Khonsar 2)	VICESkS32	50°19′	33°13′	E_1E_3	A	–	A_{III}
26	Iran (Khonsar 3)	VICESkS34	50°19′	33°13′	E_1E_3	A	–	A_{III}
27	Iran (Golpayegan)	VICESgS36	50°12′	33°26′	E_1E_3	A	–	A_{III}
28	Iran (Golpayegan 2)	VICESgS47	50°12′	33°26′	E_1E_3	A	–	A_{III}
29	Iran (Najafabad)	VICESnS37	51°22′	32°39′	E_1E_3	A	–	A_{III}
30	Iran (Semirom)	VICESaS46	51°33′	31°25′	E_1E_3	A	–	A_{III}
31	Iran (Janatabad)	VICESjS26	58°51′	28°21′	E_1E_3	A	–	A_{III}
32	Iran (Esfahan)	VICESkS27	51°41′	32°40′	E_1E_3	A	–	A_{III}
33	Iran (Buinmiandasht)	VICESbS28	50°09′	33°06′	E_1E_3	A	–	A_{III}
34	Iran (Damanefaridan)	VICESdS29	50°07′	32°05′	E_1E_3	A	–	A_{III}
35	Iran (Bardsir)	VICESbS30	56°35′	29°54′	E_1E_3	A	–	A_{III}
36	Iran (unknown)	VICUNS41	–	–	E_1	A	–	–
37	Iran (unknown)	VICUNS42	–	–	E_1	B	–	–
38	Iran (unknown)	VICUNS43	–	–	E_1	B	–	–
39	Iran (unknown)	VICUNS48	–	–	E_1E_3	A	–	A_{III}
40	Iran (unknown)	VICUNS39	–	–	E_1E_3	B	–	A_{III}
41	Iran (unknown)	VICUNS18	–	–	E_1E_3	A	–	A_{IV}
42	Iran (unknown)	VICUNS8	–	–	$E_1E_2E_3$	A	A	A_{IV}
43	Iran (unknown)	VICUNS40	–	–	E_1E_3	B	–	A_{III}
44	Iran (unknown)	VICUNS44	–	–	E_1	A	–	–
45	Iran (unknown)	VICUNS13	–	–	E_1E_3	A	–	A_{IV}
46	Iran (unknown)	VICUNS23	–	–	E_1E_3	A	–	A_{IV}
47	Russia	VICRUS105	105°19′	61°31′	E_2E_3	–	B	B_V
48	Russia	VICRUS112	105°19′	61°31′	E_2E_3	–	C	B_V
49	Russia	VICRUS113	105°19′	61°31′	E_2E_3	–	B	B_V
50	Russia	VICRUS122	105°19′	61°31′	E_2E_3	–	B	B_{VII}
51	Russia	VICRUS131	105°19′	61°31′	$E_1E_2E_3$	C	B	B_{VIII}
52	Russia	VICRUS116	105°19′	61°31′	E_2E_3	–	B	B_V
53	China	VICCHS101	104 °11′	35 °51′	E_2E_3	–	B	B_V
54	Kyrgyzstan	VICGES104	74°45′	41°12′	E_2E_3	–	C	B_V

(cont'd)

Table 1. Continued.

No.	Geographical location	Code	Longitude (°E)	Latitude (°N)	Experiment†	Morphological cluster	Anatomical cluster	Molecular cluster
55	Kyrgyzstan	VICGES108	74°45′	41°12′	E_2E_3	–	C	B_V
56	Kyrgyzstan	VICGHS133	74°45′	41°12′	$E_1E_2E_3$	C	B	B_{VIII}
57	Hungary	VICMAS134	19°30′	47°09′	$E_1E_2E_3$	C	B	B_V
58	Czech Republic	VICCHS103	15°28′	49°09′	E_2E_3	–	C	B_V
59	Czech Republic	VICCHS121	15°28′	49°09′	E_2E_3	–	B	B_{VII}
60	England	VICENS107	01°10′	52°21′	E_2E_3	–	C	B_{VI}
61	England	VICENS110	01°10′	52°21′	E_2E_3	–	C	B_{VI}
62	England	VICENS118	01°10′	52°21′	E_2E_3	–	B	B_{VI}
63	Italy	VICITS125	12°34′	51°52′	$E_1E_2E_3$	C	B	B_{VII}
64	Italy	VICITS126	12°34′	51°52′	$E_1E_2E_3$	C	B	B_{VII}
65	Slovakia	VICASS127	19°41′	48°40′	$E_1E_2E_3$	C	B	B_{VII}
66	Slovakia	VICASS128	19°41′	48°40′	$E_1E_2E_3$	C	B	B_{VII}
67	Slovakia	VICASS129	19°41′	48°40′	$E_1E_2E_3$	C	B	B_{VII}
68	Germany	VICGES119	10°27′	51°09′	E_2E_3	–	B	B_{VII}
69	East Germany	VICGES132	10°27′	51°09′	$E_1E_2E_3$	C	B	B_{VIII}
70	Netherlands	VICHOS130	05°17′	52°07′	$E_1E_2E_3$	C	B	B_{VIII}
71	Switzerland	VICSWS123	08°13′	46°49′	E_2E_3	–	B	B_{VII}
72	Switzerland	VICSWS124	08°13′	46°49′	E_2E_3	–	B	B_{VII}
73	Switzerland	VICSWS117	08°13′	46°49′	E_2E_3	–	B	B_{VI}
74	Romania	VICROS114	24°58′	45°56′	E_2E_3	–	C	B_V
75	Romania	VICROS120	24°58′	45°56′	E_2E_3	–	B	B_{VII}
76	Spain	VICSPS115	03°44′	40°27′	E_2E_3	–	B	B_{VI}
77	Morocco	VICMOS111	07°05′	31°47′	E_2E_3	–	B	B_{VI}
78	Ukraine	VICOKS109	31°09′	48°22′	E_2E_3	–	B	B_{VI}
79	America	VICAMS102	95°42′	37°05′	E_2E_3	–	B	B_V
80	Unknown	VICUNS106	–	–	E_2E_3	–	B	B_{VI}

† E_1 = Morphological experiment; E_2 = Anatomical experiment; E_3 = Molecular experiment.

Table 2. Mean values, range, and phenotypic coefficient of variation (PCV) of agronomical and anatomical traits of sainfoin accessions.

