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Original Article

Genetic Relationships among Three Yarrow Species Based on Phenotypic Traits and Peroxidase Profiling

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Abstract

Fifteen yarrow populations from different species *Achillea millefolium* L., *A. biebersteinii* L. and *A. nobilis*, from different geographical areas of Iran were studied using 24 morphological traits and peroxidase profiles. Comparison of mean values of different phenotypic traits show *A. millefolium* and *A. biebersteinii* L. had higher plant height and crown diameter; however, *A. nobilis* had higher dry matter yield and 1000-grain weight. Clustering pattern, made on the basis of different phenotypic traits, grouped the *Achillea* populations differently and gave no clear indication of origin or species. The results of peroxidase profiles revealed that the genetic diversity of *A. nobilis* samples was considerably higher than in *A. millefolium* and *A. biebersteinii*. Principal coordinate analysis revealed a clear separation between the different *Achillea* species. The results demonstrated that the study of genetic diversity and relationships among *Achillea* species using phenotypic traits and peroxidase profiles provides important information for the collection, conservation and the planning of future breeding programs.

Keywords: *Achillea*, Genetic diversity, Iran, Morphology, Peroxidase

Introduction

The genus *Achillea* (Yarrow) is one of the youngest evolutionary genera of Compositae family which is spread all around the world. More than 100 species were recognized in this genus [1]. Different species of *Achillea* are medicinal perennial herbs and native to Europe and western Asia. *Achillea* with nineteen species including seven endemic species distributed all along Iran [2].

Yarrow has medicinal and cosmetic uses, and extensively grown in drought-prone environments due to its numerous leaf and several stems developed from the rhizome [3]. Due to over collection, essentially in the flowering period, land conversion and also land degradation, the *Achillea* species are considered now at risk for local extinction, which affect greatly their financial income and subsequently their livelihoods. Many healers recognized that recently the species become very scarce and that in order to ensure the sustainable utilization and to meet the growing demand of these wild species, it has become necessary, therefore, to develop rapid methods of their commercial cultivation. A few studies have been conducted on *Achillea* species in different ecological conditions of Iran and revealed that there was considerable variation in morphological traits [4].

Knowledge of genetic variability and relationships among traits is necessary for facilitating the transfer of useful genes and maximizing the use of available germplasm resources. The extent of genetic diversity in germplasm can be assessed through morphological characterization and genetic markers. The characterized material then helps the plant breeders to select the accessions to be utilized in hybridization program [5]. Previous researchers

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in various species of *Achillea* genus established the presence of the great chemotypic variations throughout the species [6]. Consequently, to exploit this genetic diversity in a breeding program, an efficient evaluation scheme needs to be employed.

Variations in Yarrow essential oil compositions, morphological features, distributional patterns, adaptive and agronomic characters, and allozymes are well documented [7-13]. DNA profiling techniques that have been successfully used in assessing genetic diversity and relatedness of Yarrow germplasm include randomly amplified polymorphic DNA (RAPD) markers [12,14], inter-simple sequence repeat (ISSR) [15,16], SSRs [17] and AFLPs [18]. However, there is a lack of information on the genetic diversity and differentiation of Iranian Yarrow wild populations using isozyme markers. Isozyme or allozyme markers represent electrophoretically detectable forms of enzymatic proteins visualized by substrate-specific staining. Allozymes are alternative enzyme forms encoded by different alleles at the same locus, which can be used as genetic markers. Examining allozyme variation, which results from changes in protein coding DNA sequences, has been the most common technique in plant population biology [19,20]. Isozyme analysis has several advantages [21-23] as compared not only with metric characters (morphological and physiological ones), but also with the other genetic markers as well. Isozymes are mostly co-dominant with a simple Mendelian inheritance in most loci, so that the frequency of individual alleles is directly countable. Besides, isozyme patterns of most enzyme systems are independent of environmental variation and many isozyme patterns are ontogenetically stable.

Based on Peroxidase profiling, the present study aimed to compare the morphological and peroxidase diversity among the wild populations of three Yarrow species.

Material and Methods

Seed Material and Experiment Layout

Seed material of 15 wild populations of *Achillea millefolium* L., *A. biebersteinii*, synonym of *Achillea arabica* Kotschy, and *A. nobilis* L., each species five populations from different regions of Iran (Table 1), was used in the present study. The seeds provided by the Iranian Natural Resources Gene Bank (INRGB). The plants of these three species are resembled together and their obvious differences are flower color (*A. millefolium*, with white flowers; *A. biebersteinii*, with yellow flowers; and *A. nobilis*, with creamy-whitish yellow flowers).

The research was conducted on the experimental field at the INRGB. A total of 30 seedlings of each population were grown in jiffy pots for forty days before transplanting into a field in October 2010. The field trial was arranged in a randomized complete block with three replications. Each plot included 36 spaced plants (0.40 x 0.40 m). Fertilizer application rates were 100 kg/h phosphorus (P) at sowing. The field was irrigated once a week during summer. No measurements were taken in the establishment year.

During the two-year investigation (2011 and 2012), 25 phenotypic traits were observed in this research. The data were collected and analyzed for the following 25 phenotypic traits: phonological traits (day to growth start, day to flowering, day to full flowering, day to fruiting and day to seeding), agronomical traits (plant height, cm; crown diameter, cm; main inflorescence diameter, cm; dry matter yield, g; inflorescence number and 1000-grain weight, g), cauline leaves, leaves arranged along an aerial stem, traits (leaf length, cm; leaf width, cm; primary leaf segments length, cm; and secondary leaf segments length, mm), inflorescence traits (inflorescence width, cm; inflorescence length, cm; and inflorescence length/width), capitulum traits (capitulum no., capitulum length, cm; and capitulum width, cm), and basal leaf traits (leaf length, cm; leaf's petiole length, cm; and leaf's petiole width, cm). The data presented in this study are average values over two years.

Peroxidase

peroxidase was extracted using 0.1 M Tris-HCl buffer (pH 7) from young leaves, and were separated by means of PAGE electrophoresis. Enzyme separation and staining procedures were described by Thiébaut *et al*. [24].

Ata Analysis

Analysis of variance was computed on collected data for each trait morphological traits. The descriptive statistics and phenotypic correlation coefficients between traits were estimated using the SAS 9.1 software. 24 classification variables had significant ($P \leq 0.01$) variation among populations and were subsequently used for multivariate analysis. The Euclidean distances of populations

were computed on phenotypic traits and then they were used for the cluster analysis-UPGMA method using Minitab software version 14.

Peroxidase profiles from all individuals were scored based on diploid genotypes. The indices of genetic diversity, such as the observed number of bands (*Na*), percentage of polymorphic loci (*PPL*) and expected heterozygosity (*He*), were calculated using POPGENE 32 software [25] on the basis of gene frequencies. At the same time, the genetic structure within and among populations were detected using the software WINAMOVA [26] in order to partition the genetic variation among species, among populations within species and among individuals within populations. The significance of each variance component was tested with permutation tests [27]. Genetic distances were estimated according to Nei [28] and the resulting similarity matrix was subjected to principal coordinate analysis (PCA) and neighbor-joining (NJ) analysis using MEGA4 software [29]. Wright's *Fst* was used to estimate population differentiation. The rate of gene flow (*Nm*) was estimated indirectly from the proportion of total diversity that was found among populations [30, 31]. A 999 random permutation Mantel test [32] was used to assess the correlation between the calculated distance matrices (using phenotypic and total protein profile data). The Pearson correlation between the genetic index within population, phenotypic traits and ecological factors was analyzed using the SPSS 11.0 software.

