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Genetic Properties of Milk Thistle Ecotypes from Iran for Morphological and Flavonolignans Characters

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Abstract: The aim of present study was to investigate the genetic variation within and between 32 milk thistle ecotypes collected from northern (23 accessions) and southern (9 accessions) regions of Iran along with two introduced varieties, CN seeds and Budakalaszi, for morphological and flavonolignans properties. The two collections were assessed at separate field experiments. MANOVA for all the morphological traits showed significant difference between ecotypes. Univariate ANOVA verified these differences for most of the traits in the northern ecotypes (first collection) while for southern ecotypes no significant differences were obtained for the studied traits except seed yield. Among and within ecotypes genotypic coefficient of variation indicated higher level of variation among ecotypes than within ecotypes. In both of the experiments, there was a large genetic variation for silybin and silymarin quality and quantity. Cluster analysis of 34 accessions was performed for morphological traits and silymarin and silybin characteristics, separately. The resulting dendrogram based on silybin and silymarin characteristics revealed that the native accessions such as Dezfoul, Fereydounkenar and Nour, had highest flavonolignans and they were better than the foreign varieties. Also, there was no clear relationship between clustering based on morphological traits and flavonolignan compounds.

Key words: Cluster analysis, genetic variation, MANOVA, *Silybum marianum* L.

INTRODUCTION

Silybum marianum (L.) Gaertner., Milk thistle, is a plant of the Asteraceae family, grows natively in the Mediterranean (Morazzoni and Bombardelli, 1995; Leng-Peschlow, 1996) and is widespread in other regions in the world including Iran. Since the 4th century B.C., milk thistle extracts were used and became a favored medicine for hepatobiliary diseases in the 16th century (Burgess, 2003). Importance of milk thistle is for their flavonolignans which is mainly exist in the fruits (achenes). An isomeric mixture of at least three flavonolignans (silychristin, silydianin and silybin) is called silymarin. The dried fruits contain 1-4% silymarin (Murphy *et al*., 2000). There is considerable pharmacological interest for strong antihepatotoxic and hepatoprotective activity of silymarin. Recent studies have shown that silymarin acts as antichlosterolaemic agent (Krecman *et al*., 1998), also it is anticipated that milk thistle extracts could decrease liver damage caused by anti-HIV drugs (Anonymous, 2002). The silymarin flavonolignans protect liver cells against oxidative stress and tissue damage (Rodriguez-Perez *et al*., 2002; Skottova *et al*., 2004) which reduce cell death by cytotoxins and among the flavonolignans, silychristin and silydianin was reported to have best protection capacity in this regard (Dvorak *et al*., 2003). The main component of silymarin is silybin, both quantitatively (Cacho *et al*., 1999) and therapeutically. Besides the hepatoprotective activity, silybin is a useful agent in intervention of hormone refractory human prostate cancer (Zi and Agarwal, 1999).

At present, 80% of the world's population relies largely on plant based drugs for their health care needs (Anonymous, 2002). For milk thistle, American products as dietary supplements are sold in a US $ 45,000,000 industry, while the European Union regulates them as therapeutic products (Wallace *et al*., 2003b). Despite the economical and pharmaceutical values of milk thistle, efforts on domestication and breeding of this plant have been low (Ram *et al*., 2005). In order to initiate a breeding program it is necessary to study the genetic properties of the available populations. Accessions classification and identification of subsets of core accessions with respect to specific breeding purposes would be made by genetic diversity analyses (Mohammadi and Prasanna, 2003). In Iran, milk thistle is spread in different geographical

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regions, nevertheless there are no reports concerning the amount of its genetic diversity. This research report about genetic diversity of milk thistle ecotypes from different latitudes of Iran for morphological characters as well as silybin and silymarin content.

MATERIALS AND METHODS

Plant material used in this study consisted of 32 ecotypes of S. *marianum* L. These ecotypes were collected from different parts of Iran during May 2002 to June 2003. Twenty three ecotypes were collected from north and north west and the remaining from west and south west regions in 2002 and 2003, respectively (Fig. 1). The first group (23 ecotypes) together with two introduced varieties, Budakalaszi (Hungary) and CN seeds (England), were cultivated for two years, (March 2003-2004), in the Research Station of Faculty of Agriculture, University of Tabriz (38°05' N latitude and 46°17' longitude), Iran. Seeds from ten single plants of each ecotype were separately harvested in the first year and were, then, planted in one row plots as a family during the 2004 cropping season. Therefore, about 250 families composed of 25 ecotypes were analyzed for morphological traits. However, only 175 families (7 families from each ecotype) were chosen for flavonolignan assays. The experiment was performed using nested split plot design as the families were nested in ecotypes with two replications. Also, for the 2004 growing season, the remaining ecotypes (9 accessions) together with the introduced variety, Budakalaszi, as control were grown in the field. These accessions were evaluated in a Randomized Complete Block Design (RCBD) with three replications. In both experiments, the plant density was ≈66666 plant ha^{-1} using the row width of 75 cm and the plant spacing of 20 cm. Agronomic practices were performed according to the earlier reports (Hammouda *et al*., 1993; Omer *et al*., 2003).

