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Evaluating genetic diversity and structure of a wild hop (*Humulus lupulus* L.) germplasm using morphological and molecular characteristics

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Abstract *Humulus lupulus* L., is an economically and ecologically important plant species, which has suffered habitat degradation throughout the Hyrcanian forests (Northern Iran). Towards conservation and exotic breeding germplasm assessment, we conducted a survey in the Hyrcanian forests: 15 wild populations (WPs) were located and 54 samples collected. Genetic diversity and population structure were assessed by scoring sequence-related amplified polymorphism markers (SSR, ISSR, and RAPD) and morphological features. Molecular marker analysis showed that RAPDs (232) and ISSRs (77) produced more polymorphic bands compared to SSRs (64) per marker. SSRs exhibited a higher PIC average value (0.64), than RAPDs (0.24) and ISSRs (0.54). Cluster analyses based on the SSR markers to a high degree discriminated WPs based on geographical regions and were more congruent with morphologic traits than the ISSR–RAPD-based clustering. The ΔK parameter of structure analysis showed five clusters. The grouping of the WPs based on the structure analysis was congruent with the SSR clustering to some extent. The results confirmed that SSR markers are effective tools to detect the genetic diversity in hops, but employing higher number of molecular markers (more SSRs), which have a higher polymorphism and prevalence in the genome, or application of NGS SNPs in the identification and genetic relationship of hop indigenous populations, is recommended.

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Introduction

The common hop, *Humulus lupulus* L. (Cannabaceae) is a dioecious, herbaceous perennial climbing species mainly cultivated in the Northern Hemisphere primarily for female inflorescences containing glandular trichomes which accumulate secondary compounds, namely, terpenophenolics (bitter acids) and terpenes (essential oils). Hop phytochemicals provide durability, foam stability, and contribute to flavor and aroma of beer (Rohlf 1998; Zanoli et al. 2005; Zanoli and Zavatti 2008; McAdam et al. 2014). Moreover, biomedical and nutritional applications of hop constituents have been reviewed (Kavalier et al. 2011). The first report on phytochemical potential of Iranian hop collection was reported by Mafakheri and Hamidoghli (2015a), who found that these native wild hops have high ability in oxidant scavenging and are rich in polyphenolic compounds comparable with domestic, commercial cultivars. However, destructive anthropological activities such as development of industrial states and agriculture landscape, logging as well as absence of proper management programs in the last decades across the Hyrcanian forests has jeopardized habitats of wild hop populations, especially in Northern Iran. Due to the rich biodiversity of these forests, including relict species in Tertiary period forests, we add to universal conservation goals that cover a vast area between Iran and Azerbaijan (Gholizadeh et al. 2017; Soofi et al. 2018).

Hop breeders have attempted to provide farmers and brewers with the desired improved cultivars: disease and pest resistance (e.g. powdery mildew), high yield, enhanced storage stability and superior aroma and bitter quality (Seefelder et al. 2000a; Ĉerenak et al. 2011; Patzak and Henychová 2018). In this regard, wild hop germplasm and genetic diversity from under-utilized origins, can serve breeders with a crucial new gene pools to properly address crop improvement and sustainability demands (Hampton et al. 2001; Peredo et al. 2010; Xiong et al. 2016).

Molecular markers have been applied to genetic diversity estimation in hops, for example, using random amplification of polymorphic DNA (RAPD), to assess genetic diversity in hops (Abbott and Fedele 1994; Pillay and Kenny 1996; Vejl 1997; ŠUštar-Vozlič and Javornik 1999). RAPD has been used (1) in sex identification of hop (Polley et al. 1997) (2) construction of genetic linkage maps between male and female hops (Seefelder et al. 2000b) and (3) DNA profiling of hop cultivars (Brady et al. 1996) and detection the level of polymorphism in hop cultivars derived from the European and North American germplasm by Murakami (2000) and Patzak et al. (2010). ISSRs as markers were employed in hops to (1) investigate somaclonal variation (Patzak 2003), (2) identify sex-specific markers (Danilova and Karlov 2006), (3) analyze polymorphism among 10 commonly in use commercial cultivars (Patzak 2001) or 26 Russian and European cultivars (Danilova et al. 2003). SSRs due to their high accuracy, multi-allelism which is responsible for high polymorphism, codominant, high frequency, and freedom from laboratory specificity (Nybom 2004; Smykal et al. 2008; Vieira et al. 2016; Kordrostami and Rahimi 2015) have become a favored PCR technique, especially in relation to quantitative and molecular genetic variation in key features in hops (Jakše et al. 2002; Hadonou et al. 2004; Stajner et al. 2005; Bassil et al. 2008). For example, (1) Jakše et al. (2004) assessed 124 wild and cultivated accessions from North America to Europe and Asia (2) Murakami et al. (2006) evaluated genetic variability and closeness of the hop germplasm throughout the Northern hemisphere, including, recently, (3) an Italian germplasm (Mongelli et al. 2015) with SSRs application of multiple marker systems is invaluable.

In order to investigate WPs, we also used vegetative features as another suitable method (e.g. Srečec et al. 2011; Skomra et al. 2013). Morphological features have been applied to such cultivars as English Fuggles, Goldings and Czech Saaz, hops that were once chosen from WPs (Barth et al. 1994; Moir 2000). Therefore, we additionally applied morphological-based diversity assessment as complementary to molecular methods. In summary, we employed three types of molecular markers (SSR. ISSR and RAPD) in combination with morphological features to characterize genetic diversity and population structure of 15 WPs of *H. lupulus* L. in Northern Iran. The aim of this study was to evaluate genetic diversity at the population level. This study offers invaluable information on the Iranian hop collection for the very first time.

Material and methods

Study area

We collected samples in Northern Iran, mainly in Hyrcanian forests, including three provinces, Guilan, Mazandaran, and Golestan, nearly the entire *H. lupulus* L. distribution range within Iran including various elevations, − 13 to 704 m. Sites were mapped with GPS: 15 WPs were located. Geographical properties (latitude, longitude, and elevation) of all locations were documented (Fig. 1, Table 1). The means, minimum and maximum monthly temperatures, minimum and maximum humid, rainfall, sunny days and evaporation of growing seasons in the five previous years are reported (Supplemental File 1).