Results

Phenotypic Traits

Basic descriptive statistics for the morphological traits of the 15 wild populations of *Achillea millefolium*, *A. biebersteinii* and *A. nobilis* are shown in Table 2. Low to high CV values were obtained for all traits. ANOVA suggested significant differences among three *Achillea* species for all the traits except of cauline leaf width, cauline secondary leaf segments length, inflorescence width, capitulum width and basal leaf's petiole width (Table 3) Analysis of variance showed highly significant differences among 15 populations in all phenotypic traits except of primary leaf segments length and capitulum width (Table 3). Comparison of mean values of different phenotypic traits among three species show *A. millefolium* and *A. biebersteinii* samples had higher plant height and crown diameter; however, *A. nobilis* samples had higher dry matter yield and 1000-grain weight (Table 3).

The 1 Environmental data of 15 wild populations of *A. millefolium* (with M prefix), *A. biebersteinii* (with B prefix) and *A. nobilis* (with N prefix).

population	Latitude (N)	Longitude (E)	Elevation (m from see Level)	Annual average maximum temperature (ºC)	Annual average minimum temperature (ºC)	Annual average maximum humidity (%)	Annual average minimum humidity (%)	Annual average precipitation (mm)
M-KaleiABr	35˚ 52'	47˚ 01'	1180	17	8	76	49	382
M-Hamedan1	34˚ 52'	48˚ 32'	1741.5	19.74	4.24	74.33	33.11	450.7
M-Hamedan3	34˚ 52'	48˚ 32'	1741.5	19.74	4.24	74.33	33.11	450.7
M-Gorgan1	36˚ 51'	54˚ 16'	13.3	23.25	13.07	87.81	53.63	554.62
M-Gorgan2	36˚ 51'	54˚ 16'	13.3	23.25	13.07	87.81	53.63	554.62
B-Arak	34˚ 06'	49˚ 46'	1708	21	8	62	28	308
B-Salmas	38˚ 13'	44˚ 51'	1337	17.32	5.02	76.75	41	231.75
B-Shahrud1	36˚ 25'	54˚ 57'	1345.3	24.05	10	66	28.36	42.58
B-Shahrud2	36˚ 25'	54˚ 57'	1345.3	24.05	10	66	28.36	42.58
B-Minudasht	37˚ 15'	55˚ 10'	37.2	24	13	85	50	456
N-Khalkhal	37˚ 38'	48˚ 31'	1796	14.86	2.24	86.09	43.9	363.29
N-Gorgan1	36˚ 51'	54˚ 16'	13.3	23.25	13.07	87.81	53.63	554.62
N-Gorgan2	36˚ 51'	54˚ 16'	13.3	23.25	13.07	87.81	53.63	554.62
N-Rudsar	37˚ 12'	49˚ 39'	36.7	21	13	97	64	1290
N-Hamedan	34˚ 52'	48˚ 32'	1741.5	19.74	4.24	74.33	33.11	450.7

The results of phenotypic correlation showed a positive correlation between day to full flowering and plant height, main inflorescence diameter and inflorescence width, and between inflorescence width and 1000-grain weight. Dry matter yield positively correlated with plant height, and crown diameter correlated with inflorescence number and capitulum number. Basal leaf length positively correlated with inflorescence length, capitulum length and basal leaf's petiole length, however negatively correlated with inflorescence width and basal leaf's petiole (Table 4).

Euclidean distance among 15 *Achillea* populations, estimated using data on 24 phenotypic traits. The Euclidean distances matrix was subjected to agglomerative hierarchical clustering utilizing UPGMA method to construct a dendrogram (Fig. 1). 15 populations of *Achillea* were classified into two groups. Cluster I consisted of all five populations of *A. nobilis*, three populations of *A. biberstini*, and two populations of *A. millefolium*; cluster II included four populations of *A. biberstini* and three populations of *A. millefolium* Fig. 1). Besides, grouping populations according to different phenotypic data sets including phonological dates, agronomical traits, cauline leaf traits, inflorescence traits, capitulum traits and basal leaf traits did no separate different *Achillea* species (Fig. 2). Therfore *Achillea* species did not obviously discriminated by phenotypic traits. The Mantel tests indicated that there was no significant associated relationship between genetic distance and geographic distance among populations in *Achillea* species ($P > 0.05$ for three species alone, and all populations combined).

Peroxidase

In *Achillea* species 3 loci, PX-A with 6 alleles (Rm: 0.51, 0.56, 0.6, 0.65, 0.69 and 0.75), PX-B with 6 alleles (Rm: 0.31, 0.36, 0.45, 0.51, 0.56 and 0.6) and PX-C with 2 alleles (Rm: 0.15 and 0.2) were recognized (Table 5). Among the six alleles were observed at locus PX-A, two of them (named 3 and 4) were observed in the all three species. Alleles 5 and 6 were specific to *A. biberstini*; and alleles 1 and 2 were practically exclusive to *A. millefolium* and *A. nobilis* (Table 5). Six alleles were identified at locus PX-B in *Achillea* species. The isozymes coded for these alleles showed migrations close to the products of locus PX-A. The allele PX-B/1, /2 and /3 overlaps with the allele PX-A/3, /4 and /5. In *A. bieberstini* none of these three PX-B alleles was observed. Alleles 6, 2 and 1 were specific to *A. biberstini*, *A. nobilis* and *A. millefolium*, respectively. PX-C, was always monomorphic in the three *Achillea* species. The pooled values of *Ne* and *He* were higher in the *A. nobilis* samples than the *A. millefolium* and *A. biberstini* samples (Table 6). The population N-Gorgan1 (from *A. nobilis*) had the highest level of variability (*Na*, *Ne*, *I* and *He* values: 3, 2.734, 1.013 and 0.612, respectively), whereas population B-Salmas (from *A. biberstini*) had the lowest level of variability (*Na*, *Ne*, *I* and *He* values: 1.667, 1.667, 0.462 and 0.333, respectively).

Genetic distances among populations of different *Achillea* species were calculated (Table 7) and ranged from 0.011 between populations N-Gorgan2 and N-Khalkhal (both from *A. nobilis*) to 0.970 between population M-Hamedan3 (from *A. millefolium*) and population B-Salmas (from *A. bibersetinii*) with an average 0.431 (Table 7). Genetic distances among populations were used to perform principle coordinates analysis (Fig. 3). According to the analysis populations were grouped on the basis of species. The first three principle coordinates explain 87% of the total variation among populations/species. Fig. 3 shows that the three *Achillea* species are clearly separated from each other. Over all patterns of genetic differentiation was also examined using NJ analysis (Fig. 4). The obtained tree had long terminal branches, suggesting well differentiated populations/species groups. The Mantel tests indicated that there was no significant associated relationship between genetic distance and geographic distance among populations in *Achillea* ($P>0.05$).