Silymarin was extracted by standard method (Quaglia *et al*., 1999; Wallace *et al*., 2003b). Flavonolignans content was measured by HPLC using a Knauer system which composed of K1001 pump, K2501 UV detector and Marathon autosampler. Silymarin components were separated by Perfectsil Target (125 m×4.0 mm, 5 µ ID) with a flow rate of 1 mL min^{-1} and the substances were detected by absorption at 288 nm. The mobile phase was applied in a gradient manner (Table 1). Data collection and processing were done digitally by Chromgate software.

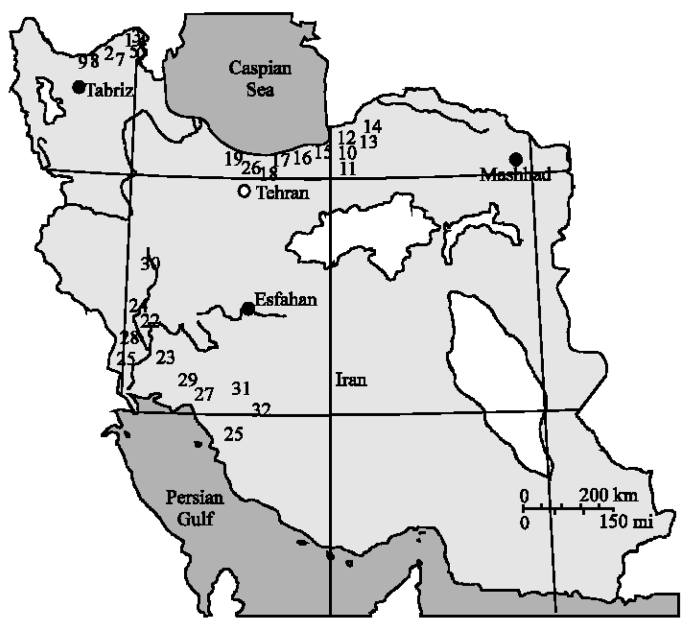

Fig. 1: Geographic distribution of sampling localities of milk thistle in Iran. 1-Pars Abad (30 m), 2-Ghara Khieh (240), 3-Babak (60), 4-Bilesavar (100), 5-Rouhkandi (30), 6-Anjirlou (150), 7-Ghara Aghaj (600), 8-Tatar (320), 9-Gharachilar (420), 10-Gorgan (200), 11-Naharkhoran (420), 12-Aghghala (110), 13-Azad Shahr (120), 14-Gonbad (120), 15-Kordkouy (100), 16-Behshahr (90), 17-Sari (400), 18-Ghaemshahr (20), 19-Nour (30), 20-Mahmoud Abad (20), 21-Fereydoun Kenar (40), 22-Dezful (180), 23-Mollasani (20), 24-Andimeshk (200), 25-Abpakhsh (20), 26-Hamidieh (0), 27-Behbahan (300), 28-Shoush (85), 29-Ramhormoz (90), 30-Jolge Khalaj (780), 31-Nour Abad (980), 32-Ghaemieh (800)

Table 1: Program of solvent systems in HPLC analysis of flavonolignan compounds in milk thistle

Time (min)	Acetic acid (1%)	Acetonitrile (%)	Methanol (%)	Water (%)
00.00	63	15	22	0
07.50	63	15	22	0
08.50	40	20	40	0
15.00	40	20	40	0
15.30	0	0	0	100
24.00	0	0	0	100
24.30	63	15	22	0
30.00	63	15	22	0

Morphological characters measured were: plant height, seed weight/capsule, seed number/capsule, capsules number/plant, 1000 seed weight, flowering date, capsules diameter, seed yield and yield (kg ha^{-1}). Beside these characters silybin and silymarin content (%) were also recorded. Before data analysis, Kolmogorov-Smirnov test (Steel and Torrie, 1980) was performed to test the normality of errors. For all the traits the distribution was normal, except 1000 seed weight which logarithmic transformation was applied. For assessing differences between ecotypes, Multivariate Analysis of Variance (MANOVA) was used at first considering all the morphological characters measured., then univariate analysis was done to determine variation among and within populations and also to calculate genetic parameters including heritability, genetic gain (with 5% selection intensity) computed from total phenotypic variance, genotypic and phenotypic coefficient of variation for within, between and total variation of the ecotypes (Allard, 1960; Wricke and Weber, 1986). Cluster analysis was performed on morphological characters and also on silymarin and silybin content, separately, for grouping the accessions. All statistical analyses were conducted by SPSS 11.5 software.