Morphological traits					Anatomical traits				
	Range					Range			
Traits	Min.	Max.	Mean	PCV	Traits	Min.	Max.	Mean	PCV
Days to flowering (DF)	13	67	33.4	28.1	Vessel diameter (mm) (VD)	0.002	0.12	0.06	0.36
Plant height (cm) (PH)	14	90	53.1	40.5	Sieve diameter (mm) (SD)	0.01	0.82	0.03	0.48
No. of shoots plant^{-1} (SPP)	6	102	31.4	59.6	Width of phloem (mm) (WP)	0.014	0.16	0.03	0.57
No. of nodes shoot^{-1} (NNS)	4	9	6.62	16.2	Xylem/phloem ratio (XPR)	0.8	1.75	0.14	0.29
Panicle length (cm) (PL)	3	13.5	6.34	28.2	Width of upper cuticle (mm) (WUC)	0.03	0.12	0.04	0.46
Dry matter yield (g plant^{-1}) (DMY)	9.33	378.3	51.2	71.5	Width of lower cuticle (mm) (WLC)	0.08	0.16	0.05	0.5
Fresh matter yield (g plant^{-1}) (FMY)	28	605.6	167.3	73.2	Width of leaf collenchymas (mm) (WOLC)	0.08	0.41	0.23	0.28
Percentage of dry matter yield (PDMY)	33.3	62.3	40.36	39.4	Leaf hair (LH)	33	355	162.5	36
Percentage of leaf (POL)	12	90	61.5	34.8	Legume length (mm) (LL)	4.79	7.11	6.01	9.7
Percentage of shoot (POS)	8	87	38.3	44.5	Legume width (mm) (LW)	3.77	4.05	4.53	5.45
Palatability (leaf shoot^{-1}) (P)	0.4	4.02	2.8	36.7	Legume length/width ratio (LLW)	0.82	1.4	1.33	11.8
Crown diameter (cm) (CD)	3.5	12	7.4	17.9	Seed length (mm) (SL)	2.83	4.35	3.7	11.6
Resistance to powdery mildew (RPM)	1	5	4	44.7	Seed width (mm) (SW)	2.33	4.35	3.04	14.5
					Seed thickness (mm) (ST)	1.38	2.34	2.06	11.3
					Seed length/width ratio (SLW)	0.97	1.44	1.23	21.4

Anatomical and Seed-related Traits Analysis

For anatomical assessments, half of the accessions were randomly chosen from the germplasm (Table 1) at the flowering stage and the highest leaf and stem of three random plants from each accession were sampled and preserved in a 1:1:1 solution of ethanol:water:glycerol. Cross-sectioning of the samples was done with a sharp razor blade and stained following the Edward (1956) staining method. Stained samples were mounted on microscope slides and examined under a Nikon ECLIPSE E600 microscope (400×). Eight anatomical traits were investigated, including vessel diameter (VD), sieve diameter (SD), width of phloem (WP), xylem/phloem ratio (XPR), width of

Table 3. Information of inter-simple sequence repeats primers, number of total bands (NB), number of polymorphic bands (NPB), percentage of polymorphic bands (PPB), polymorphism information content (PIC), marker index (MI), and resolving power (RP) obtained in 75 accessions of sainfoin.

No.	Sequence (3′–5′)	Annealing temperature	Size range	NPB/NB	PPB	PIC	MI	RP
		°C			%			
1	$(CA)_8G$	52	400–1300	9/10	90	0.34	3.03	4.37
2	$(TC)_8C$	56	350–1400	9/10	90	0.29	2.57	3.92
3	$(TC)_8G$	54	300–1100	6/7	85.71	0.42	2.50	3.92
4	$(AC)_8G$	48	400–1100	11/13	84.62	0.33	2.62	4.88
5	$CA)_8$–RT	46	300–1300	9/12	75	0.35	3.11	4.61
6	$(GA)_8$–RT	51	250–1350	17/17	100	0.38	6.52	9.17
7	$(AC)_7$–DBD	50	200–1300	6/9	66.67	0.45	2.70	2.27
8	$(AG)_7C$	52	200–1100	10/11	90.91	0.24	2.36	2.99
9	$(GA)_8$–SC	57	200–1200	12/14	85.71	0.39	4.71	7.09
10	$(AC)_8C$	48	250–1400	11/13	84.62	0.35	3.85	5.95
11	$(AG)_8$–SG	56	300–1100	11/12	91.67	0.36	3.97	5.78
12	$(GA)_8$–SG	58	200–1100	11/12	91.67	0.44	4.85	8.03
13	$(GA)_8$–WT	47	350–1000	13/14	92.86	0.38	4.95	7.41
14	$(CT)_8$–RG	51	250–1100	11/12	91.67	0.41	4.48	6.88
15	$(GA)_8C$	50	200–1300	12/13	92.31	0.34	4.12	6.40
16	$(AC)_8C$	54	200–1350	13/16	81.25	0.36	4.70	7.39
17	$(GA)_8$–YT	52	200–800	10/11	90.91	0.41	4.07	6.45
18	$(GA)_8$–YC	54	150–1100	9/12	75	0.22	1.97	2.37
19	$(AG)_8$–YT	54	150–1200	18/19	94.74	0.43	4.47	12.08
20	$(GACA)_4$	50	300–1300	10/11	90.91	0.33	5.78	4.85
21	$(GA)_8$–RC	51	300–1400	17/18	94.44	0.3	5.02	6.96
22	$(GACA)_5$	55	300–1400	8/9	88.89	0.33	2.67	4.05

lower cuticle (WLC), width of upper cuticle (WUC), width of leaf collenchymas (WOLC), and number of leaf hairs (LH). Additionally, seven morphological traits related to seed and legume were examined with dial calipers (v.osk 9888) on a random sample of 30 seeds of each accession: seed length (SL), seed width (SW), seed thickness (ST), seed length/width ratio (SLW), legume length (LL), legume width (LW), and legume length/width ratio (LLW) (Table 2).

Molecular Analysis

Young leaf tissues from 75 accessions were harvested and DNA was extracted following the Murray and Thompson (1980) method. Of the 45 ISSR primers screened, 22 produced a higher number of reproducible bands and were selected for the ISSR analysis (Table 3). Polymerase chain reaction (PCR) was performed in a 15-μL total volume solution containing 20 ng total DNA, 1.5 10× PCR buffer, 1.5 mM $MgCl_2$, 0.3 mM dNTP, 2 pM of each primer, and 1 U *Taq* DNA polymerase. The amplification was done in a thermocycler (Bio-Rad) with the following program: initial denaturation at 94°C for 4 min, 35 cycles of 94°C for 1 min, specific angling temperature (Table 3) 45 sec, 72°C for 2 min, and final extension step at 72°C for 7 min. Amplified DNA fragments were separated in a 1.5% agarose gel at 100 W for 2 h in 1× TBE buffer (100 mM Tris–Borate, pH 8.0, 2 mM EDTA) and stained with ethidium bromide.