Sampling the germplasm, and plant material

Fifty-four samples were obtained from 15 WPs (Table 1). Only aspects of female plants with reproductive structures are reported. Gene flow and seed dispersal are under continued study in the WPs, but not reported here. Populations are defined as any group of organisms of a species existing in a specific space and functioning actively as part of biotic community, thus, the hop individuals presented at a particular area were considered as 'a population' (Odum and Barrett 1971). For further morphological evaluation (leaves and cones), and DNA extractions (leaves), samples were kept in an ice-box and carried to the laboratory, and stored under − 80 °C until DNA was extracted. A part of harvested cone hops in September 2016 was dried similar to the methods described by Hofmann et al. (2013), then stored in 4 °C. For plant systematic identification, from each population, a voucher specimen was prepared and deposited in Herbarium of

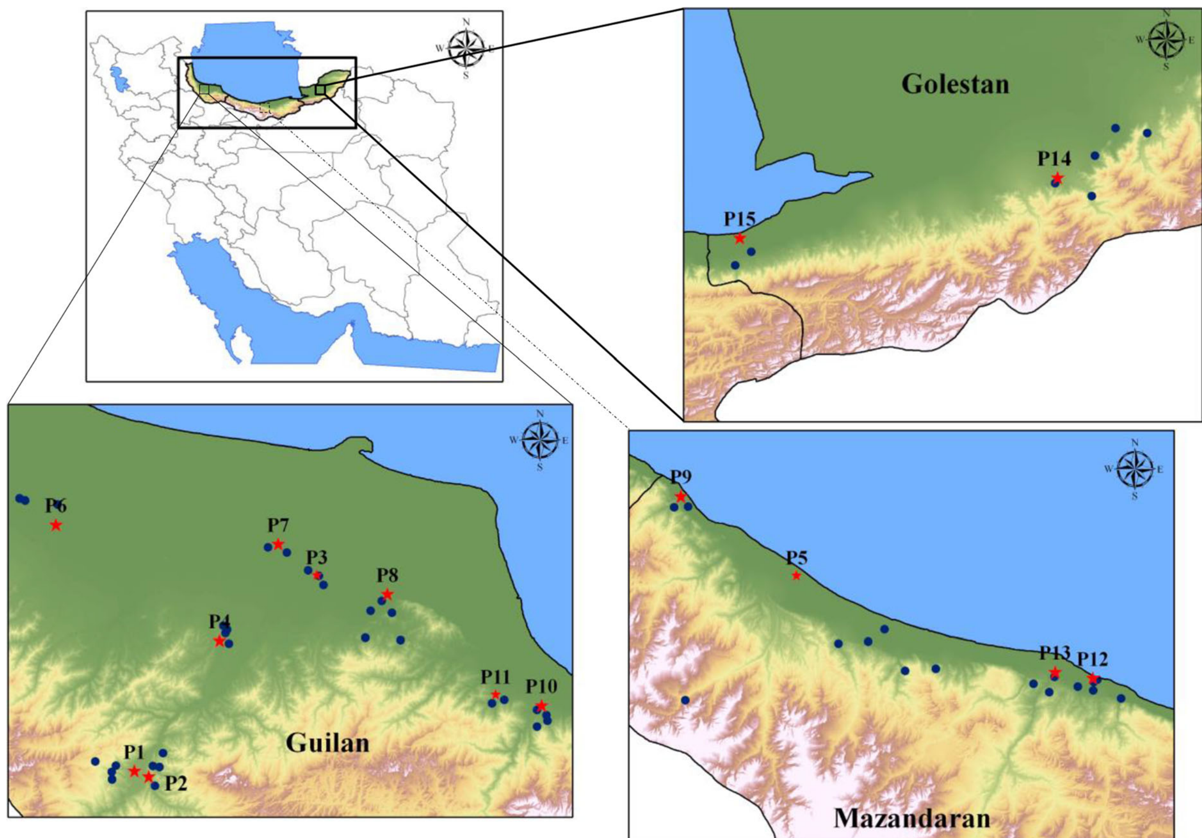

Fig. 1 Regions in three provinces Northern Iran, in which 15 WPs (marked red star, 1–15) spotted and 54 samples were taken from each population (blue dots). (Color figure online)

Table 1 List of the 15 studied WPs and their geographical origin

Origin	Population code	Sample size (n)	Latitude(°N)	Longitude(°W)	Elevation (m)
Guilan					
Rostamabad	P1	4 (G1–G4)	36° 54′ 1.19″ N	36° 54′ 1.19″ N	704
Totkabon	P2	4 (G5–G8)	36° 55′ 0.09″ N	49° 33′ 40.02″ E	516
Someh Sara	P3	3 (G9–G11)	37° 20′ 58.59″ N	49° 18′ 11.98″ E	− 13
Saravan	P4	4 (G12–G15)	37° 8′ 32.15″ N	49° 40′ 32.78″ E	50
Lahijan	P6	3 (G21–G23)	37° 11′ 25.69″ N	49° 59′ 58.35″ E	− 3
Rahimabad	P7	2 (G24–G25)	37° 0′ 23.92″ N	50° 20′ 45.83″ E	126
Ranekouh	P8	5 (G26–G30)	37° 2′ 57.17″ N	50° 14′ 41.49″ E	48
Kisom	P10	4 (G34–G37)	37° 14′ 13.88″ N	49° 50′ 42.32″ E	− 7
Kochsfhan	P11	2 (G38–G39)	37° 16′ 30.27″ N	49° 47′ 22.78″ E	− 1
Mazandaran					
Ramsar	P9	3 (G31–G33	36° 53′ 54.62″ N	50° 39′ 27.39″ E	50
Shahsavar	P5	5 (G16–G20)	36° 54′ 30.95″ N	50° 39′ 43.04″ E	− 9
Nowshahr	P12	5 (G40–G44)	36° 38′ 40.24″ N	51° 30′ 38.96″ E	− 6
Chalus	P13	3 (G45–G47)	36° 37′ 46.47″ N	51° 24′ 17.49″ E	77
Golestn					
Aliabad-e-Katul	P14	5 (G48–G52)	37° 0′ 31.56″ N	55° 7′ 45.01″ E	276
Bandar-e Gaz	P15	2 (G53–G54)	36° 42′ 22.55″ N	53° 56′ 42.76″	138

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Morphological analysis

Seventeen morphological traits of Iranian hop germplasm were evaluated, 13 traits from cones (width, length, cone size, cone shape, cone intensity of green color, cones fresh and dry weight (100 cones), and bracts (width and length, bract ratio width/length, length apex of bract and length of bracts), one trait each from leaves (size of blade), main shoots (anthocyanin coloration of main shoot), flowering habit and, growth habit for which values of descriptors are defined in a table (Supplemental Table S1). Descriptors are based on UPOV (2006), Rígr and Faberová (2000) and Čeh et al. (2012). Thirty leaves and 30 cones collected per sample were measured by a digital caliber with precision 0.01 mm. “Dwarf” is defined as having average main bine internodes of less than 6 cm.

DNA extraction, SSR analysis

Fresh leaves were used for DNA extraction with a CTAB method (Saghai-Maroof et al. 1984). The quality and quantity of the DNA were evaluated by analysis on a 1% agarose gel. Twenty SSR markers were selected based on Stajner et al. (2005). Fourteen SSR primer-pairs were selected that yielded clear and visible bands within the expected range and without presence of non-specific bands (Supplemental Table S2).

PCR conditions were as follows 10 µl PCR reactions contained 0.4 mM of each primer, 10 µM deoxyribonucleotides, 50 mM KCl, 10 mM TRIS–HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, 50–100 ng of DNA, and 1 unit of *Taq* polymerase. The PCR was performed with a profile of 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, at 55 °C for 1 min, at 72 °C for 2 min, and finally for 5 min at 72 °C for the final extension. PCR conditions were as described in Panaud et al. (1996). Taq DNA polymerase (1 U) and DNA (20 ng). The PCR was performed with a profile of 95 °C for 2 min, followed by 45 cycles at 94 °C for 1 min, at 42 °C for 1 min, at 72 °C for 2 min, and finally for 5 min at 72 °C for the final extension. Next, a total of 10 µl PCR products were run on 1.5% agarose gels. Products were also examined on sequencing gels, a total of 3 µl PCR products were denatured and run on 6%

polyacrylamide denaturing gels, and marker bands were revealed using the silver staining as that described by Panaud et al. (1996).