AMOVA was used to estimate and partition the total peroxidase variance among species, among populations within species, and within populations, as well as to test the significance of partitioned variance components using a permutation procedure. Variation among species accounted for 52% of the total variation, among populations within species only 11% of the total variation, and within populations 38% of the total variation (Table 8). The variation for all three sources was significant ($P=0.01$). Although there are moderate morphological differences among the species, the variation accounted for the species (52%) was larger than that of within populations (38%) and between populations within species (11%).

Table 2 Mean, maximum, minimum standard error, and average values of local and exotic for 24 phenotypic traits in the 15 wild populations of *A. millefolium* (with M prefix), *A. biebersteinii* (with B prefix) and *A. nobilis* (with N prefix)

Variable	*A. millefolium*					*A. bieberstinii*					*A. nobilis*				
	Mean	Min.	Max.	Std. Dev.	CV	Mean	Min.	Max.	Std. Dev.	CV	Mean	Min.	Max.	Std. Dev.	CV
Phonological traits															
Day to growth start	76.33	67.00	90.00	7.18	9.40	69.00	67.00	90.00	6.08	8.82	67.00	67.00	67.00	0.00	0.00
Day to flowering	125.33	116.00	130.00	5.72	4.56	119.73	116.00	130.00	5.20	4.35	124.40	123.00	130.00	2.90	2.33
Day to full flowering	134.87	130.00	137.00	2.45	1.81	126.80	123.00	134.00	4.39	3.47	132.93	130.00	137.00	3.06	2.30
Day to fruiting	156.13	146.00	167.00	7.49	4.80	154.67	152.00	160.00	3.90	2.52	148.53	146.00	160.00	4.17	2.81
Day to seeding	170.80	159.00	182.00	7.87	4.61	174.00	167.00	182.00	6.38	3.67	164.20	159.00	173.00	4.65	2.83
Agronomical traits															
Plant height (cm)	84.80	69.80	102.30	10.66	12.57	50.29	35.40	69.60	9.31	18.51	80.49	50.50	109.20	19.41	24.12
Crown diameter (cm)	68.29	51.80	87.50	10.32	15.11	70.68	50.20	122.00	23.28	32.93	55.62	27.00	97.80	22.90	41.17
Main inflorescence diameter (cm)	8.21	6.00	9.80	1.15	13.99	7.49	5.60	9.40	1.22	16.26	7.98	6.80	10.00	0.96	11.97
Dry matter yield (g)	433.06	262.50	729.17	145.04	33.49	342.78	240.00	525.00	91.79	26.78	572.50	150.00	1162.50	336.61	58.80
Inflorescence number	26.00	14.00	40.00	10.21	39.25	48.47	26.80	64.60	13.70	28.26	31.20	11.00	57.00	19.93	63.86
1000- grain weight (g)	0.14	0.12	0.16	0.02	11.66	0.09	0.05	0.18	0.05	58.29	0.18	0.04	0.51	0.18	100.82
Cauline leaf traits															
Cauline leaf length (cm)	6.6	4.0	10.1	2.1	31.4	6.3	3.5	10.0	1.9	30.9	5.4	2.5	9.0	2.0	36.4
Cauline leaf width (cm)	3.2	0.5	7.0	2.0	63.6	2.9	1.0	5.0	1.5	50.7	1.3	0.5	3.0	0.8	57.8
Primary leaf segments length (cm)	3.0	1.0	9.0	2.9	99.7	5.5	1.0	8.0	1.9	35.3	2.5	1.0	6.0	1.6	64.1
Secondary leaf segments length (mm)	1.9	1.0	5.0	1.3	69.0	2.4	1.0	4.2	0.9	36.9	3.6	1.0	8.0	1.8	50.1
Inflorescence traits															
Inflorescence width (cm)	8.3	5.0	11.0	1.5	18.6	7.3	5.0	11.0	1.9	25.6	8.7	6.0	11.0	1.5	17.3
Inflorescence length (cm)	3.2	1.6	7.0	1.7	53.6	3.7	2.0	5.5	0.9	24.6	2.0	1.0	3.0	0.6	29.6
Inflorescence length/ width	0.4	0.2	1.0	0.2	58.4	0.5	0.3	0.9	0.2	33.7	0.2	0.1	0.3	0.1	31.0
Capitulum traits															
Capitulum no.	45.1	16.0	95.0	24.6	54.4	58.8	21.0	105.0	24.2	41.2	42.7	18.0	106.0	24.3	56.9
Capitulum length (cm)	6.1	5.0	8.0	1.0	17.0	5.1	3.0	8.0	1.4	26.5	4.0	2.0	5.0	0.9	22.7
Capitulum width (cm)	2.5	2.0	5.0	0.8	32.7	2.5	1.0	5.0	1.2	46.5	2.0	1.0	3.0	0.5	26.7
Basal leaf traits															
Basal leaf's petiole length (cm)	9.2	5.0	16.9	2.9	32.0	7.7	2.5	12.0	3.1	40.8	6.3	3.0	12.0	2.8	43.7
Basal leaf's petiole width (cm)	1.1	1.0	2.0	0.3	24.2	1.3	0.5	2.0	0.5	40.6	1.5	1.0	3.0	0.6	41.7
Basal leaf length (cm)	32.0	23.0	54.0	8.5	26.5	28.4	12.5	58.0	13.6	47.9	20.8	11.0	30.0	5.4	25.8

Table 3 Evaluation of data on 24 phenotypic traits in 15 wild populations of *A. millefolium* (with M prefix), *A. biebersteinii* (with B prefix) and *A. nobilis* (with N prefix).