Statistical Analysis

Analysis of variance and determination of the correlation coefficients for morphological and anatomical traits were performed using SAS statistical software (SAS Institute, 1999). The relationship between geographical characteristics of the accessions (altitude and longitude) with morphological and anatomical traits were also estimated (data not shown). Principal components analysis and cluster analysis (based on Euclidean dissimilarity matrix and Ward's method) were performed after standardization data (*Z* score) using Statistical Package for Social Science software (SPSS version 19.0). Phenotypic coefficients of variation (PCV) were calculated using the following models:

$$PCV = (\sigma_p/\mu) \times 100$$

where σ_p and μ are phenotypic variance and mean of the traits, respectively.

For molecular analysis only sharp and precise bands were scored as 1 for present and 0 for absent as data matrix. The polymorphism information content (PIC), resolving power (Rp), and marker index (MI) of each ISSR marker were computed using the following formulas:

$$PIC_i = (2f_i \times [1 - f_i])$$
(Roldan-Ruiz et al., 2000)

$$Rp_i = \sum|(1 - [2 \times |0.5 - f_i|)]$$
(Prevost and Wilkinson, 1999)

$$MI_i = PIC_i \times N_i \times \beta_i$$
(Powell et al., 1996)

Table 4. Group means of morphological and anatomical traits in each cluster obtained in a sainfoin collection.

Morphological cluster				Anatomical cluster			
	Group				Group		
Traits	A	B	C	Traits	A	B	C
Days to flowering	33.6b†	34.5b	38.1a	Vessel diameter (mm)	0.005b	0.005b	0.01a
Plant height (cm)	54.9b	61.7a	46.3c	Sieve diameter (mm)	0.019b	0.02b	0.05a
No. of shoots plant^{-1}	29.5b	40.9a	31.1b	Width of phloem (mm)	0.02b	0.03ab	0.048a
No. of nodes shoot^{-1}	6.5a	6.4ab	6.2b	Xylem/phloem ratio	0.86b	0.86b	1.1a
Panicle length (cm)	7.9a	8.3a	7.4b	Width of upper cuticle (mm)	0.03b	0.03b	0.05a
Dry matter yield (g plant^{-1})	38.7b	52.1a	37.3b	Width of lower cuticle (mm)	0.039b	0.043b	0.06a
Fresh matter yield (g plant^{-1})	107.3b	164.2a	100b	Width of leaf collenchyma (mm)	0.180b	0.20b	0.33a
Percentage of dry matter yield	37.6a	32.7b	39.6a	Leaf hair	159.1ab	177.1a	132.8b
Percentage of leaf	64.5b	64.5b	72.2a	Legume length (mm)	6.15a	4.8b	4.31b
Percentage of shoot	33.4a	35a	27b	Legume width (mm)	6.3a	4.4b	4.2b
Palatability	1.8b	1.8b	3.6a	Legume length/width ratio	0.97a	1.1a	1.1a
Crown diameter (cm)	0.3b	0.33a	0.31ab	Seed length (mm)	3.5b	3.8a	3.5b
Resistance to powdery mildew	4.6a	3.6b	2.5c	Seed width (mm)	2.6b	3.0a	3.3a
				Seed thickness (mm)	2.1a	2.1a	2.1a
				Seed length/width ratio	1.3a	1.3a	1.1b

† For each trait, means with different letters are significant at $p = 0.05$.

where i is the ith primer, f_i is the frequency of the amplified allele, $(1 - f_i)$ is the frequency of the null allele, PIC_i is the polymorphism information content of the ith primer, N_i is the total bands for the ith primer, and β_i is percentage polymorphic bands of the ith primer. Genetic similarity among all accessions was calculated according to Jaccard (J) similarity index, using the similarity of qualitative data (Simqual) routine of NTSYSpc version 2.02 (Rohlf, 1998), then cluster analysis (complete linkage method) and principal coordinate analysis were performed. Comparison between classification result utilizing morphological, anatomical, and ISSR data was performed using the Mantel test (Mantel, 1967). The cophenetic correlation coefficient was generated by means of the COPH routine to check the goodness of fit between the clusters in the dendrogram and the similarity coefficient matrix. The accessions were divided into eight groups based on different geographical areas and diversity statistics, including gene flow (Nm), gene differentiation coefficient (Gst), Nei and Li's index (H), and Shannon's index (I), and percentage of polymorphic loci was estimated using Popgen software version 1.32 (Yeh et al., 1999). Analysis of molecular variation among the mentioned groups was performed using Arliquin software version 3.0 (Excoffier et al., 2005).

RESULTS

Agro-morphological Analysis

Results of the analysis of variance showed that except for PL (data not shown), large and significant ($p < 0.01$) differences were found among accessions for all the measured traits, indicating high levels of variation in the studied germplasm. The mean, range, and coefficient of variation (CV) were calculated for 15 agro-morphological traits and are presented in Table 2. The FMY (73%), DMY (71%), and PH (59%) had the highest CV and showed the highest variability among the morphological traits. The lowest variability for these traits belonged to NNS (16%) and CD (17.9%) (Table 2).

The dendrogram based on morphological distance matrix of 57 accessions excluded in Table 1. On this basis, the accessions were clustered in three groups (A, B, and C) and the mean value of each group is presented in Table 4. Group A contained 37 Iranian accessions characterized by low mean value for CD, DMY, P, DF, and high RPM (Table 4). The second group (B) consisted of eight Iranian accessions with moderate RPM, high values for PH, CD, SPP, PL, and DMY. The mean value for POL and P was low for the accessions in this group (Table 4). The third cluster (Group C) contained 11 accessions characterized by the highest POL and P, and lowest DMY and RPM (Table 4). This cluster was completely comprised of exotic accessions but one Iranian accession (VICAZaS2) was also located in this cluster.

The first three principal components cumulatively explained 62% of all variation. The first principal component explained 30% of the total variation, with two traits (PDMY and P) being the major contributors according to the corresponding eigenvector value (data not shown). Apparently, the first principal component differentiated the exotic accessions (Group C) with high POL and P from native accessions (Groups A and B) that had lower values for these two traits. The second principal component justified 22% of the total variation and mainly differentiated the accessions according to DMY and SPP (data not shown). According to the second principal component, Iranian accessions were separated into two groups (Groups A and B) (Fig. 1), which had significant differences based on DMY and its components (SPP, PH, and CD).