For ISSR analysis, 14 primers were used (Supplemental Table S3) to amplify each individual. PCR conditions were as described in Morales et al. (2011). In summary, 25 µl PCR reactions contained 40 ng of genomic DNA, 2.5 µl of 1 × PCR buffer, 1.5 mM of $MgCl_2$, 200 µM of each dNTP, 10 µmol of primer, 1 U *Taq* polymerase and water. The PCR was performed with a profile of 95 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, at 50 °C for 45 s, at 72 °C for 2 min, and finally for 7 min at 72 °C for the final extension. Ten µl PCR products were run on 1.5% agarose gels.

For RAPD analysis, five primers were used (Supplemental Table S3) to amplify the individuals. PCR conditions were as described in Morales et al. (2011). In summary, 10 µl PCR reactions contained PCR buffer (1 ×), $MgCl_2$ (2.5 mM), dNTPmix (0.2 mM), primer (25 ng), Taq DNA polymerase (1 U) and DNA (20 ng). The PCR was performed with a profile of 95 °C for 2 min, followed by 45 cycles at 94 °C for 1 min, at 42 °C for 1 min, at 72 °C for 2 min, and finally for 5 min at 72 °C for the final extension. Ten µl PCR products were analyzed with 1.5% agarose gels.

Data analysis

For each primers combination (SSR, ISSR and RAPD markers), the total number of bands was determined, and only the polymorphic ones were taken into account to estimate the percentage of polymorphic bands (PPB). Polymorphic information content) (PIC), was calculated as reported by Lynch and Walsh (1998). For SSR, PIC was calculated according to Nei (1973): $PIC = 1 - \sum p_{ij}^2$ where p_{ij} is the frequency of the jth allele for ith locus across all alleles at a locus.

For RAPD and ISSR, polymorphism information content was calculated according to Anderson et al. (1993): $PIC = 1 - \sum_{1}^{k} p_i^2$ where k is the total number of alleles detected for a given marker locus and Pi is the frequency of the ith allele in the set of genotypes investigated.

Cluster analysis

SSRs bands were scored on the molecular weight of the bands and designated A, B, C, etc. following Gao et al. (2017), Ćurčić et al. (2017) and Yelome et al. (2018). For SSRs Bray–Curtis dissimilarity values were calculated to reveal the relationships among samples based on the scores. The hierarchical clustering software Cluster version 3.03 was used for computing the tree and the calculated tree was visualized using Java TreeView version 1.1.6.4. RAPD and ISSR bands were scored as 1 for their presence or 0 for their absence to generate a matrix. The genetic similarity among genotypes was calculated using simple matching genetic distance (SM = m/n), where 'm' is the number of matches and 'n' is the total number of variables. Clustering analysis was obtained using the un-weighted pair group method of arithmetical average (UPGMA) algorithm. All of these computations were carried out using NTSYS 2.02 software. To determine how accurately the dendrograms represent the estimates of genetic similarity among the genotypes, a cophenetic matrix was generated for each dendrogram and compared with the corresponding similarity matrix by the Mantel matrix correspondence test (Mantel 1967). The same Mantel statistic was used to compare the similarity matrices as well as the dendrograms produced by the SSR, RAPD and ISSR techniques. All these procedures were performed by appropriate routines in NTSYSpc version 2.0 (Rohlf 1998).

Results

Morphological features

ANOVA for 17 morphological features assessed among the WPs revealed significant differences among the traits at 1 and 5% probability levels. The highest and lowest CV (coefficient of variation) were observed in leaf size of blade (11.29%) and degree of opening of bracts (0.1%), respectively (Tables 2, 3).

Comparison of means between WPs in Table 4 showed that the populations from Bandar-e Gaz and Aliabad-e-Katul had the highest value for cone width (2.12 and 2.15 cm, respectively) and cone length (2.79 and 2.76 cm, respectively), bract width (0.72 and 0.70 cm, respectively) and bract length (1.06 and

Table 2 Analysis of variance of the studied morphological variables in 15 WPs

S.oV	*df*	MS of traits																
		X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13	X14	X15	X16	X17
WPs	14	3.687**	0.34**	0.58**	154.36**	4.93**	3.96**	0.009**	0.029**	11.66**	0.49*	5.566**	8.35**	2.49*	2.5*	11.85*	4.22**	6.32*
Error	39	0.036	0.002	0.007	0.56	0.057	0.1	0.0008	0.0007	0.0005	0.0001	0.27	0.00001	0.0001	0.0006	0.0005	0.001	0.12
CV%		5.59	2.93	3.77	1.91	2.91	5.82	1.39	3.11	0.2	0.3	11.29	0. 5	0.1	0.15	0.26	0.5	7.71

ns,*, **: non-significant and significant at 5% and 1% probability levels, respectively

WPs: wild population; CV: coefficient of variation; X1: Cone shape; X2: cone width; X3: cone length; X4: cone fresh weight; X5: cone dry weight; X6 bract shape; X7: bract width; X8: bract length; X9: main shoot anthocyanin, X10: growth type; X11: blade size; X12: flowering time; X13: degree of opening of bract; X14: intensity of green color; X15: Bracts length of apex; X16: width/length ratio; X17: cone size

1.00 cm, respectively), cone fresh weight (49.94 and 48.40 gr, respectively) and cone dry weight (10.30 and 10.17 gr, respectively), while the populations from Rostamabad and Totkabon had the lowest values for these traits. The bract size value varied in six populations (Rostamabad, Totkabon, Someh Sara, Saravan, Rahimabad and Lahijan,) exhibited score "small", while only two populations were "large" (Bandar-e Gaz and Aliabad-e-Katul), the other seven populations had "medium" bract size. The growth type did not vary notably between populations, most of populations were "normal" except for Rostamabad and Totkabon populations which were "dwarf". Similarly, time of flowering was not markedly variant between populations; although eleven populations showed "late" (September) flowering habit and only two populations were "early" (July) flower (Rostamabad and Totkabon). Conversely, leaf size of blade varied greatly amongst the studied populations from "small" (Rostamabad, Lahijan, Ranekouh, Kochsfhan) to "large" (Bandar-e Gaz, Aliabad-e-Katul). The degree of opening of bracts as another important character exhibited a great variation, which many of populations had "closed" cones (Chalus, Nowshahr, Bandar-e Gaz, Aliabad-e-Katul, Ranekouh and Rahimabad). In 12 populations the "intensity of green color" were medium, and other three ones were "low" (Rostamabad, Totkabon and Someh Sara). The descriptive values for "length of apex" in bract were "long" in populations of Kisom and Kochsfhan, "very short" in Rostamabad and Totkabon, and other population were "short" or "medium". Populations commonly had "medium" and "large" cone width/length ratio, excluding Lahijan which was "small".