Pop.	Day to growth start	Day to flowering	Day to full flowering	Day to fruiting	Day to seeding	Plant height (cm)	Crown diameter (cm)	Main inflorescence diameter (cm)	Dry matter yield (g)	Inflorescence number	1000- grain weight (g)	Cauline leaf length (cm)	Cauline leaf width (cm)	Primary leaf segments length (cm)	Secondary leaf segments length (mm)	Inflorescence width (cm)	Inflorescence length (cm)	Inflorescence length/ width	Capitulum no.	Capitulum length (cm)	Capitulum width (cm)	Basal leaf's petiole length (cm)	Basal leaf's petiole width (cm)	Basal leaf length (cm)
M-	86a	128a	135ab	160ab	173bc	83.7bc	77.1bc	7.9a-e	586.1bc	19.0d-f	0.13c	8.4a	5.0a	1.97a	2.3a-	8.3a-	2.3cd	0.3de	24.7c	7.0a	3.3a	11.0a	1.3bc	33.2ab
M-	74bc	125a	133bc	160ab	173bc	90.8a-c	71.4b-	7.8a-e	458.3cd	35.0bc	0.12c	7.5ab	3.0a-c	3.00a	3.3a-	7.7a-	6.3a	0.8a	52.7a-	6.3ab	2.2a	9.5 a-c	1.0bc	43.9a
M-	67c	128a	133bc	165a	182a	80.0bc	74.3bc	7.2c-e	452.8cd	14.0ef	0.14c	6.5a-c	3.3ab	2.73a	1.0c	7.0bd	2.8bd	0.4cd	69.3ab	5.7a-	2.7a	8.5 a-c	1.0bc	27.7bc
M-	81ab	121ab	137a	146e	162de	91.6a-c	65.9b-	9.0ab	366.7de	40.0b	0.16b	4.1bc	3.7ab	3.67a	1.0c	9.0a-	2.4cd	0.3de	39.0a-	5.3a-	2.0a	10.7ab	1.0bc	27.5bc
M-	74bc	125a	137a	150de	164de	77.7cd	52.7d-	9.2a	301.4de	22.0de	0.16b	6.5a-c	0.8d	3.4a	2.0a-	9.3ab	2.3cd	0.2de	40.0a-	6.0a-	2.3a	6.3a-d	1.0bc	27.5bc
B-Arak	67c	121ab	130cd	152cd	167c-	57.4ef	84.3b	6.8de	436.1c-e	55.4a	0.06d	6.8ab	1.7b-d	5.0a	2.2a-	7.7a-	4.2b	0.6bc	69.7ab	6.0a-	3.4a	8.0a-c	1.7ab	23.7bd
B-Salmas	67c	116b	128d	157bc	177ab	57.1ef	101.2a	8.3a-d	376.4de	57.1a	0.18b	5.0a-c	2.0b-d	5.3a	2.3a-	8.7a-	3.3bc	0.4c-	57.0a-	4.3bd	2.5a	2.8d	1.3bc	13.5d
B-	69c	116b	123e	157bc	177ab	40.3g	55.1d-	6.5e	298.6de	51.8a	0.05d	5.7a-c	3.3ab	6.2a	2.7a-	6.3cd	4.0b	0.7ab	69.7ab	5.0a-	2.2a	9.3 a-c	1.3bc	41.7a
B-	75bc	125a	123e	155b-	176ab	45.8fg	58.4c-	6.7de	322.8de	51.3a	0.05d	7.5ab	2.7b-c	5.7a	2.9a-	5.7d	4.0b	0.7ab	64.3a-	5.4a-	2.3a	9.6 a-c	0.8c	42.1a
B-	67c	121ab	130cd	152cd	173bc	50.7fg	54.4d-	9.2a	280.0de	26.8cd	0.10c	6.3a-c	5.0a	5.3a	1.7bc	8.3a-	3.1bd	0.3de	33.3a-	5.0a-	2.3a	8.6 a-c	1.3bc	20.9bd
N-	67c	128a	137a	146e	167c-	54.1e-g	29.5g	8.1a-e	238.9e	11.0f	0.51a	5.3a-c	1.0cd	3.1a	2.7a-	10.3a	1.8cd	0.2e	26.0bc	4.7bd	2.3a	5.3cd	1.7ab	20.0cd
N-Gorgan1	67c	125a	135ab	155bd	169b-	103.1a	83.6b	7.8a-e	1116.7a	50.0a	0.05d	6.3a-c	2.7b-d	1.8a	4.3ab	9.3ab	2.3cd	0.2de	48.3a-	2.7d	1.7a	9.3 a-c	1.3bc	23.0bd
N-Gorgan2	67c	123ab	133bc	146e	159e	66.2de	39.4fg	7.4a-e	355.6de	11.0f	0.18b	5.3a-c	1.0c-d	3.2a	4.0ab	8.7a-	1.7d	0.2de	33.0a-	4.2bd	1.7a	5.7b-d	2.3a	17.3cd
N-Rudsar	67c	123ab	130cd	150de	162de	85.63bc	76.8bc	8.1a-e	472.22cd	57.00a	0.11c	3.00c	0.83d	2.73a	2.3a-	7.7a-	2.0cd	0.3de	72.7a	4.0cd	2.3a	6.7a-d	1.0bc	26.3bc
N-	67c	123ab	130cd	146e	164de	93.43ab	48.8ef	8.5a-c	679.17b	27.00cd	0.04d	7.00ab	1.00cd	1.50a	4.7a	7.3bd	2.0cd	0.2de	33.7a-	4.3bd	2.0a	4.7cd	1.3bc	17.3cd
A.	76a	125a	135a	156a	171b	84.80a	70.68a	8.21a	433.1b	26.0c	0.14b	6.6a	3.2a	2.9b	8.6a	8.6a	3.2a	0.39a	37.7b	5.9a	2.4a	10.2a	1.0a	30.1a
A.	69b	120a	127c	155a	174a	80.49a	68.29a	7.49b	342.8c	48.5a	0.09c	6.3a	2.9a	5.5a	7.4a	8.3a	3.2a	0.46a	50.6a	5.1a	2.8a	6.8b	1.5a	28.5a
A. nobilis	67b	124b	133b	149b	164c	50.29b	55.62b	7.98ab	572.5a	31.2b	0.18a	5.4a	1.3b	2.5b	8.3a	7.4a	1.9b	0.24b	36.1b	3.8b	1.7a	5.7b	1.7a	21.0b

Table 4 Pearson correlation analysis for the relationships between phenotypic and genetic parameters of 15 wild populations of *A. millefolium* (with M prefix), *A. biebersteinii* (with B prefix) and *A. nobilis* (with N prefix)