Anatomical Analysis

There were significant differences among accessions for most of the anatomical characteristics. The mean value, range, and PCV for anatomical traits are presented in

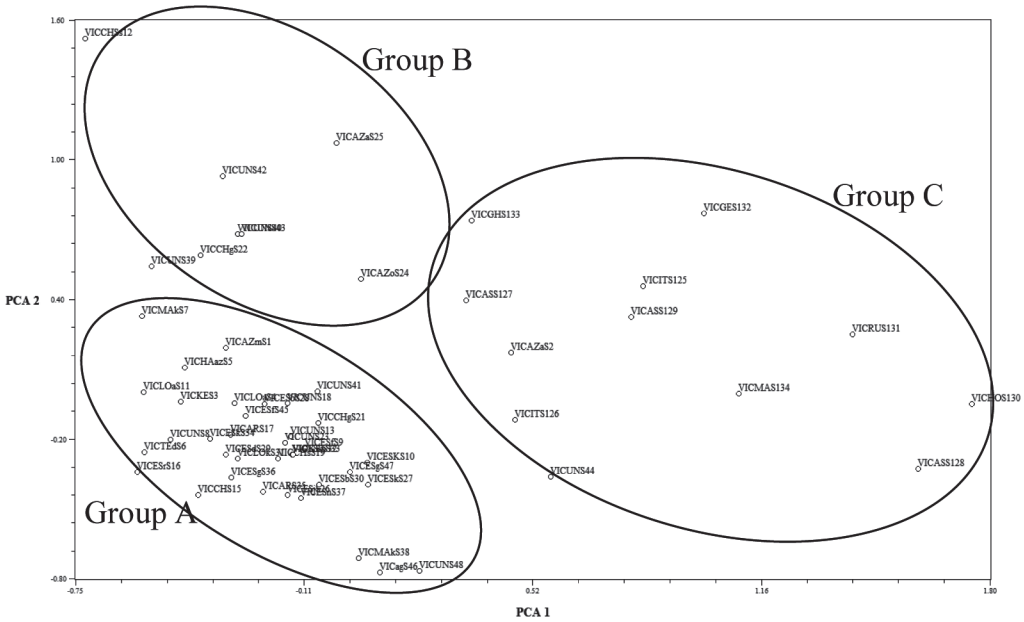

Figure 1. Two-dimensional representation of genetic relationships based on principal components analysis among the 56 *Onobrychis viciifolia* accessions evaluated for 15 agro-morphological traits. The first principal component (PCA1) differentiated exotic accessions (Group C) from Iranian accessions (Groups A and B). In the first component, the eigenvector corresponding to leaf percentage and palatability was the major contributor factor. According to the second principal component (PCA2), Iranian accessions were separated into two groups (Groups A and B), which had significant difference based on dry matter yield and its components (number of shoots per plant, plant height, and crown diameter).

Table 2. On this basis the anatomical traits can be divided into three sets including leaf-, legume-, and seed-related traits. All leaf-related traits, excluding LH (PCV = 36%), had very low PCV (from 0.28–0.57%). For other traits the PCV ranged from 5% for LW to 21% for SLW (Table 2).

The dendrogram based on the cluster analysis of the anatomical assessments is summarized in Table 1. According to this table, the 10 accessions from Group A (all originating from Iran) were characterized by high LL, LW, and SLW (Table 4). Group B contained the highest number of accessions (27 exotic) characterized by high value of LH, SL, and SLW (Table 4). Group C contained seven exotic accessions that were characterized by high values in most of the leaf-related traits, including VD, SD, XPR, WUC, WLC, and WOLC (Table 4).

Molecular Analysis

The 22 chosen ISSR primers amplified 275 bands, of which 243 (88%) were polymorphic across the 75 accessions (Table 3). The amplified DNA ranged from 150 to 1200 bp in size (Table 3). The percentage of polymorphic bands ranged from 67 to 100% for primers $(AC)_7$–DBD and $(GA)_8$–RC, respectively. The polymorphism information content value ranged from 0.22% [$(AG)_8$–YC] to 0.45% [$(AC)_7$–DBD] with an average of 0.36. The marker index, with an average of 3.9, ranged from 2.0 to 6.5 for $(GA)_8$–YC and $(GA)_7$–RT primers, respectively. For resolving power index highest and lowest value belonged to $(AG)_8$–TC and $(AC)_7$–DBD, respectively (Table 3).

Jaccard similarity coefficient between the accessions ranged from 0.053 (VICARS35 vs. VICASS128) to 0.87 (VICKEbS33 vs. VICLOkS31) with an average of 0.45 (data not shown). The complete linkage cluster analysis based on Jaccard similarity matrix is shown in Table 1 and Fig. 2. The breakdown of accessions in the dendrogram (Fig. 2) of molecular data is shown in Table 1, indicating that all 75 accessions are divided into two main groups (Iranian group [A] and exotic group [B]). This grouping was confirmed by principal coordinate analysis (Fig. 3). Moreover, Iranian accessions (Group A) were divided into four subclusters mainly in agreement with their geographical distributions (Fig. 2). These four subclusters included A_I with 3 accessions from Northwest Iran, A_{II} with 5 accessions from West Iran, A_{III} from Central Iran with 19 accessions, and A_{IV} from the middle Zagrous Mountains with 14 accessions (Table 1 and Fig. 2). Exotic Group B was also divided into four subclusters, including B_I from Asia and Eastern Europe with 11 accessions, B_{II} from Western and Southern Europe with 8 accessions, B_{III} from Central and Eastern Europe with 11 accessions, and B_{IV} from Central and Northern Europe containing 4 accessions (Table 1 and Fig. 2).

Analysis of molecular variation revealed a noticeable difference between the two main groups (Iranian and exotic accessions), with 49% of the total genetic variability related to this difference (Table 5). A difference was also observed among (20% for Iranian and exotic groups) and within subclusters (80% for Iranian and exotic groups) (Table 5).