Correlation analysis indicated strong positive and negative correlations among morphological characteristics (Table 5). Positive correlation between cone shape and cone width, cone length, bract width, bract length, leaf size of blade and width/length ratio was observed. It is interesting to note that a significant negative correlation between cone shape and all the other measured features was observed, except for cone size, width/length ratio, degree of opening of bracts, time of flowering, leaf size of blade and main shoot anthocyanin. In addition, we observed a positive correlation between time of flowering and growth type. There was also a strong positive correlation between cone fresh weight and cone dry weight and the yield related traits such as cone size, cone width,

Table 3 The statistical characteristics of the studied traits in 15 WPs

Traits	CS	CW	CL	CFW	CDW	BS	BW	BL	MSA	GT	LSB	TF	DOB	IGC	LA	WL	CZ
Min	2	1.08	1.4	28.42	6.45	4	0.54	0.73	3	1	3	3	1	3	1	3	3
Max	5	2.15	2.79	49.94	10.3	7	0.72	1.06	7	2	7	7	3	5	7	7	7
Mean	3	1.63	2.23	39.63	8.31	5.39	0.64	0.83	5	1	5	5	2	5	5	5	5
Variance	1.04	0.10	0.16	43.92	1.39	1.17	0.00	0.01	3.12	0.12	1.63	2.17	0.70	0.69	3.35	1.26	1.83
Stand. dev	1.02	0.31	0.40	6.63	1.18	1.08	0.05	0.09	1.77	0.35	1.28	1.47	0.83	0.83	1.83	1.12	1.35
Skewness	0.04	− 0.01	− 0.83	− 0.10	0.19	− 0.01	− 0.74	1.30	0.60	− 2.40	0.48	− 1.63	0.27	− 1.67	− 0.11	0.11	0.39
Kurtosis	− 1.03	− 0.11	0.52	− 0.67	− 0.63	− 1.30	0.44	1.27	− 1.49	4.35	− 0.11	1.32	− 1.50	0.90	− 0.48	0.38	− 0.43
CV%	37	25.14	26.31	24.85	20.23	27.25	7.92	18.22	48	26	31	30	53	24	51	28	31

Min: minimum; Max: maximum; Stand. dev: standard division; CV: coefficient of variation CS: cone shape; CW: cone width (cm); CL: cone length (cm); CFW: cone fresh weight (gr); CDW: cone dry weight (gr); BS: bract size (cm); BW: bract width (cm); BL: bract length (cm); MSA: main shoot anthocyanin, GT: growth type; LSB: leaf size of blade; TF: time of flowering; DOB: degree of opening of bracts; IGC: intensity of green color; LA: length of apex; WL: ratio width/length; CZ: cone size

cone length, bract width, bract length, and leaf size of blade and width/length ratio.

Based on the cluster analysis, the WPs were divided into three distinct groups (Fig. 2). In the first group, the populations from Rostamabad and Totkabon were clustered together, while these two populations had the smallest values for cone width and cone length, bract width and bract length, cone fresh weight and cone dry weight. The second cluster, had two sub-clusters. In the first sub-cluster, the WPs from Bandar-e Gaz and Aliabad-e-Katul were grouped, and these two populations had the highest values for cone width, cone length, bract width, bract length, cone fresh weight and cone dry weight. The second sub-cluster had two populations from Kisom and Kochsfhan, and these WPs, in terms of cone width, cone length, bract width, bract length, cone fresh weight and cone dry weight, had the highest value after Bandar-e Gaz and Ali-abad. The third cluster consisted of the rest of the populations (Table 4), which had middle values of the descriptors and quantitative yield associated characteristics.

Molecular analysis

SSR analysis

A total of 64 score-able SSR markers were produced using fourteen molecular markers with an average of 4.57 bands per marker (Table 6). The polymorphism percentage (P %) was 100% for each pair of SSR primers. The polymorphism information content value ranged from 0.50 (Hl-AGA1) to 0.97 (Hl-GT24) with a mean of 0.64. The total number of bands from SSRs was 64. In this study, average expected (H_e) and observed (H_o) heterozygosis estimated 66% and 55%, respectively (Table 7). Cluster analysis based on SSR data using Bray–Curtis dissimilarity analysis placed WPs in five groups. The first and second groups each contained 1 WP from Bandar-e Gaz (P15) and Rahimabad (P7), respectively, that were out-grouped (Fig. 3). The populations from Mazandaran province: Shasavar, Ramsar and Noshahr (P5, P9 and P12, respectively) and 1 WP, Ranekouh (P8) from Guilan, clustered together as third group. The fourth group encompassed the highest number of WPs (7) from Guilan province including Rostamabad, Totkabon, Someh Sara, Saravan, Lahijan, Kisom, Kochsfhan (P1, P2, P3, P4, P6, P10 and P11, respectively). The last

Table 4 Mean comparison of the studied traits in 15 WPs

Population code	Origin	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	X12	X13	X14	X15	X16	X17
P1	Rostamabad	5	1.13	1.50	28.42	6.45	3	0.54	0.73	7	1	3	3	3	3	1	5	3
P2	Totkabon	5	1.08	1.40	28.66	6.57	3	0.54	0.74	7	1	5	3	3	3	1	5	3
P3	Someh Sara	4	1.46	1.97	35.49	7.21	3	0.62	0.78	3	2	5	7	2	3	3	7	5
P4	Saravan	4	1.52	2.08	36.14	7.36	3	0.59	0.76	3	2	5	7	2	5	3	7	5
P5	Shahsavar	3	1.72	2.35	40.27	7.73	5	0.66	0.82	3	2	5	7	2	5	5	5	5
P6	Lahijan	4	1.57	2.40	37.11	8.20	3	0.64	0.77	3	2	3	7	2	5	3	3	3
P7	Rahimabad	4	1.47	2.05	35.53	8.30	3	0.63	0.77	3	2	5	7	1	5	5	5	5
P8	Ranekouh	4	1.45	2.12	36.04	8.52	5	0.63	0.79	3	2	3	7	1	5	5	5	5
P9	Ramsar	3	1.68	2.33	40.90	7.93	5	0.66	0.83	5	2	4	7	2	5	5	5	5
P10	Kisom	2	1.83	2.33	46.93	9.45	5	0.67	0.89	5	2	5	7	3	5	7	5	3
P11	Kochsfhan	2	1.97	2.55	47.12	9.57	5	0.67	0.90	5	2	3	7	3	5	7	5	3
P12	Nowshahr	3	1.63	2.42	41.54	8.28	5	0.65	0.84	3	2	4	7	1	5	5	5	4
P13	Chalus	3	1.68	2.45	41.93	8.58	5	0.66	0.84	3	2	5	7	1	5	5	5	5
P14	Aliabad-e-Katul	2	2.12	2.79	48.40	10.17	7	0.70	1.00	7	2	7	5	1	5	3	7	7
P15	Bandar-e Gaz	2	2.15	2.76	49.94	10.30	7	0.72	1.06	7	2	7	5	1	5	3	7	7