	Day to growth start	Day to flowering	Day to full flowering	Day to fruiting	Day to seeding	Plant height (cm)	Crown diameter (cm)	Main inflorescence diameter (cm)	Dry matter yield (g)	Inflorescence number	1000- grain weight (g)	Cauline leaf length (cm)	Cauline leaf width (cm)	Primary leaf segments length (cm)	Secondary leaf segments length (mm)	Inflorescence width (cm)	Inflorescence length (cm)	Inflorescence length/ width	Capitulum no.	Capitulum length (cm)	Capitulum width (cm)	Basal leaf's petiole length (cm)	Basal leaf's petiole width (cm)
Day to flowering	0.26																						
Day to full flowering	0.27	0.55*																					
Day to fruiting	0.17	0.10	-0.27																				
Day to seeding	0.01	-0.05	-0.46	0.87**																			
Plant height (cm)	0.24	0.42	0.60*	-0.02	-0.35																		
Crown diameter (cm)	0.09	-0.25	-0.14	0.57	0.36	0.26																	
Main inflorescence diameter (cm)	0.15	0.02	0.58*	-0.42	-0.40	0.39	-0.11																
Dry matter yield (g)	-0.06	0.23	0.19	0.14	-0.07	0.69*	0.43	-0.05															
Inflorescence number	-0.12	-0.59*	-0.57*	0.12	0.12	-0.12	0.63*	-0.26	0.21														
1000- grain weight (g)	-0.06	0.31	0.51	-0.30	-0.17	-0.18	-0.43	0.24	-0.41	-0.51													
Cauline leaf length (cm)	0.34	0.41	-0.06	0.46	0.44	0.00	0.00	-0.25	0.21	-0.26	-0.28												
Cauline leaf width (cm)	0.51*	-0.03	-0.06	0.54*	0.55*	-0.04	0.24	0.00	0.03	-0.03	-0.27	0.37											
Primary leaf segments length (cm)	-0.12	-0.66*	-0.67*	0.11	0.38	-0.86**	0.05	-0.30	-0.60*	0.46	-0.13	-0.09	0.20										
Secondary leaf segments length (mm)	-0.29	0.08	-0.14	-0.26	-0.28	0.21	-0.22	-0.19	0.52*	0.01	-0.17	0.23	-0.40	-0.35									
Inflorescence width (cm)	0.00	0.19	0.82**	-0.44	-0.48	0.28	-0.13	0.62*	0.09	-0.36	0.66*	-0.29	-0.21	-0.38	-0.02								
Inflorescence length (cm)	0.08	-0.21	-0.43	0.53*	0.50	-0.21	0.31	-0.39	-0.14	0.40	-0.33	0.39	0.29	0.46	-0.05	-0.48							
Inflorescence length/ width	0.11	-0.25	-0.63*	0.53*	0.54*	-0.38	0.26	-0.63	-0.23	0.48	-0.36	0.31	0.26	0.57*	-0.10	-0.69*	0.92**						
Capitulum no.	-0.32	-0.32	-0.61*	0.45	0.39	-0.20	0.51*	-0.59*	0.00	0.69*	-0.45	-0.20	-0.11	0.42	-0.21	-0.64*	0.47	0.63*					
Capitulum length (cm)	0.64*	0.24	0.09	0.38	0.28	-0.13	0.02	-0.08	-0.43	-0.27	0.00	0.54*	0.39	0.14	-0.50	-0.22	0.43	0.42	-0.05				
Capitulum width (cm)	0.28	0.09	-0.06	0.44	0.36	-0.22	0.42	-0.25	-0.13	0.06	-0.03	0.38	0.27	0.16	-0.48	-0.18	0.22	0.27	0.20	0.69*			
Basal leaf's petiole length (cm)	0.62	0.24	0.05	0.37	0.23	0.16	0.11	-0.23	0.17	0.07	-0.36	0.34	0.73	0.03	-0.28	-0.30	0.33	0.38	0.09	0.42	0.18		
Basal leaf's petiole width (cm)	-0.36	-0.11	0.15	-0.40	-0.40	-0.24	-0.33	-0.18	-0.03	-0.34	0.33	-0.09	-0.30	-0.04	0.37	0.38	-0.31	-0.32	-0.36	-0.24	-0.06	-0.38	
Basal leaf length (cm)	0.50	0.16	-0.32	0.49	0.39	-0.09	0.01	-0.45	-0.13	0.20	-0.31	0.38	0.38	0.24	-0.12	-0.58	0.65	0.76	0.36	0.52	0.12	0.71	-0.53

*, **: significant at 0.05 and 0.01 level, respectively.

Table 5 Allelic frequencies of three peroxidase loci in 15 wild populations of *A. millefolium* (with M prefix), *A. biebersteinii* (with B prefix) and *A. nobilis* (with N prefix).

locus	PXC		PXB						PXA					
Allele	2	1	6	5	4	3	2	1	6	5	4	3	2	1
Relativemobility	0.15	0.2	0.31	0.36	0.45	0.51	0.56	0.6	0.51	0.56	0.6	0.65	0.69	0.75
M-Kaleibar	0.500	0.500	0	0.111	0.111	0.167	0	0.611	0	0	0.500	0	0.500	0
A. millefolium														
M-Hamedan1	0.500	0.500	0	0.125	0	0.375	0	0.500	0	0	0.500	0	0.375	0.125
M-Hamedan3	0.500	0.500	0	0.071	0.214	0	0	0.714	0	0	0.286	0.214	0.500	0
M-Gorgan1	0.550	0.450	0	0	0	0.500	0	0.500	0	0	0.400	0.100	0.400	0.100
M-Gorgan2	0.500	0.500	0	0	0.167	0.222	0	0.611	0	0	0.389	0.111	0.389	0.111
Pooled	0.510	0.490	0	0.059	0.118	0.221	0	0.603	0	0	0.412	0.088	0.441	0.059
A. bieberstinii														
B-Arak	0.500	0.500	0.500	0.500	0	0	0	0	0.286	0.071	0.500	0.143	0	0
B-Salmas	0.500	0.500	1.000	0	0	0	0	0	0.500	0	0.500	0	0	0
B-Shahrud1	0.500	0.500	0.550	0.150	0.300	0	0	0	0.500	0	0.150	0.350	0	0
B-Shahrud2	0.500	0.500	0.500	0.500	0	0	0	0	0.300	0.150	0.250	0.300	0	0
B-Minudasht	0.500	0.500	0.571	0.071	0.357	0	0	0	0.375	0	0.438	0.188	0	0
Pooled	0.500	0.500	0.569	0.278	0.153	0	0	0	0.400	0.044	0.356	0.200	0	0
A. nobilis														
N-Khalkhal	0.500	0.500	0	0	0.500	0	0.500	0	0	0	0.429	0	0.429	0.143
N-Gorgan1	0.500	0.500	0	0.333	0.167	0	0.500	0	0	0	0.357	0.143	0.286	0.214
N-Gorgan2	0.500	0.500	0	0.050	0.400	0	0.550	0	0	0	0.500	0.050	0.350	0.100
N-Rudsar	0.500	0.500	0	0.111	0.444	0	0.444	0	0	0	0.444	0.167	0.278	0.111
N-Hamedan	0.500	0.500	0	0	0	0.438	0.563	0	0	0	0.556	0.222	0.167	0.056
Pooled	0.500	0.500	0	0.088	0.313	0.088	0.513	0	0	0	0.464	0.119	0.298	0.119

Table 6 The mean number of alleles across three loci (*Na*), the effective number of alleles (*Ne*), Shanon index (*I*), number of Private alleles (*Np*), number of locally common alleles with frequency <=50% (*Nlc*), the average expected heterozygosity (*He*) and percentage of polymorphic loci (*PPL*) across the 15 wild populations of *A. millefolium* (with M prefix), *A. biebersteinii* (with B prefix) and *A. nobilis* (with N prefix)

Population	*Na*	*Ne*	*I*	*Np*	*Nlc*	*He*	*PPL*
A. melifolium							
M-Kaleibar	2.667	2.116	0.825	0	2	0.525	100
M-Hamedan1	2.667	2.308	0.881	0	2	0.563	100
M-Hamedan3	2.667	2.143	0.829	0	1	0.52	100
M-Gorgan1	2.667	2.307	0.858	0	2	0.552	100
M-Gorgan2	3	2.425	0.95	0	2	0.574	100
Pooled	3.333	2.330	0.952	1	0	0.565	100
A. bibershtaini							
B-Arak	2.667	2.267	0.852	1	3	0.548	100
B-Salmas	1.667	1.667	0.462	0	2	0.333	66.67
B-Shahrud1	2.667	2.314	0.889	0	2	0.563	100
B-Shahrud2	2.667	2.591	0.913	1	3	0.578	100
B-Minudasht	2.667	2.3	0.871	0	2	0.558	100
Pooled	3.000	2.466	0.950	3	0	0.582	100
A. nobilis							
N-Khalkhal	2.333	2.193	0.797	0	1	0.537	100
N-Gorgan1	3	2.734	1.013	0	1	0.612	100
N-Gorgan2	3	2.249	0.877	0	1	0.55	100
N-Rudsar	3	2.544	0.972	0	1	0.593	100
N-Hamedan	2.667	2.18	0.833	0	2	0.534	100
Pooled	3.333	2.557	1.016	1	0	0.597	100