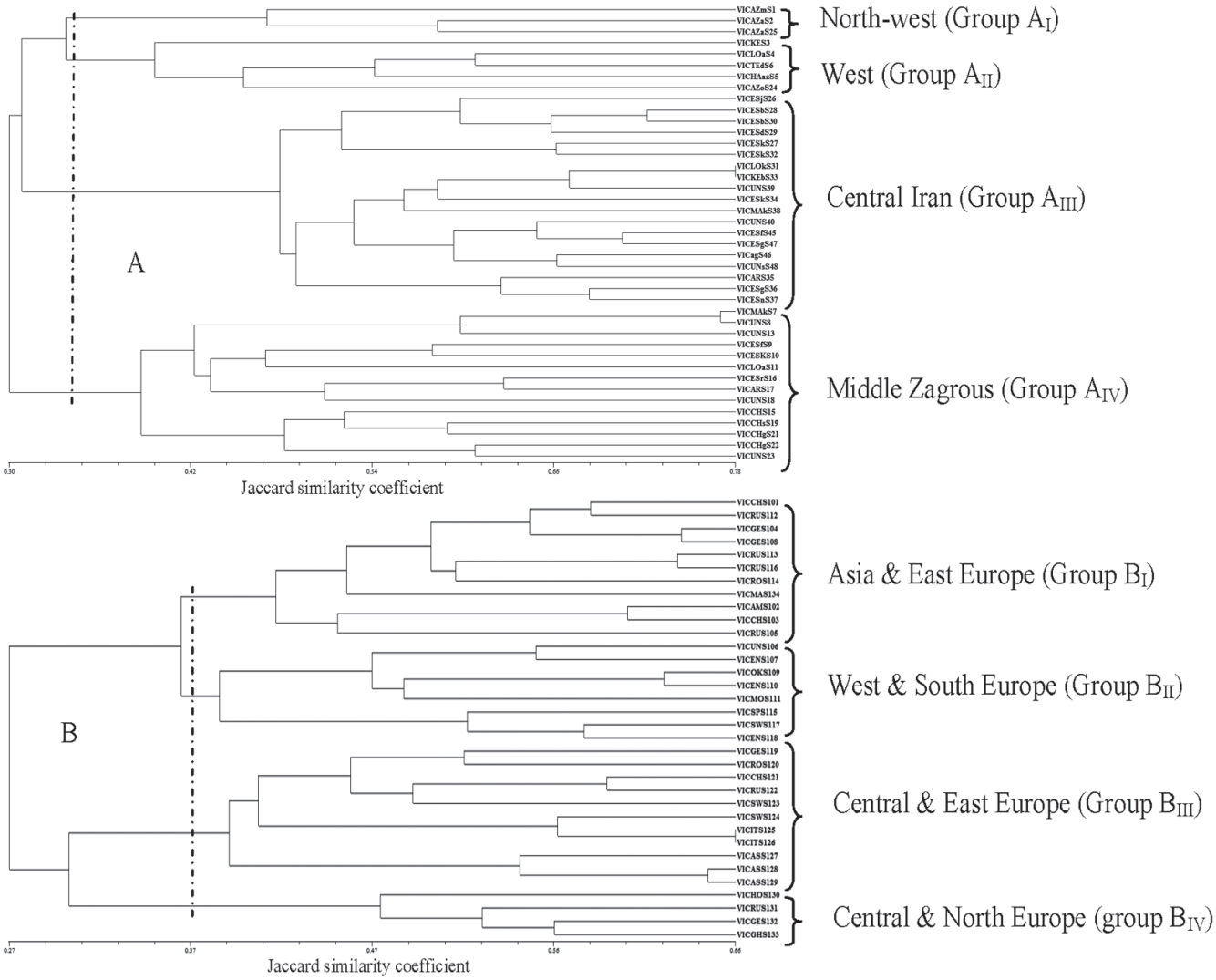

Figure 2. Association between 75 *Onobrychis viciifolia* accessions revealed by complete linkage model and Jaccard similarity coefficient based on 243 polymorphic inter-simple sequence repeats loci. A = dendrogram of Iranian accessions and B = dendrogram of exotic accessions.

Diversity statistics were estimated to compare levels of genetic diversity between the main groups (Iranian and exotic) and among subclusters (geographical regions) (Table 6). Results showed that among subclusters in the Iranian group, middle Zagrous Mountains (H = 0.29, I = 0.44, and p = 81%) had the highest level of genetic variability, while Northwest Iran subcluster (H = 0.23, I = 0.34, and p = 53%) possessed the lowest level. For exotic accessions, Asia and Eastern Europe (H = 0.41, I = 0.46, and p = 85%) and Central and Northern Europe (H = 0.23, I = 0.45, and p = 79.53%) subclusters had the highest and lowest variability, respectively (Table 6).

Correlation between Traits

Phenotypic and genotypic correlation between different variables is presented in Table 7. The DMY had significantly positive correlation with FMY, PH, SPP, NNS, PL, POL, POS, and CD, while its association with P and PDMY was negative. Most of these associations were supported by genotypic correlation coefficients. The exception was genetic association of DMY and PDMY, which was positive (r_g = 0.54), indicating the effect of environment on this association. The PH had high correlation with all traits, with the exception of DF, SPP, and POS. Moreover, SPP was significantly correlated with CD, and CD was correlated with PH, SPP, and DMY (Table 7).

There was no strong phenotypic association between DMY with anatomical leaf-related traits. The best correlation was observed among DMY with WP (r_p = 0.32), which was supported by high genetic correlation (r_g = 0.61). The P as well as RPM had strong correlation with VD and SD, which was supported by genotypic correlations. The VD had positive correlation with PH, PL, RPM, and P, and negative correlation with DF. The XPR had positive correlation with WOLC (r_p = 0.45 and r_g = 0.65) (Table 7). The DF, PH, SPP, NNS, CD, VD, SD, and P were positively correlated with altitude.