RESULTS

Differences between ecotypes with respect to all the morphological characters were determined at first by MANOVA in both experiments. The results of multivariate analysis indicated that there were significant differences between the ecotypes in the first experiment at 0.01 probability level (Table 2). In the second experiment (southern ecotypes), except Pillai's trace, others (Wilks lambda, Roy's greatest root and Hotteling's trace) were significant at 0.05 probability level (Table 3). Although MANOVA was significant in both experiments, univariate ANOVA for the second experiment showed no significant difference among ecotypes for all the morphological traits except seed yield while in the first experiment the ecotypes were significantly differed for some traits including 1000 seed weight, flowering date, capsule diameter and seed weight/capsule. To estimate the genetic variation among ecotypes in univariate analyses, the data from the both experiments were included. Means of the studied characters and the genetic parameters such as heritability, genetic gain and genotypic coefficient of variation, for 23 ecotypes

Table 2: Multivariate analysis of variance for morphological traits in the milk thistle ecotypes collected from north and north west of Iran (experiment 1)

Effects	Pillai's trace	Wilks' lambda	Hotelling's trace	Roy's greatest root
Rep	0.430**	0.570**	0.755**	0.755**
Ecotype	2.042**	0.056**	4.771**	3.048**
Rep*Ecotype	1.243**	0.247**	1.583**	0.483**
Family (Ecotype)	4.168**	0.003**	9.054**	1.700**

** Significant at 0.01 probability level

Table 3: Multivariate analysis of variance for morphological traits in the milk thistle ecotypes collected from west and south west of Iran (experiment 2)

Effects	Pillai's trace	Wilks' lambda	Hotelling's trace	Roy's greatest root
Rep	1.174*	0.144*	3.753*	3.021*
Ecotype	3.217^{ns}	0.003*	14.464**	7.510**

* and ** Significant at 0.05 and 0.01 probability level, respectively, ns: non significant

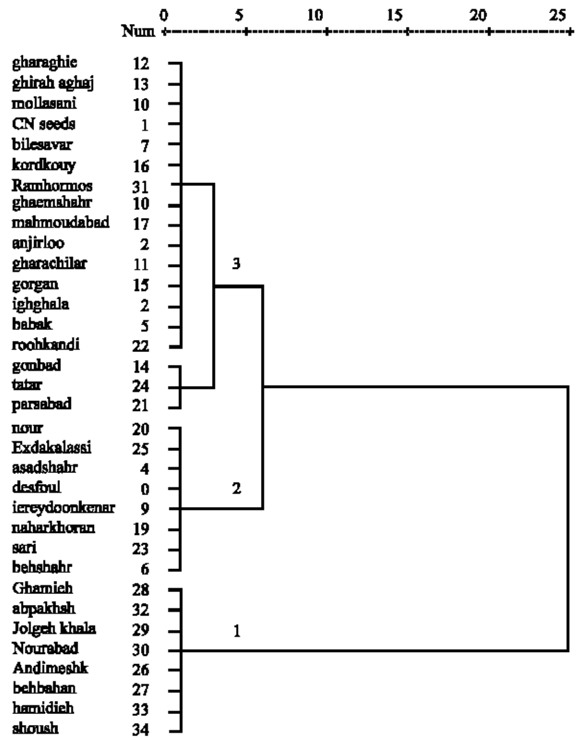

Fig. 2: WARD's clustering based on squared Euclidean distances among 34 milk thistle accessions for morphological traits. 1, 2 and 3 are the cluster numbers

Table 4: Morphological and flavonolignan properties of milk thistle in the ecotypes collected from north and north west of Iran (experiment 1)