Table 7 Pair-wise values for Nei's genetic distances (below diagonal) and Pair wise values for *Fst* (above diagonal) of 15 wild populations of *A. millefolium* (with M prefix), *A. biebersteinii* (with B prefix) and *A. nobilis* (with N prefix)

	M-KaleiABr	M-Hamedan1	M-Hamedan3	M-Gorgan1	M-Gorgan2	B-Arak	B-Salmas	B-Shahrud1	B-Shahrud2	B-Minudasht	N-Khalkhal	N-Gorgan1	N-Gorgan2	N-Rudsar	
M-KaleiABr		0.014	0.021	0.028	0.010	0.137	0.251	0.165	0.149	0.140	0.096	0.093	0.093	0.090	0.113
M-Hamedan1	0.036		0.050	0.008	0.014	0.120	0.233	0.154	0.133	0.131	0.099	0.083	0.092	0.088	0.084
M-Hamedan3	0.051	0.139		0.059	0.017	0.159	0.283	0.156	0.155	0.149	0.116	0.107	0.115	0.101	0.145
M-Gorgan1	0.072	0.022	0.154		0.019	0.143	0.254	0.159	0.149	0.143	0.114	0.100	0.109	0.101	0.087
M-Gorgan2	0.024	0.041	0.041	0.048		0.134	0.242	0.141	0.139	0.124	0.093	0.087	0.091	0.081	0.101
B-Arak	0.546	0.496	0.678	0.637	0.617		0.121	0.057	0.011	0.046	0.148	0.093	0.130	0.110	0.128
B-Salmas	0.812	0.770	0.970	0.851	0.834	0.168		0.104	0.138	0.083	0.257	0.226	0.242	0.226	0.245
B-Shahrud1	0.782	0.792	0.684	0.802	0.693	0.176	0.163		0.039	0.017	0.144	0.117	0.138	0.111	0.153
B-Shahrud2	0.680	0.628	0.698	0.728	0.701	0.036	0.221	0.119		0.053	0.158	0.095	0.142	0.117	0.140
B-Minudasht	0.585	0.595	0.615	0.655	0.551	0.133	0.097	0.052	0.164		0.119	0.107	0.111	0.090	0.130
N-Khalkhal	0.357	0.413	0.403	0.466	0.356	0.653	0.847	0.635	0.780	0.449		0.037	0.005	0.009	0.079
N-Gorgan1	0.385	0.370	0.438	0.456	0.400	0.373	0.810	0.563	0.418	0.483	0.109		0.024	0.019	0.054
N-Gorgan2	0.353	0.391	0.418	0.455	0.363	0.548	0.784	0.614	0.676	0.425	0.011	0.073		0.005	0.058
N-Rudsar	0.358	0.394	0.382	0.451	0.344	0.474	0.772	0.490	0.551	0.355	0.025	0.064	0.014		0.057
N-Hamedan	0.413	0.292	0.585	0.280	0.385	0.531	0.765	0.716	0.633	0.551	0.237	0.175	0.167	0.185	

Table 8 Analysis of Molecular Variance (AMOVA) of the 15 wild populations *Achillea* species based on peroxidase profile

Source	df	SS	MS	%	Prob.
Among species	2	95.592	47.796	52%	0.010
Among Pops/species	12	29.947	2.496	11%	0.010
Within Pops	135	88.682	0.657	38%	0.010
Total	149	214.222	50.949		

The degree of population differentiation (*Fst* values, Table 7) ranged between 0.005 (between populations N-Gorgan2 and N-Khalkhal, and N-Gorgan2 and N-Rudsar, all from *A. nobilis*) to 0.283 (between M-Hamedan3, from *A. millefolium,*and B-Salmas, from *A. biebersteinii*). As expected, all populations in each species were found related, while higher values were observed between *A. biebersteinii* and the other *Achillea* species.

All pairwise *Fst* values (Table 7) were significant (p<0.01), demonstrating significant genetic differentiation. The Pairwise Population *Fst* (via frequency) values were used to estimate the number of individuals that migrated between each pair of sampling source (populations/species) per generation. Estimates of *Nm* reflected the cross allele movement generated from the assignment test, revealing limited gene flow among species and considerable gene flow within. The general properties of assignment indices as a function of interspecies dispersal rate are illustrated in Fig. 5, showing the intersection between the three species. The strength of the study is revealed by the percentage of the correctly assigned individual populations into their corresponding species. All *Achillea* species samples were classified within the appropriate group (Table 9). The strength of the study was revealed by the high percentage (82%) of *Achillea* samples that were assigned correctly into their corresponding species; furthermore, there was an intersection among the three *Achillea* species (Fig. 5).

Correlation coefficients among pairwise genetic and phenotypic distance matrices were calculated using Mantel's test. Regression and correlation analysis between genetic and phenotypic distances showed no significant correlation (P>0.05). Pierson correlative analysis showed that there was no significant correlation either between genetic diversity and latitude (p=0.9408) and/or longitude (p=0.3368).

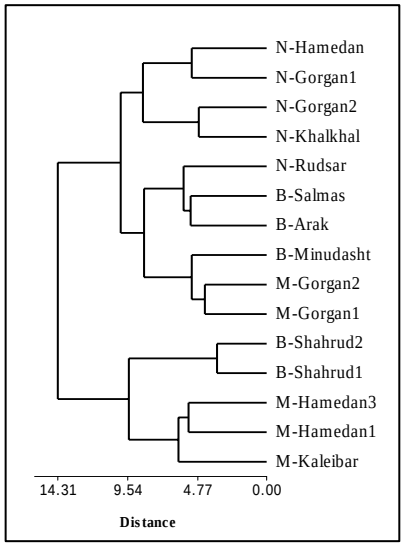

Fig. 1 Phenogram of the 15 wild populations of *A. millefolium* (with M prefix), *A. biebersteinii* (with B prefix) and *A. nobilis* (with N prefix) based on phenotypic traits.