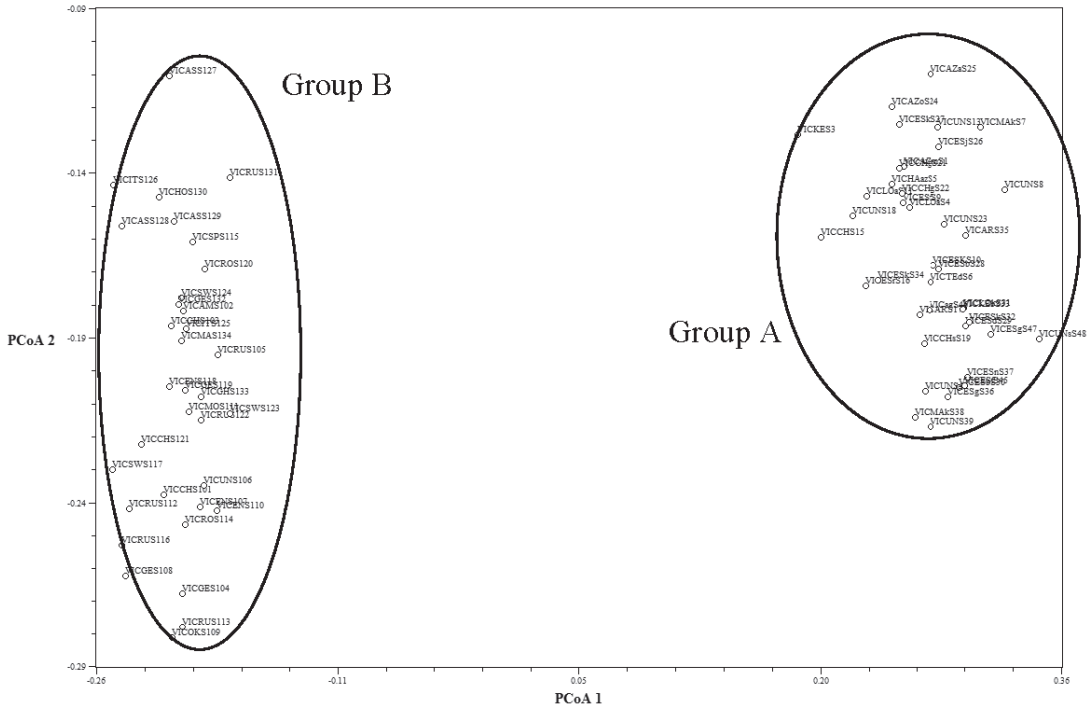

Figure 3. Two-dimensional representation of genetic relationships based on principal coordinate analysis (PCoA) among 75 *Onobrychis viciifolia* accessions based on inter-simple sequence repeats markers. Group A = Iranian dendrogram and Group B = exotic dendrogram.

Table 5. Analysis of molecular variance of 75 *Onobrychis viciifolia* accessions from two main groups (Iranian and exotic accessions).

Source of variation		df	Mean square	Variance component	Ratio of variance	*p*-value†
Group level	Between groups	1	1023.6	35.26	48.91	<0.001
	Within groups	6	403.699	5.33	10.18	<0.001
	Between accessions	67	1462.5	21.82	41.63	<0.001
Iranian group	Between clusters	3	171.3	4.42	19.79	<0.001
	Within clusters	37	662.9	17.91	80.21	<0.001
Exotic group	Between clusters	3	228.8	6.2	19.58	<0.001
	Within clusters	30	765.2	25.5	80.42	<0.001

† Levels of significance are based on 1000 permutations.

Table 6. Diversity statistics from inter-simple sequence repeats markers analyzed in 75 sainfoin accessions.

Group	H†	I	P	Gst	Nm
			%		
Iranian	0.37	0.54	96.77	0.26	1.4
Northwest	0.23	0.34	53.54	–	–
West	0.26	0.4	78.74	–	–
Central Iran	0.28	0.41	70.08	–	–
Middle Zagrous	0.29	0.44	81.89	–	–
Exotic	0.39	0.57	98.39	0.26	1.4
Asia and Eastern Europe	0.41	0.46	85.96	–	–
Western and Southern Europe	0.28	0.41	74.27	–	–
Central and Eastern Europe	0.31	0.33	54.34	–	–
Central and Northern Europe	0.23	0.45	79.53	–	–

† H = Nei's diversity index; I = Shannon's diversity index; P = percentage of polymorphic loci; Gst = gene differentiation coefficient; Nm = gene flow.

Comparison between Agro-morphological, Anatomical, and Molecular Classification

The ISSR data matrix based on Jaccard dissimilarity was significantly correlated with agro-morphological data based on the Euclidean distance coefficient (Mantel test with 1000 random permutations, $r = 0.5$ and $p = 0.0001$). The correlation between anatomical and agro-morphological data was significantly positive ($r = 0.2$, $p = 0.01$). However, there was not a significant correlation between anatomical and molecular data ($r = 0.04$, $p = 0.99$).

DISCUSSION

Various breeding efforts have successfully improved the agronomic performance of both *Medicago* and *Trifolium* species, but little research has been directed toward improving sainfoin varieties in Asia and Europe. In the perennial forage crops, planning and conducting breeding programs requires information as to the level of the genetic

Table 7. Phenotypic (above diagonal) and genotypic (below diagonal) correlation between different traits of sainfoin accessions.

	VD†	SD	WP	XPR	WOLC	LH	DF	PH	SPP	NNS	PL	FMY	DMY	POS	POL	P	PDMY	CD	RPM
VD	1	0.77**	0.46**	0.31*	0.70**	−0.31*	−0.52**	0.58**	−0.07	0.21	0.52**	0.29	0.28	−0.19	0.63**	0.66**	−0.64**	0.13	0.53*
SD	0.99	1	0.53**	0.52**	0.73**	−0.3*	−0.22	0.47**	−0.16	0.12	0.35*	0.28	0.13	−0.30*	0.41**	0.57**	−0.37*	0.11	0.47**
WP	0.90	0.67	1	−0.23	0.35*	0.02	−0.01	−0.01	0.33*	0.03	0.51**	0.19	0.32*	0.10	0.09	−0.07	−0.02	0.48*	0.14
XPR	0.67	0.60	−0.001	1	0.45**	−0.26	0.11	0.35*	−0.01	0.33*	0.26	0.31	0.15	−0.35*	0.15	−0.27	−0.13	0.10	0.32*
WOLC	0.59	0.89	0.56	0.65	1	−0.21	0.06	0.40**	0.18	0.36*	0.56**	0.24	0.22	−0.16	0.44**	0.44**	−0.17	0.32*	0.43**
LH	−0.27	−0.21	−0.01	−0.33	−0.24	1	−0.22	0.02	0.12	0.01	−0.15	0.12	0.17	0.05	0.06	−0.13	−0.13	0.18	−0.20
DF	−0.04	0.34	0.32	0.35	0.47	−0.09	1	−0.20	0.08	−0.08	−0.13	0.01	−0.1	−0.30*	0.43**	−0.47**	−0.18	0.01	−0.44**
PH	0.72	0.55	0.27	0.37	0.57	−0.12	0.21	1	0.19	0.39**	0.67**	0.52**	0.54**	−0.20	0.60**	−0.63**	−0.68**	0.30*	0.61**
SPP	−0.83	−0.31	0.24	−0.27	0.06	0.15	0.01	0.20	1	0.02	0.16	0.55**	0.49**	−0.19	0.12	−0.04	0.03	0.33*	−0.13
NNS	1.09	0.59	0.40	0.39	−0.02	0.61	0.33	0.53	0.29	1	0.44**	0.31*	0.37*	−0.07	0.19	−0.39**	−0.32*	0.11	0.32*
PL	−5.47	−3.77	1.25	−4.71	−4.76	0.72	−0.34	−1.01	−0.40	−0.56	1	0.46**	0.49**	−0.22	0.37*	−0.43**	−0.52**	0.37*	0.27
FMY	−0.63	−0.45	0.73	−0.24	0.31	0.14	0.01	0.30	0.70	0.19	−0.65	1	0.95**	−0.28	0.35*	−0.36*	−0.35*	0.45**	0.01
DMY	−0.24	−0.33	0.61	−0.33	0.24	−0.18	−0.01	0.08	0.26	0.21	−0.15	0.46	1	0.35*	0.34**	−0.35*	−0.41**	0.52**	0.09
POS	0.88	0.54	0.49	0.11	0.43	0.08	0.21	0.61	−0.11	0.49	−0.35	0.08	0.36	1	−0.2	0.18	0.21	−0.14	0.05
POL	−0.88	−0.54	−0.48	−0.12	−0.43	−0.07	−0.21	−0.62	0.15	−0.48	0.36	−0.08	0.32	−0.90	1	0.80**	−0.76**	0.19	0.55**
P	0.89	0.56	0.54	0.10	−0.43	−0.15	−0.20	−0.45	−0.11	−0.49	0.34	−0.09	−0.43	−0.91	0.89	1	0.8**	−0.08	−0.66**
PDMY	0.60	0.17	−0.10	−0.10	−0.16	−0.46	0.03	−0.04	−0.51	−0.15	−0.15	−0.44	0.54	−0.32	0.28	0.44	1	−0.10	−0.61**
CD	0.60	0.11	0.8	0.03	0.55	0.18	0.09	0.68	0.69	0.17	−0.5	0.10	0.15	0.67	−0.65	−0.04	0.5	1	0.05
RPM	0.40	0.50	0.38	0.23	0.38	−0.25	0.25	0.28	−0.77	0.65	−0.02	−0.33	−0.11	0.47	−0.4	−0.01	0.4	0.04	1