X1: Cone shape; X2: cone width (cm); X3: cone length (cm); X4: cone fresh weight (g); X5: cone dry weight (g); X6: bract size (cm); X7: bract width (cm); X8: bract length (cm); X9: main shoot anthocyanin, X10: growth type; X11: size of blade; X12: time of flowering; X13: degree of opening of bracts; X14: intensity of green color; X15: length of apex; X16: width/length ratio; X17: cone size

Table 5 Pearson's correlation coefficient (r) among morphological parameters from 15 WPs

Traits	CS	CW	CL	CFW	CDW	BS	BW	BL	MSA	GT	LSB	TF	DOB	IGC	LA	W/LR	CZ
CS	1																
CW	− 0.958**	1															
CL	− 0.891**	0.950**	1														
CFW	− 0.989**	0.981**	0.929**	1													
CDW	− 0.890**	0.921**	0.878**	0.928**	1												
BS	− 0.776**	0.713**	0.619*	0.749**	0.663**	1											
BW	− 0.916**	0.940**	0.952**	0.939**	0.896**	0.673**	1										
BL	− 0.878**	0.918**	0.806**	0.909**	0.897**	0.828**	0.844**	1									
MSA	− 0.129	0.131	− 0.094	0.121	0.174	0.505	− 0.037	0.434	1								
GT	− 0.647**	0.692**	0.801**	0.679**	0.618*	0.154	0.779**	0.424	− 0.582*	1							
SB	− 0.421	0.493	0.362	0.454	0.423	0.438	0.435	0.631*	0.336	0.188	1						
TF	− 0.371	0.343	0.501	0.370	0.274	− 0.147	0.475	0.003	− 0.834**	0.882**	− 0.179	1					
DOB	0.218	− 0.346	− 0.507	− 0.297	− 0.399	− 0.179	− 0.504	− 0.329	0.336	− 0.552*	− 0.383	− 0.326	1				
IGC	− 0.659**	0.684**	0.795**	0.685**	0.686**	0.360	0.725**	0.458	− 0.351	0.784**	0.106	0.656**	− 0.497	1			
LA	− 0.646**	0.498	0.534*	0.591*	0.522*	0.281	0.591*	0.264	− 0.430	0.680**	− 0.168	0.762**	− 0.087	0.678**	1		
W/LR	− 0.243	0.324	0.161	0.269	0.205	0.217	0.177	0.448	0.260	0.145	0.707**	− 0.138	− 0.244	− 0.123	− 0.223	1	
CZ	− 0.386	0.541*	0.524*	0.446	0.461	0.389	0.557*	0.591*	0.068	0.459	0.739**	0.086	− 0.754**	0.331	− 00.073	0.695**	1

CS: cone shape; CW: cone width (cm); CL: cone length (cm); CFW: cone fresh weight (g); CDW: cone dry weight(g); BS: bract size (cm); BW: bract width (cm); BL: bract length (cm); MSA: main shoot anthocyanin, GT: growth type; LSB: leaf size of blade; TF: time of flowering; DOB: degree of opening of bracts; IGC: intensity of green color; LA: length of apex; W/LR: width/length ratio; CZ: cone size

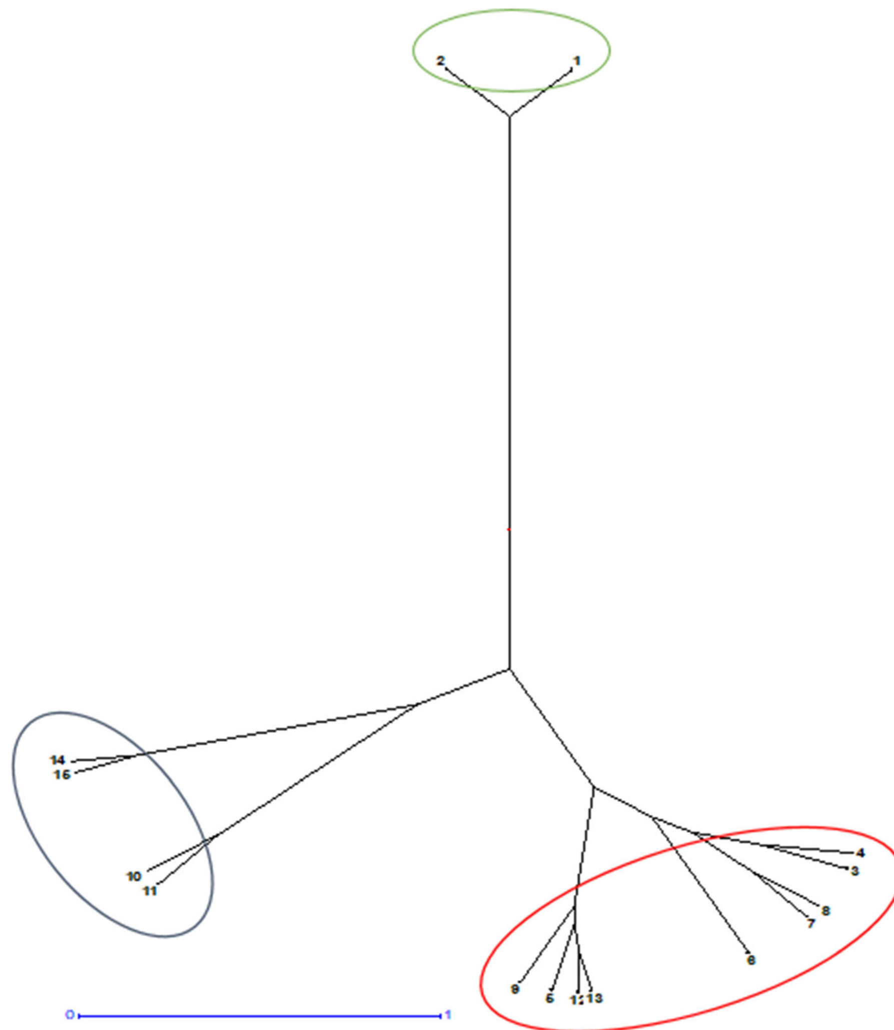

Fig. 2 Dendrogram of the 15 WPs using squared Euclidean distances based on 17 morphological characteristics

cluster had two WPs 1 from Mazandaran (P13: Chalus) and Golestan (P14: Aliabad-e-Katul). The similarity coefficient among the WPs were between 44 and 92%.