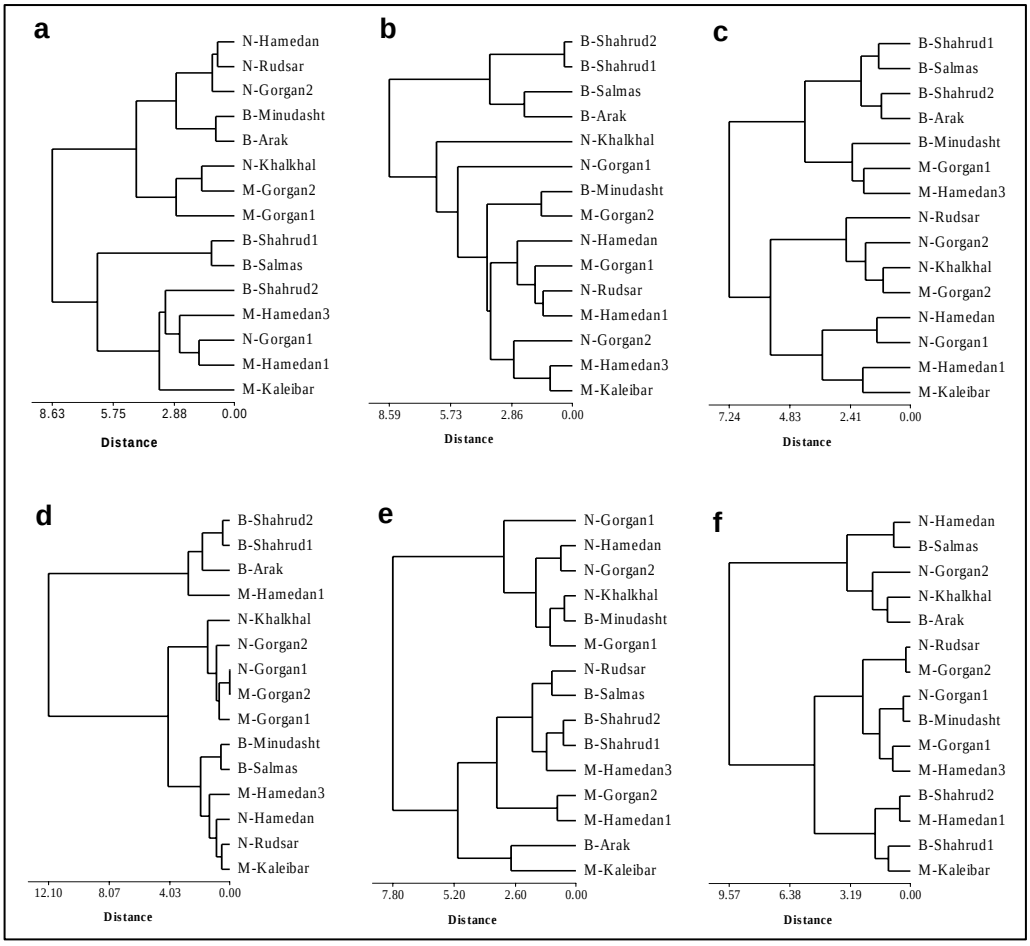

Fig. 2 Phenogram of the 15 wild populations of *A. millefolium* (with M prefix), *A. biebersteinii* (with B prefix) and *A. nobilis* (with N prefix) based on different phenotypic traits: a, phonological traits (day to growth start, day to flowering, day to full flowering, day to fruiting and day to seeding); b, agronomical traits (plant height, crown diameter, main inflorescence diameter, dry matter yield, inflorescence number and 1000 - grain weight); c, cauline leaf traits (cauline Leaf length, cauline leaf width, Primary leaf segments length and secondary leaf segments length); d, inflorescence traits (inflorescence width, inflorescence length and inflorescence length/width); e, capitulum traits (capitulum no., capitulum length and capitulum width); and f, basal leaf traits (basal leaf's petiole length, basal leaf's petiole width, basal leaf length).

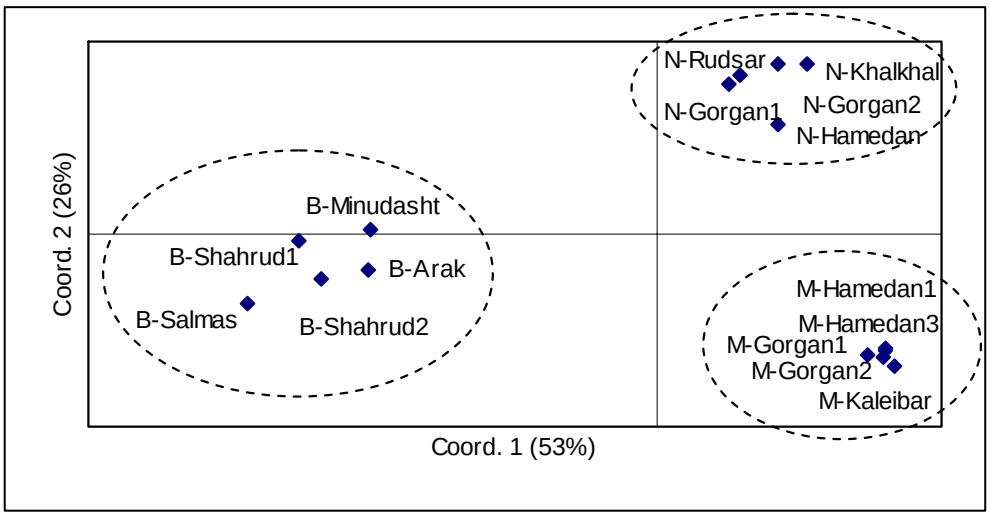

Fig. 3 Two-dimensional graph of the 15 wild populations of *A. millefolium* (with M prefix), *A. biebersteinii* (with B prefix) and *A. nobilis* (with N prefix) based on the ordination scores of the principal coordinate analysis.

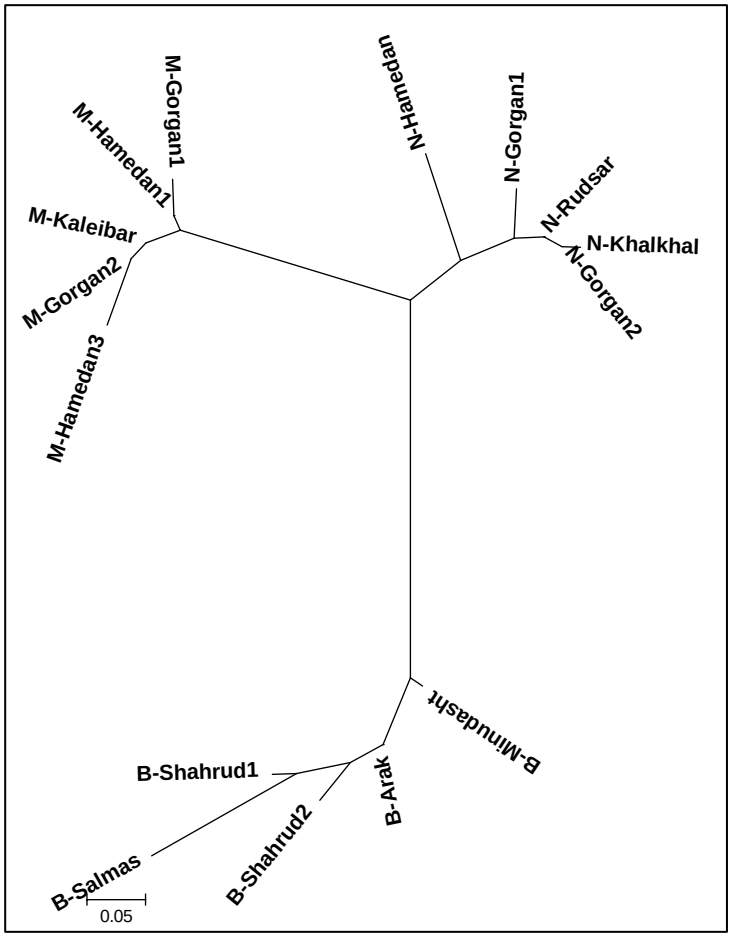

Fig. 4 Dendrogram of 15 wild populations of *A. millefolium* (with M prefix), *A. biebersteinii* (with B prefix) and *A. nobilis* (with N prefix) obtained by the Neighbor-Joining clustering method.