* Significant at $p = 0.05$.

** Significant at $p = 0.01$.

† Agro-morphological and anatomical traits abbreviations as indicated in Table 2.

variation for the various characters and their genetic relationship (Majidi et al., 2009). Genetic studies on sainfoin have been slow, and limited knowledge is available on the level of genetic diversity of its germplasm. In this study, for the first time, different accessions were analyzed from a worldwide collection of cultivated sainfoin using morphological, anatomical, and ISSR markers.

Agro-morphological Analysis

Results of the present study showed that wide range of genetic diversity was observed in the studied germplasm, especially for DMY, P, DF, PH, and RPM. This diversity was expected given the wide range of geographic origins and cultivation status of the germplasm, which reflects the impact of climate, landscape, agricultural use, and history on the accessions' phenotypes. A similar agronomic evaluation on a small germplasm from central Italy has also shown considerable diversity in similar traits (Negri and Cenci, 1988). Delgado et al. (2008) reported considerable variation for some morphological traits of Spanish sainfoin accessions. This wide range of sainfoin diversity had been also reported by Carbonero (2011).

Negative correlation found between DF and RPM may indicate that late-flowering accessions are less resistant to this disease (Table 7). Also, DF had negative correlation with P, indicating that early-flowering accessions could produce relatively higher value of leaf/stem ratio (P). However, negative correlation of P with DMY indicated that breeding programs need to overcome this inverse association using suitable selection indices. This negative correlation might be caused by lignin increase in plant shoots in late reproductive stage and falling of old leaves (Sheaffer et al., 2000). The positive correlation found between PH and RPM may indicate that taller plants of sainfoin are more resistant.

According to cluster analysis, confirmed with principal components analysis, three different main groups were found with the exotic accessions, mostly originating from Europe, clustered in one group (Group C) and clearly separated from Iranian accessions in the other two groups (Groups A and B). This reflects the impact of geographic, climate, landscape, agricultural use, and history on the accessions' phenotypes. In this grouping, Iranian accessions (Groups A and B) mainly presented sainfoin populations with early DF, high PH, high NNS, high PL, high DMY, high RPM, and low P. Most European accessions (Cluster C) were characterized by late in DF, high P, and low in RPM and DMY. In this study, some traits were associated with geographic coordinates of collection site. For example, latitude was associated with DF, PH, SPP, and P. In general, a population coming from a lower latitude had late DF, higher PH, and lower palatability than those collected at a higher latitude. Taylor and Smith (1995) and Dias et al. (2008) in white clover (*Trifolium repens* L.) reported that early-flowering populations were predominant at lower latitude. Similar patterns of association among geographical characteristics have been reported by Greene et al. (2004) for red clover and Drobna (2010) for *Lotus*.

Anatomical Analysis

Anatomical characterization has been used to study genetic variation of crop plants, but in sainfoin reports are limited. In this study some leaf-, legume-, and seed-related traits were measured on sainfoin accessions. The plant vascular system has been subjected to numerous studies, not only for its responsibility in transportation but also for constraints in the distribution of resources within the plant (Zwieniecki et al., 2003; Chen et al., 2006). Chen et al. (2006) suggested that the anatomical and chemical characteristics of the foliar vascular bundles are different when the common reed (*Phragmites communis* Trin.) adapted in the long term to different habits. With the exception of LH, leaf-related traits had lower variation in studied germplasm. Hofreiter and Tillich (2002) believe that environmental factors affect the anatomical traits less than morphological traits. However, legume- and seed-related traits showed considerable variation in our study. Karamian et al. (2012) reported that anatomical traits of genus *Onobrychis* showed considerable variation within section. Variation in xylem and phloem has also been reported by Equiza and Tognetti (2002) in wheat (*Triticum aestivum* L.) and Chen et al. (2006) in reed ecotypes. In the present study, different groups of accessions based on cluster analysis of anatomical characters showed high variation leading to different mechanisms of water and solute transport in each group.

Correlations between traits are needed to determine whether selection for one trait will have an effect on another. In addition, selection could be practiced on a highly heritable trait that correlates with a more complex trait (Majidi et al., 2009). There was significant and positive correlation between VD, SD, WP, and WOLC with P. This may indicate that plants with a wider vascular system and better transportation of substance are more palatable, and selection based on these traits may lead to more palatable varieties. The WP had positive association with VD and SD. Sevanto et al. (2011) reported that variation in WP is mainly caused by changes in the radial flow rate of water between the xylem and phloem. This expression of radial water flow between the xylem and phloem has been reported by others (Minchin and Lacointe, 2005; Ohya et al., 2008).