Characters	Mean	Heritability (%)	Genetic gain as % of mean	Cvg[a] Within	Cvg[a] Between	Cvg[a] Total	CVp[b] Within	CVp[b] Between	CVp[b] Total
1000 seed weight (g)	16.98±1.23	85.68	8.68	0.000	4.5570	4.557	1.551	4.672	4.923
Flowering date (day)	111.19±2.91	90.19	5.18	0.319	2.6320	2.651	0.661	2.712	2.792
Capsule diameter (cm)	4.29±0.36	51.29	4.19	0.613	2.7770	2.844	1.956	3.456	3.971
Seed weight/capsule (g)	1.48±0.48	68.38	21.44	2.731	12.2910	12.591	7.680	13.147	15.226
Silybin content (%)	6.67±2.34	75.70	111.04	20.328	68.3457	71.305	39.963	68.345	79.172
Silybin yield (kg ha^{-1})	0.43±0.15	81.05	129.63	7.152	13.4277	15.214	19.126	21.252	28.592
Silymarin content (%)	23.13±5.46	34.47	21.40	6.143	62.4982	62.799	36.261	62.498	72.255
Silymarin yield (kg ha^{-1})	1.44±0.25	56.87	31.09	0.000	17.7695	17.769	24.719	20.300	31.986

[a]Genotypic coefficient of variation (%) [b]Phenotypic coefficient of variation (%)

Table 5: Morphological and flavonolignan properties of milk thistle in the ecotypes collected from west and south west of Iran (experiment 2)

Characters	Mean	Heritability (%)	Genetic gain as % of mean	CVg[a]	CVp[b]
Seed yield (kg ha^{-1})	883.870±200.16	46.33	51.15	36.47	53.56
Silybin content (%)	4.584±2.643	27.24	63.76	18.25	23.97
Silybin yield (kg ha^{-1})	0.520±0.246	52.12	70.17	55.18	82.33
Silymarin content (%)	14.157±6.265	45.69	63.61	51.59	67.60
Silymarin yield (kg ha^{-1})	1.520±0.383	29.16	39.31	55.03	45.95

[a]Genotypic coefficient of variation (%), [b]Phenotypic coefficient of variation (%)

together with the two introduced varieties (the first experiment) are presented in Table 4. Also, genotypic and phenotypic coefficients of variation between and within populations are shown. There was considerable genetic variation between and within the studied ecotypes. Broad sense heritabilities for flowering date, 1000 seed weight, silybin content and yield and seed weight/capsule were high and for capsule diameter, silymarin content and yield were moderate. Genetic gain was considerable for silybin and silymarin yield and content, seed weight per capsule and 1000 seed weight. Seed weight/capsule as an important yield component showed high heritability in this study.

Genetic parameters of the ecotypes from second experiment for seed yield, silybin and silymarin content and yield are shown in Table 5. Contrary to the northern ecotypes, genetic parameters such as heritability (46%) and genotypic coefficient of variation (36.47%) of seed yield were medium in the southern ecotypes.

Thirty four accessions were grouped using Ward's algorithm on morphological traits, silybin and silymarin. Clustering based on morphological traits separated the accessions into three separate groups (Fig. 2). The collected ecotypes from southern parts were assigned in cluster 1 and cluster 2 contained most of the northern ecotypes. Cluster analysis based on silybin, also revealed three clusters. Members of the second cluster, Dezfoul, Fereydounkenar and Nour had highest mean of silybin content (1.10%) (Fig. 3). Average of silybin content of the first and third clusters were 0.66 and 0.24%, respectively. Foreign varieties (Budakalaszi and CN seeds) were included in the third cluster which had lowest mean of the flavonolignan component. Grouping the ecotypes using silymarin also provided three groups which approximately

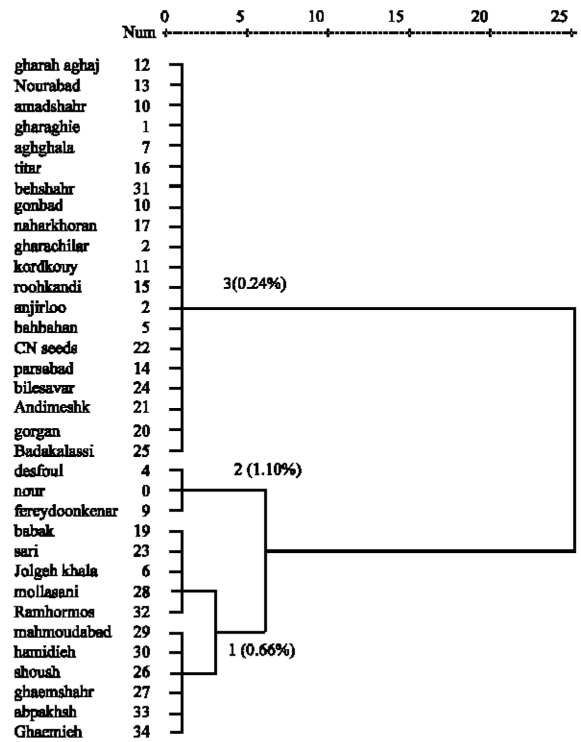

Fig. 3: WARD's clustering based on squared Euclidean distances among 34 milk thistle accessions for silybin content. The digits indicate the cluster numbers and cluster means for silybin percent (within parenthesis)

agreed with the grouping based on silybin (Fig. 4). In fact three members of the second cluster (with the mean of 2.21%), Fereydounkenar, Dezfoul and Nour, were same as the second cluster of grouping of ecotypes using