ISSR and RAPD Analysis

A total of 232 score-able markers were produced by fourteen polymorphic ISSRs with an average of 16.6 bands per marker, while RAPDs had less bands (70), the mean number of bands per marker was 14 (Table 6). The PPB for ISSRs and RAPDs were 71.6% and 91.9%, respectively, which in this index RAPDs were notably more informative than ISSRs. For ISSR primer combination, the PIC value ranged from 0.26 to 0.35 with a mean of 0.30. In RAPDs, the PIC value varied from 0.20 to 0.30 with an average of 0.24. Cluster analysis through the UPGMA method with simple matching distances placed WPs in 4 groups in which the first group (1) had 4 WPs, 75% belong to Guilan area (Totkabon (2), Someh Sara (3) and Kochsfahan (11) and 25% to Golestan region (Bandar-e Gaz (15), and WPs of this group had similarity coefficient between 0.36 and 0.68 which consisted of WPs of 4 parts of the two areas. The second group (2) included 3 WPs, P6 and P7 from Guilan (Rahimabad and Lahijan) and P9 from Mazandaran (Ramsar), respectively, which the similarity coefficient was between 0.40 and 0.71. The third group (3), as the biggest group, included 5 WPs which the

Table 6 Details of RAPD, ISSR and SSR primers with various parameters revealing the discriminatory power of each primer

Primers	TNB	NPB	PPB	PIC	Allele size (bp)
RAPD					
RAPD-g11	13	12	92.3	0.204	150–1500
RAPD-m2	6	6	100	0.233	300–750
RAPD-m3	11	10	90.9	0.209	200–1000
RAPD-a2	17	13	76.4	0.256	300–1750
RAPD-a10	23	23	100	0.304	100–2000
Total	70	64	–	–	
Average	14	12.8	91.9	0.24	
ISSR					
ISSR440	17	13	76	0.26	160–980
ISSR425	19	12	63	0.35	150–100
PRI-1	14	11	78	0.24	150–1000
PRI-4	14	6	43	0.27	200–1500
PRI-5	18	11	61	0.32	100–2000
PRI-7	19	19	100	0.31	150–1500
PRI-9	24	12	50	0.33	300–1000
ISSR-2	12	10	83	0.31	200–800
ISSR-5	17	8	47	0.27	300–1750
ISSR-7	14	8	57	0.30	100–1800
ISSR-9	15	15	100	0.28	150–1500
ISSR-10	19	13	69	0.28	300–750
ISSR-11	13	13	100	0.32	200–1000
ISSR-12	17	12	71	0.29	300–1800
Total	232	163	–	–	
Average	16.6	11.6	71.6	0.30	
SSR					
HlGT24	5	5	100	0.97	270–310
Hl-ACA2	4	4	100	0.55	260–272
Hl-ACA3	4	4	100	0.69	250–258
Hl-AGA1	4	4	100	0.50	215–232
Hl-AGA4	3	3	100	0.59	195–240
Hl-AGA6	5	5	100	0.62	190–230
Hl-AGA7	3	3	100	0.52	190–220
HlGA22	6	6	100	0.67	165–184
HlGA23	5	5	100	0.70	165–182
HlGA24	5	5	100	0.63	145–170
HlGA27	4	4	100	0.65	138–190
HlGA31	9	9	100	0.73	134–167
HlGA35	2	2	100	0.51	120–135
HlGA36	5	5	100	0.64	117–137
Total	64	64	–	–	–
Average	4.57	4.57	100	0.64	

TNB: total number of bands; NPB: number of polymorphic bands; PPB: percentage of polymorphic bands; PIC: polymorphism information content

Table 7 Genetic diversity indices of eight genic SSRs evaluated using 15 WPs

Marker	N	H_O	H_e	HW
HlGT24	5	0.48	0.34	0.06
Hl-ACA2	4	0.53	0.56	0.11
Hl-ACA3	4	0.65	0.77	0.00
Hl-AGA1	4	0.31	0.57	0.00
Hl-AGA4	3	0.73	0.75	0.82
Hl-AGA6	5	0.63	0.64	0.88
Hl-AGA7	3	0.82	0.74	0.69
HlGA22	6	0.57	0.70	0.15
HlGA23	5	0.73	0.72	0.51
HlGA24	5	0.44	0.68	0.00
HlGA27	4	0.47	0.71	0.00
HlGA31	9	0.65	0.75	0.02
HlGA35	2	0.49	0.68	0.02
HlGA36	5	0.52	0.56	0.01
Mean	4.57	0.57	0.66	–

nA: number of alleles per locus; H_o: observed heterozygosities; H_e: expected heterozygosities, HW: Hardy–Weinberg test

WPs from Guilan (Rostamabad (1) and Saravan(4), Mazandaran (Nowshahr (12) and Chalus (13) and Golestan (Aliabad-e-Katul (14) were clustered together, and in the fourth group (4) WPs from Guilan (Ranekouh (8) and Kisom (10)) and Mazandaran (Shahsavar (5) were clustered together; the similarity coefficient was 0.41 and 0.74. Therefore, in ISSR-RAPD dendrograms, WPs, two by two, had less similarity and most of WPs grouped together with similarity coefficient between 0.41 and 0.65 (Fig. 4).

Structure analysis

STRUCTURE yielded K and ΔK and the two-dimensional graph is presented in Fig. 5, showing a