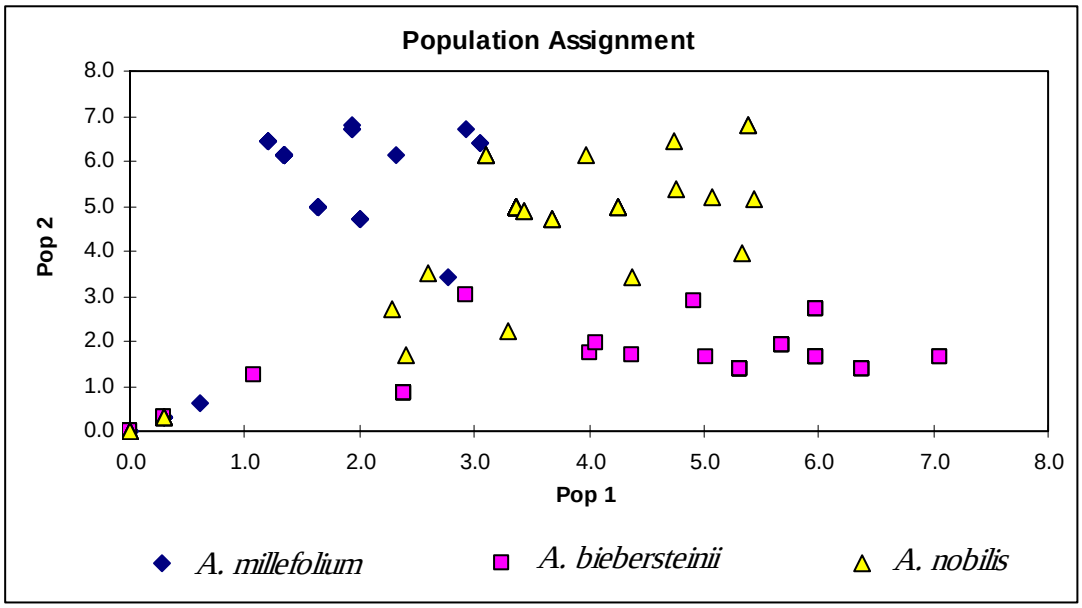

Fig. 5 Log expected frequencies (assignment indices) of genotypes drawn from *A. millefolium*, *A. biebersteinii* and *A. nobilis* samples.

Discussion

Knowledge of genetic variation and genetic relationship among genotypes in plant germplasm are important considerations for efficient utilization of germplasm resources. The peroxidase profiles and phenotypic traits utilized in the present study revealed moderate levels of variation in 15 Iranian wild populations of *Achillea* compared to previous studies [14,33]. The quantitative characters as expected were more variable, and the within and between species variation was more detectable. For almost all characters with a quantitative genetic control, breeding for increasing or reducing a given phenotypic value would be possible. Parallel to our findings, significant variation was observed with respect to morphological, phonological, biological and molecular properties between populations in previous studies [7-13, 16-18, 33-34]. The reason for this variation detected within populations may be related to genetic structure, which is probably due to the heterozygosity of cross-pollination of *Achillea* species [35]. The cross-pollination mechanism, sexual reproduction, high seed ratio and incompatibility to produce offspring of the many plant species could have resulted in the accumulation of abundant genetic variation during the long evolutionary history [36]. This indicated that improvement through simple selection for these traits is possible. However, broadening the genetic base from diverse sources is recommended to include most of the genetic determinants of these traits [5,37].

On morphological analysis three *Achillea* species did not obviously discriminated by each other. This is because of similar phenotypic traits among them. But peroxidase profiles confirmed the presence of a much more pronounced and significant (P<0.01) differentiation among species based on AMOVA (account for 52% among species). An additional partial exclusion Bayesian-based assignment test showed that in *A. millefolium*, *A. biebersteinii* and *A. nobilis* overall 81% of the individuals were assigned to their species and 19% were assigned to another species. Previously, AFLP [18] and ISSR and RAPD analysis [14, 33] also could differentiate different *Achillea* species.

The pair-wise genetic distances of *A. millefolium* (6%), *A. biebersteinii* (10%) and *A. nobilis* (10%) populations in the present study was in comparison with that in studies on *A. millefolium* populations (4% [38]), and also other *Achillea* species (17% in *A. fragrantissima*, [10]; 15-40% in *A. tenuifolia* and *A. santolina*, [14,33]). Mantel tests for isolation by distance confirmed no correlation between pairwise genetic differentiation (*Fst*) and geographical distances among populations. The neighbor-joining tree grouped the populations of different species into discrete clusters. The moderate proportion of bands shared between the three species does suggest either introgression or shared ancestral polymorphisms between these species. In contrary with other studies in *Achillea* species [10,14,15,17,18,33], the results of this work

implied that the genetic diversity of studied species was the result of the joint effects of one or several ecological factors, i.e., the ecological factors do not play an important role in influencing the peroxidase profiles polymorphism of studied species. This study provides evidence that peroxidase marker polymorphisms are an informative and suitable approach to evaluation of phylogenic relationships in wild population of *Achillea* species.

Other hand, allelic peroxidase variants varied between different populations without any special tendency. It is therefore imperative for conservation planners, when designing conservation strategies for wild populations of *Achillea* species in Iran, to ensure that as many as possible separate populations are targeted for conservation rather than a few selected populations [22,39]. It would be beneficial to find ways to strengthen the gene flow between populations to maintain the natural genetic variation of *Achillea*. Considering the high genetic differentiation among the wild populations, preservation of only a few populations may not adequately protect the genetic variation within the species in Iran. Therefore, several populations throughout the entire range of the species in the country should be considered for conservation. Although *Achillea* had not been listed as a species of conservation concern for Iran, it is an important medicinal species endemic to Iran. Therefore, the conservation and further reasonable utilization of the germplasm resources of this species is an urgent task. Further studies are required to reveal whether there are other factors that cause genetic variation in *Achillea* species.

Conclusion

The 15 *Achillea* populations showed a wide range of morphological variability. Comparison of mean values of different phenotypic traits show *A. millefolium* and *A. biebersteinii* had higher plant height and crown diameter; however *A. nobilis* had higher dry matter yield and 1000-grain weight.

The polymorphism observed in peroxidase profiles among the *Achillea* species in the present study demonstrated the effectiveness of this method in determining genetic variation. The peroxidase profiles used in the study were found to be highly informative for revealing the genetic diversity among the genotypes studied, thus suggesting their potentiality in future genetic diversity analysis and also in identifying medicinal efficient genotypes. Availability of unique profiles present in different species which are indicated in species specific diagnostic markers) together with genetic dissimilarly data would be very useful for improvement of the species through conventional breeding methodologies as well as molecular breeding approaches such as marker assisted selection.

The study confirmed that genetic and morphological diversity work in different ways to determine the relationships among species. To effectively exploit germplasms, we should utilize both methods for identification of elite genotypes for domestication and breeding programs. The information obtained in this study will be useful in the management of wild *Achillea* species collection. The identity of *Achillea* species is difficult to establish based on morphological traits alone. We have demonstrated that peroxidase profiles can be effectively used to recognize certain accessions of *Achillea* species. Information about the genetic similarity of *Achillea* species can provide valuable insights into their systematic classification and can guide and improve the effectiveness of the breeding process.

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