Molecular Analysis

Accuracy of fingerprinting methods strongly depend on the quality of DNA obtained (Carbonero, 2011). The Murray and Thompson (1980) method was successfully used to extract the genomic DNA for ISSR analysis in sainfoin. Twenty-two ISSR primers yielded 239 reproducible and polymorphic bands, indicating that this technique is a powerful molecular marker tool for analysis of genetic diversity in sainfoin. In higher plants (seed plants), ISSR analysis has been used to detect the organization, frequency, and level of polymorphism, in which dinucleotide motifs are more common than tri-, tetra-, and pentanucleotide motifs and within dinucleotides class the poly (GA) motifs is more variable than poly (GT) (Nagaoka and Ogihara, 1997; Pradeep Reddy et al., 2002; Wang et al., 2006). Our results showed that there was no (GT) motif amplified in sainfoin accessions studied. Moreover poly (GA)–anchored ISSR motif produced more bands than poly (AG) motif. These results indicated that the poly (GA) motif is the most frequent dinucleotide class in sainfoin genome.

Results of diversity parameters showed that the level of genetic diversity for Iranian sainfoin accessions was as high as the accessions from the rest of the world. This may indicate that Iranian accessions are located in the primary diversity center of sainfoin. Moreover, Asian and Eastern Europe accessions were more diverse than the accessions from other regions, supporting the idea that Asia and Eastern Europe may be the main center of diversity for this species. It has been also reported by Yildiz et al. (1999) that Iran and Anatolia appear to be the center of diversity of this species.

All accessions clustered into two main clusters (Iranian and exotic), each divided into four subclusters. This clear separation is mainly due to presence of specific and exclusive alleles in each group. Results of analysis of molecular variation indicated that almost 20% of variation existed between these two main groups. This was confirmed with genetic differentiation coefficient (Gst = 0.26) and the limited interarea gene flow (Nm = 1.4). According to Nei (1978) when Gst is higher than 0.15, there is strong genetic differentiation between two populations. Wright (1931) proposed strong differentiation between populations when Nm is higher than 1 and less than 4. High-genetic diversity was also found among accessions within each area (Iranian and exotic). Based on the ISSR data, accessions with a similar geographic origin were generally grouped in the same cluster. However, some accessions did not reveal any correlation between geographic origin and genetic diversity, which can be due to heterogeneity, the dominant nature of ISSR markers, and emigration in cross-pollinated crops (Roldan-Ruiz et al., 2000).

Within Iranian accessions, those derived from west of the Zagrous Mountains (Northwest [Group A_I] and West [Group A_{II}]) had more similarity compared with the other two groups. It can also be assumed that the isolation by Zagrous Mountains and low seed exchange is the main reason for separation of accessions belonging to central Iran from accessions of North and West Iran. Within exotic accessions, cluster analysis showed that Asian and Eastern European accessions were mainly clustered together (Group B_I). Results support the hypothesis that sainfoin accessions in European countries originated from an area between Asia and Eastern Europe. Demdoum et al. (2012) used SSR markers on a European sainfoin germplasm and

reported that part of seed demand is met with imports from Eastern European countries.

High genetic differentiation among populations can be closely related to different facts such as long lifetime, genetic drift, effective size, population structure, breeding regime, and population isolation (Feng et al., 2009; Li et al., 2010). In this study the high level of population differentiation may be complying with theoretical prediction from an "isolation by distance" model (Wright, 1943; Su et al., 2009). In this model total population is assumed to be divided into subgroups, each breeding at random within itself. Pfeifer and Jetschke (2006) reported that geographic isolation influenced genetic variation by limiting the number of gene flows via both pollen and seed. Although no clear study has been done on seed or pollen dispersal in sainfoin, the morphology of seed and bee pollination suggest that they could not disperse long distances. The observed diversity patterns suggested that rare gene flow among areas within each ecotype existed, and this low gene flow probably led to genetic diversity. Li et al. (2009) reported that different physical conditions can lead to variation in fruit ripening and flowering asynchrony. Zong et al. (2008) proposed that long distance of population caused genetic diversity in *Dysosma pleiantha* (Hance) Woodson population.

Relationship between Different Markers

The relationship between morphological and molecular markers has been the subject of several studies (Fufa et al., 2005, 2008). In this study anatomical data had low association with morphological traits, which may be due to a small number of anatomical traits and different effective rate of natural selection on morphological and anatomical traits. The ISSR data significantly correlated with morphological ones ($r = 0.5^{\star\star}$), consistent with the findings of Fu et al. (2008) in *Dianthus* but was not in agreement with Dias et al. (2008) in *Trifolium*. However, there was no coincidence between the ISSR data and anatomical assessments in the present study. Several reasons may account for the discordance of the anatomical and ISSR trait markers. First, the anatomical variation observed may be due to different combinations of alleles producing similar phenotypes and may be associated with environmental effects and therefore are not proportional to the underlying genetic differences (Johns et al., 1997). Second, some of the characteristics with adaptive value may have accumulated in specific habitats subjected to similar ecological conditions (Steiner and Los Santos, 2001). Third, the evolutionary rates of anatomical and morphological traits with adaptive value and those originating from selectively neutral DNA can be different (Linhart and Grant, 1996). Although molecular markers are more reliable than morphological traits for assessing genetic diversity and genetic relationship (Fu et al., 2008), morphology still has the advantage of providing a direct tool for gauging plant performance.

CONCLUSION

In the present study, the sainfoin germplasm used revealed a wide range of variability in morphological, anatomical, and ISSR characteristics that can be exploited for genetic studies and breeding programs. Genetic diversity for Iranian accessions was as high as the accessions from the rest of the world, indicating that they are located in the primary center of diversity. Also, Iranian accessions were significantly higher based on DMY and its components. Accessions VICLOaS11, VICLOaS12, and VICLOaS44 had the highest forage yield, which can be included into future breeding programs. Accessions with lowest similarity (e.g., VICARS35 and VICASS128) may be useful for developing an ISSR linkage map population. The high association of morphological and ISSR markers but low correlation of anatomical and ISSR data indicated that these approaches provide complementary information for sainfoin breeding. The cross-pollinated nature of sainfoin makes breeding efforts generally focus on the development of synthetic cultivars and improved heterogeneous populations. Therefore, information generated from different data sets can be used to select parental combinations more precisely for development of superior synthetic varieties. Dias et al. (2008) suggested that a breeding program can be started with any morphological trait without inbreeding depression when morphological diversity confirmed the molecular diversity. In large breeding programs, since the identification of diversity based on morphological characters is costly and time consuming and may be influenced by environmental effects or epistatic interactions, selection based on molecular marker diversity may be an appropriate means to improve first-generation progenies.

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