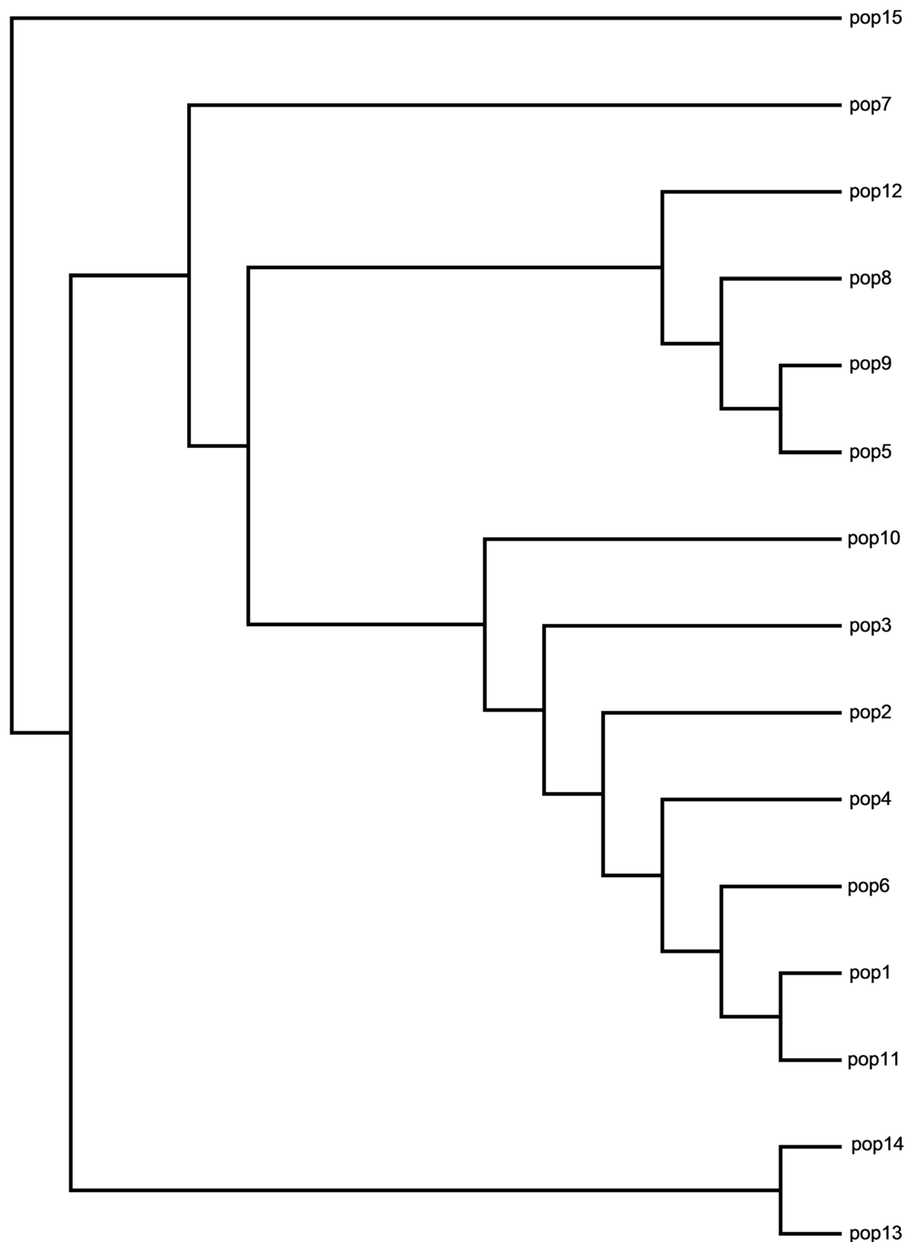

Fig. 3 Dendrogram of the 15 WPs based on SSR markers

two-way graph for determining the optimal K. The best K, which is the peak of the curve, was 5. Supplemental Table S4 shows the placement of each genotype in each cluster. S10, G2, G10, G13, G14, G16, G18 G46 and G47 WPs were clustered in group 1; G4, G16, G20, G21 G28 and G36 WPs were clustered in group 2; G3, G24, G26, G29, G31, G32, G37, G39 and G54 WPs were clustered in the 3rd group; G7, G9, G22, G25 and G30 WPs were clustered in the 4th group and G42, G44, G48, G50, G51, G52 WPs were clustered in the last group. The bar plot diagram for the population structure is shown in Fig. 6. Of the 4 WPs in cluster 1, 50% belonged to Guilan and 50% belonged to Mazandaran. In the cluster 2, 20% of the WPs belonged to Mazandaran and 80% belonged to Guilan Province. In cluster 3, 11% of the WPs belonged to Golestan Province, 22% of them belonged to Mazandaran Province and 67%

Fig. 4 Dendrogram of the 15 WPs based on ISSR and RAPD markers

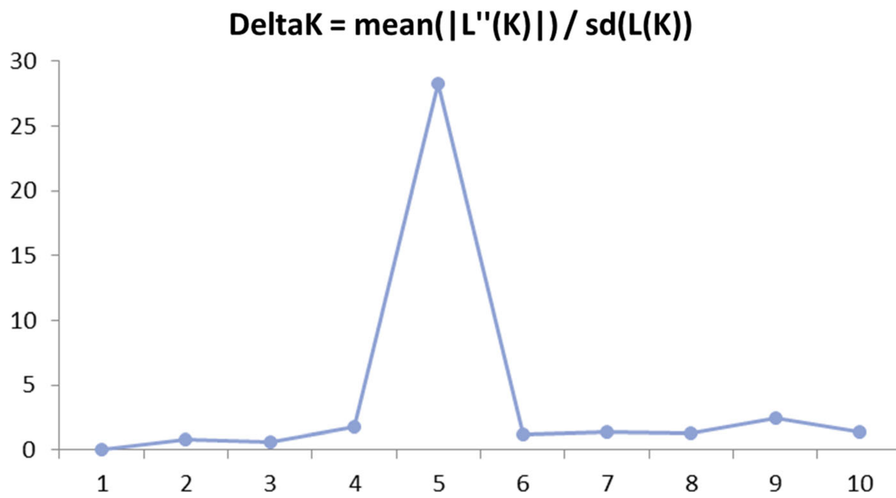

Fig. 5 Values of ΔK, with its modal value detecting a true K of the four groups (K = 5)

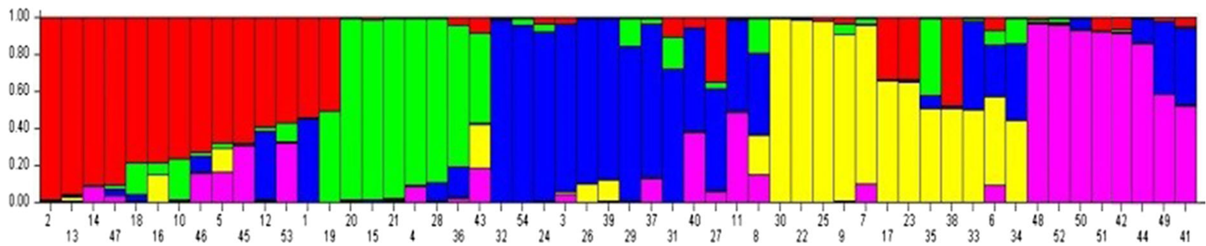

Fig. 6 Projected genetic STRUCTURE of 54 hop individuals with K = 5 clusters. Red cluster (**a**), green cluster (**b**), Blue cluster (**c**), yellow cluster (**d**), Purple cluster (**e**). The y-axis shows the proportion membership into various clusters. (Color figure online)

belonged to Guilan Province. In cluster 4, all the WPs belonged to Guilan Province. Interestingly, in the last cluster, 33% of the WPs were from Mazandaran Province and 67% were from Golestan Province.

Discussion

Identification of genetic diversity as well as natural population relationships and structure are necessary steps toward rational exploitation a new genetic reservoir and pave the way to conserve a germplasm properly, therefore, we attempted to reveal the possible genetic diversity in *H. lupulus* L. wild germplasm in Northern Iran for the first time. We detected the structure of WPs through two well-proven approaches, including morphological and molecular analysis which frequently have been employed for hops (McAdam 2013; Mongelli et al. 2015; Turchetto et al. 2016). In this study, a large degree of variation among populations were observed for most yield components. Therefore, due to the high genetic diversity for different agronomic traits among native hop populations, especially in terms of yield and yield components, potential to breed cultivars for flower yield, growth characteristics and other yield characteristics exists.

In the process of examining plant genetic diversity, evaluating the correlation of traits is critical perquisite for selection of superior WPs (Ali et al. 2003). Therefore, simple correlation was used to obtain information about the relationship between traits. Strong correlation between cone length, cone width, bract width, bract length, leaf size of blade and width/length ratio was observed. Due to morphological complexity, breeders may employ our various measures in selecting source plants (Yimram et al. 2009). Our results showed a wide range diversity in the vegetative traits of Iranian hops. Similarly Mongelli et al. (2015) observed a significant variation in evaluated morphological features in an Italian hop germplasm.