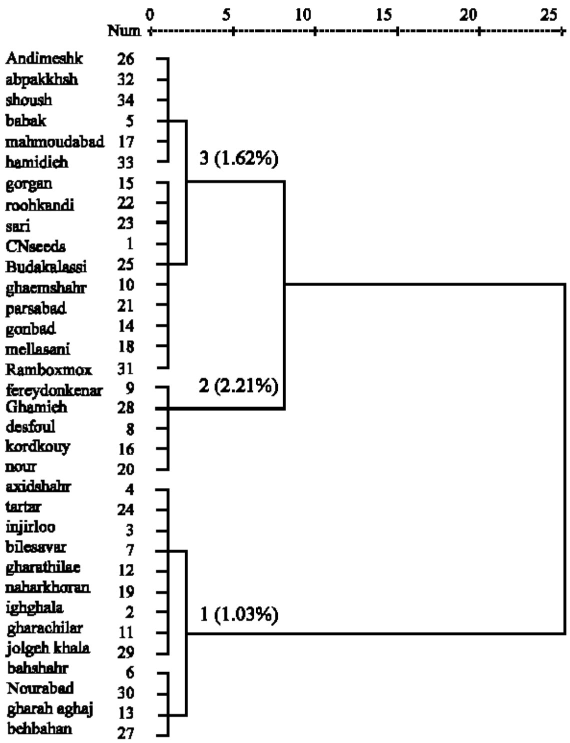

Fig. 4: WARD's clustering based on squared Euclidean distances among 34 milk thistle accessions for silymarin content. The digits indicate the cluster numbers and cluster means for silymarin percent (within parenthesis)

silybin component (Fig. 3). The member of third cluster had moderate silymarin content (1.62%) which included introduced varieties.

DISCUSSION

There were two important reasons for conducting a multivariate analysis (Sharma, 1996): 1) If all the univariate tests are independent, then overall type I error will be much higher than the chosen alpha and 2) It is possible that the multivariate test is significant even though none of the univariate tests are significant. The high estimates of genetic parameters such as genetic gain and heritability for some morphological and flavonolignan characters at first experiment ecotypes suggested a good potential for improving these traits in a breeding program. Also, seed weight/capsule could be consider as a essential trait, therefore, to increase seed yield by indirect selection in segregating generations. Ram *et al.* (2005) reported that seed yield/plant and capsules/plant had highest genotypic and phenotypic coefficient of variation.

Silybin and silymarin content and yield were lower than those reported in earlier studies (Hetz *et al.*, 1995; Kazmierczak and Seidler-Lozykowska, 1997; Wallace *et al.*, 2003a). Flavonolignan accumulation in seeds depends on the stage of flower development and is maximum at the late flowering time (Carrier *et al.*, 2002). Also, the relative amounts of silymarin compounds change according to the weather and climatic conditions (Hetz *et al.*, 1995; Morazzoni and Bombardelli, 1995). Therefore, these differences may be caused by different genetic material and partly by different climatic conditions in which plants were grown.

Comparing genotypic coefficient of variation between and within ecotypes indicates that the largest proportion of existing variation for most of the characters under study belongs to between ecotypes. Hetz *et al.* (1993) found that milk thistle is a self fertile species with a low outcrossing ratio (1.06-2.79%). According to propagation system, genetic variation is expected to be higher among populations than within (Jaradat *et al.*, 2004). Contrary to the northern ecotypes, good genetic variation of seed yield in the southern ecotypes stated the possibility of improving seed yield in these populations by an efficient selection programs.

Clustering based on morphological traits indicated that the ecotypes within each area were more similar with respect to morphological traits than between areas. It seems that morphological traits are somewhat affected by climatic conditions. The grouping on the flavonolignan compounds stated that some native ecotypes performed better than introduced varieties for both silybin and silymarin content which may be attributed to unfavorable climatic conditions at the experimental site. Although there was genetic diversity among ecotypes for flavonolignan compounds but no concordance was obtained between geographical regions of ecotypes and their grouping based on silymarin and silybin contents. Further experiments, therefore, should be conducted in several years and locations to assess more precisely the capability of the ecotypes and varieties under study.

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