Clustering patterns of populations based on morphological characters inclined to be consistent with geographical origins. This finding was in accordance with Hartings et al. (2008) observations on maize, and also Liu et al. (2016) on *Ulmus lamellose*. Moreover, the dispersion pattern of a species may impact genetic diversity, because typically plants with less dispersion have less genetic variability (Hamrick and Godt 1996). Even though hop populations in Northern Iran are in decline, geographic distribution ranges from east to west of Hyrcanian forests, which predisposes morphological and genetic diversity. Most of the wild hop populations are late flowering, unlike the commercial varieties, suggesting lack of domestication in the WPs. Russian native hops, especially in the main hop growing areas, are early flowering: in order to cope with the constraint cold climate (less than 100 days to vegetation phase), domestic hops have undergone breeding selection for such commercial agronomics (information from official booklet of Research and Technological Institute of Hop Production, Chuvashia, Russian Federation). Another example which as Skomra et al. (2013) reported, domestic hops have shorter branches in comparison with other wild hop collections in Europe. Short branches are correlated to early maturation, however, the short branch pattern has been observed in some late-maturing lines. On the contrary, our inquiries from local people (where WPs were sited) and literature search in the available early documentation of hop in Northern Iran, gave no indication of neither common use of hop nor its cultivation. Understanding the scope of genetic variation and genetic structure of the gene pool is a criterion for managing and efficient use of germplasm

resources. Population structure analysis using the STRUCTURE software allows the population to be segmented into subgroups with different structures. The subgroups are genetically distinct from each other, thus admixed WPs can be identified (Dadras et al. 2014). New genetic technologies, especially DNA sequencing, has led to the development of new methods for measuring the similarity and genetic variation of plant species and populations (Xu et al. 2010). The results of molecular cluster and structure analysis showed that the genetic diversity of collected WPs does not follow their geographic distribution origins: no correlation between the collection site and the genetic distance between the native populations; and the WPs collected from an area were not necessarily similar and had a great genetic distance. Solouki et al. (2008) reported similar results on *Matricaria chamomilla*, in which genetic variation in masses were not matched to geographical distribution. Similarly, resultant of experiment conducted on *Jatropha curcas* by Vasquez-Mayorga et al. (2017) to assess the genetic diversity in its Costa Rican germplam revealed that accessions were grouped separately from their sample sites. Other studies reported lack of uniformity between the genetic origin and the collection localities in their observations (Ambrosi et al. 2010; Maghuly et al. 2015).

RAPD-ISSR markers have been applied for detecting genetic variability in *H. lupulus*; for example, ŠUštar-Vozlič and Javornik (1999) investigated 65 hop cultivars by RAPD, and observed the polymorphism of 38.6%. Patzak (2001), also used RAPD, ISSR, STS and AFLP to analysis diversity in ten hop varieties and compared the outcomes, in which RAPD and ISSR polymorphism were 32.6% and 42.3% respectively. In the both studies above, the polymorphism was significantly lower in comparison to our Iranian wild hop collection using the similar markers; RAPD (71.6%) and ISSR (91.9%).

SSR markers indicated high diversity in hop populations: our results were the same or even higher than to level of variation that Bassil et al. (2008) reported in European hop accessions, but the indices that they reported in American wild hops were higher than our Iranian WPs. For our WPs, the polymorphism level revealed by SSRs was higher in comparison to RAPDs and ISSRs, which indicates that SSRs are highly informative and useful markers for hops. Clustering patterns for SSR diversity was observed to successfully separate WPs based on their geographical origins, particularly for Guilan and Mazandaran WPs where mostly clustered together. Suitability of SSRs over other markers also reported in several cases in other plants (Goulão and Oliveira 2001; Palombi and Damiano 2002; Belaj et al. 2003).

STRUCTURE analysis produced 5 groups which they grouped similar to the SSRs distance-based clustering. Commonly, the genetic structure of a germplasm depends on factors (geographical distribution, dispersal paradigm, population size and gene flow) which vary by historical and biological background (Loveless and Hamrick 1984; Lynch and Walsh 1998; Nybom 2004). Ecological circumstances are generators of selective pressures which impose changes at population level that eventually causes distinction between them. Consequently, a reliable prediction of genetic diversity in a plant germplasm cannot be achieved without contemplating the environmental components (Ohsawa et al. 2008; Wang et al. 2018). Ecological factors varied in the locations of WPs, in the respect of not only yearly precipitation, temperature, and light intensity, but the soil features (physically i.e. clay, silt, sand, moss percentage, and chemically, i.e., macro and micro element, pH, EC) (data not shown, available upon request). Based on our field survey, hops in Iran seem fairly adaptable to the wide range of soil properties.

For our WP locations, the climatological elements had varied significantly, as a case in point, yearly average temperature and precipitation in Rostamabad were 16.2 °C and 950 mm, respectively, while in Aliabad-e-Katul, average values were 17.9 °C and 445 mm, respectively. Further, elevation in our survey varied from − 13 (Someh Sara) to 714 (Rahimabad). Detectable changes in plant diversity (i.e. richness and phonotypical variation) often occur in elevations above 700 m (Bonanomi et al. 2016; Xu et al. 2017), and it's generally accepted that with increasing elevation the height of plants shrink (Boscutti et al. 2018). We observed the 'dwarf' growth habit in areas with the highest elevations (714 and 520 m), and speculate that elevation might have a key role. Two WPs in the highest elevation showed the lowest quantity of stature and floral components, perhaps, conditioned by the negative impacts that increasing in elevation has on dry matter, volume or height (Worrell 1987). Soil components in the collection sites were highly variable; for example, pH and nitrogen, both of

which potentially affect plant recruitment, were 4.8 and 0.48%, respectively in Lahijan whereas in Rostamabad they were 6.8 and 0.93%, respectively (data not shown, available upon request). Molecular analyses did not distinguish populations according to their geographical region suggesting gene flows between populations (Martins et al. 2006; Liu et al. 2013; Yang et al. 2016; Vasquez-Mayorga et al. 2017). Although vegetative traits are prone to be interpreted prejudicially (Ravi et al. 2003; Baránek et al. 2006; Yook et al. 2014; Zhang et al. 2018) assessing plant morphological variation as a basic procedure still allows discrimination of plant populations in a convenient and cost-saving way. Tackling erosion of genetic diversity is the core of saving endangered plant species (Avise and Hamrick 1996). Our results can be used in conservation programs to slow down or halt declining *H. lupulus* populations in the Hyrcanian forests in a most effective way. First and foremost, detecting the frequency of populations and diversity of germplasm now, allows tracking of deterioration taking place in each locality over time, recognize the populations that are in danger the most and require the protection urgently. Secondly, by being aware of the wide range of climatic and ecologic factors where hop populations remain can help recover those populations, even reintroduce hop to the sites that once had hops, and this population can be propagated efficiently ex situ under in vitro condition Mafakheri and Hamidoghli (2015b), and provides the suitable propagation material to repopulate the hop populations.

Conclusions

A large spectrum of diversity was demonstrated in the Iranian hop germplasm, which offers a unique opportunity to access a new reservoir of suitable alleles that breeders can utilize to cope with novel challenges in developing new cultivars. Evaluating a germplasm through relying on morphological features and using different molecular methods (SSR, ISSR, and RAPD) can generate a reliable information on such previously unexplored germplasm, but it is recommended, first, to use more efficient molecular markers (more SSRs and SNPs), which have a high polymorphism and exist abundantly in the genome, in the identification and genetic relationship of hop indigenous populations. Also, we are profiling hop secondary metabolites as the third approach to differentiate between populations.

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Author contributions MM and PM conceived and designed research. MM and MK conducted experiments. MK, MM, MR, and PM analyzed data. MK, PM and MM wrote the manuscript. All authors read and approved the manuscript.

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