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Morphological, Molecular and phytochemical Variation in Some Thyme Genotypes

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ABSTRACT
Thyme is an important medicinal plant in cosmetic, pharmaceutical and food industries. The first step for breeding of thyme is evaluating of genetic variation and relationship between thyme's accessions. Thirteen accessions of Thyme medicinal plant were studied in the aspect of morphology, chemical and molecular variation. ANOVA showed significant differences between accessions for total characterization tested. The dendrogram constructed on the basis of morphology similarities showed two major clusters. In order to evaluate the genetic variation, the genomic DNA extracted using modified medicinal CTAB protocol. The evaluation of the quality of DNA was examined with electrophoresis. Twenty primers were used for PCR analysis and only 9 primers showed clear bands. Out of 149 bands, 83/22% were the polymorphism. The data were analyzed with SPSS and POPGENE programs and the dendrogram was drawn based on UPGMA and showed three major clusters. In order to evaluate the chemical variation, essential oil was obtained by Clevenger unit. ANOVA showed a significant differences between accessions for total characterization test. Dendrogram for chemical variation showed two major clusters. Chemical and morphological traits' matrices were formed using Statistical V5.5A software and were compared with genetic similarity matrices using GenAlex 6.1 software.
Keywords:Thymus, marker, genetic, essential oil, RAPD

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INTRODUCTION

Thyme is a medicinal herb of the genus *Thymus* and family of Lamiaceae [1]. That is one of the oldest medicinal plants and possesses various applications in the food, pharmaceutical and cosmetic industries [2]. The pharmacological properties and biological activity of the thyme are attributed to the presence of essential oils. Thus, special attentions nowadays have been given to characterize and evaluate the volatile compounds and essential oils present in this plant [3]. The aromatic and medicinal properties of *Thymus* have made it one of the most popular medicinal herbs. *Thymus* essential oil is among the world's best ten essential oils [4]. At present, the demand of essential oils for this herb is raised for perfumery, cosmetic and medicinal use deprived of any breeding programs to select proper cultivars. In traditional herbal medicine, *Thymus* species are greatly used as tonic, antiseptic, antitussive and carminative [5].Two important species of *Thymus, Thymus daenensis* and *Thymus kotschyanus*, in Iranian folk medicine, are more greatly used as fresh or dried for these objectives [4].Wild relatives of crops are usually used as principle resource of genetic variation for new breeding programs, while wild medicinal plants are actually in danger of extinction. Therefore, genetic relationship of these plants is a critical subject [6, 4]. Knowledge about the genetic basis of medicinal plants populations threatened by extinction is an essential factor to perform the conservation programs. The unique genetic makeup of plant populations not only discriminates them from other populations, but also defines their ability to adapt to changing conditions and, potentially, to create new species. Many conservationists would discuss that the conservation of genetic diversity is the foundational basis of all conservation efforts because genetic diversity is requisite for evolutionary adaptation, and such adaptation is the key to the long-term survival of any species [7]. The habitat fragmentation and the spatial distance of populations increase genetic drift

and differentiation among them and reduce their future adaptation to environmental changes [8]. Hybridization is very common in *Thymus* due to cross pollination. Bees are the most insects which visit the flower of this plant and have an important role in transferring of pollen. Thereby, high morphological variations present in the thyme population [9]. All selections are based on variation in the plant breeding. Selection requires genetic diversity and high genetic diversity widens the range of selection. The characterization and classification of germplasm allow breeders to avoid duplication. Heterosis depends on the genetic distance between parents and to investigate the genetic distance, cultivars and varieties should be classified [10]. Genetic diversity in plant species through morphological and biochemical traits has always been common. Due to environmental effects on the gene expression, the morphological assessment may not be a reliable method to determine the genetic differences. The differences that are present in the DNA (DNA markers) have been widely used in the classification of organisms, genetic diversity and mapping [11].The aim of this study was to classify the Iranian native thyme using morphological and molecular techniques and to determine the variation in the essential oil content among those varieties.

MATERIAL AND METHODS

Plant material collection

The 13 accessions of thyme were collected from Khorasan Razavi Agricultural and Natural Resources Research Center in Mashhad (Table 1).

Table 1 Genotypes used as plant materials

Number	Accession	Geographical origin
1	*T. kotschyanus*	Zanjan
2	*T.transcaucasicus*	Zanjan
3	*T. kotschyanus*	West Azarbaijan
4	*T.pubescens*	Qazvin
5	*T. kotschyanus*	Qazvin
6	*T. kotschyanus*	Khorasan Razavi
7	*T.pubescens*	East Azarbaijan-Maragheh
8	*T. Vulgaris*	Khorasan Razavi
9	*T. lancifolius*	Fars
10	*T. lancifolius*	Kordestan
11	*T.pubescens*	Zanjan
12	*T. vulgaris*	Markazi
13	*T. kotschyanus *T. Trautveteri*(Hybrid)	West Azarbaijan

Morphological variation

Biometric measurements of the plants were made during the vegetation period including number days to two, four, six and eight leaves, height of plant, maximum diameter crown area of canopy, minimum diameter crown area of canopy, crown area of canopy area, number of stem in each plant, germination percentage, germination speed, number of days to start flowering, number days to 50% of flowering. The samples were taken and shooting parts of the plant were weighed and dried in the shade. After drying process, the samples were weighed again [12].

Phytochemical variation

The leaves of each accession were dried individually in the oven at 60^{o}C for 3 days. Subsequently, the essential oil content was extracted by hydrodistillation for 2.5 hours using a Clevenger [13]-type apparatus by water distillation [12]. The resulting essential oil was dried over anhydrous sodium sulfate and stored at 4^{o}C. The volume, weight and the percentage of the extracted essential oil for each accession were determined.

Genetic variation

One gram of leaf tissue from each accession was placed in a porcelain mortar chilled with liquid nitrogen and ground with a pestle. DNA extraction was performed as Khanuja method [14]. PCR mixtures (25 µl) contained $MgCl_2$, PCR buffer, dNTP, primer (Table. 2), Taq DNA polymerase (Cinna Gen, co, Iran) and 50ng templates genomic DNA. The DNA amplification was carried out using an Eppendorf thermocycler gradient programmed as follows: initial denaturation at 94°C for 3 min, 35 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C and 10 min at 72°C for a final extension. The amplification products were electrophoreses on 1.2% agarose gels with 0.5X TBE buffer for 40minutes at a voltage of 90. Gels were stained with ethidium bromide and visualized under UV light and the size of amplified products was

determined by comparison with lambda DNA digested with *ECOR I*(size range from 250to 1000) used as DNA size marker.

Table 2 Primers used for PCR amplification

Number	Primer code	Primer sequence (5´ → 3´)
1	OPS 17	TGG GGA CCA A
2	OPC 8	TGG ACC GGT G
3	OPJ 21	ACG AGG GAC T
4	OPA 9	GGG TAA CGG C
5	OPI 14	TGA CGG CGG T
6	OPP7	GTC CAT TGA C
7	OPU19	GTC AGT GCG C
8	OPJ18	TGG TCG CAG A
9	JOP19	CAC T GGA CAC

Data analysis

The data obtained from essential oil content and morphological assessments were analyzed in a balanced completely randomized design using SAS (ver. 9) and SPSS (ver. 16)s. Comparison of means was performed using Duncan method at 5% level. Cluster analysis was carried out using Ward [15] method and a dendrogram was constructed using SPSS (ver. 16) software. For molecular data, all the accessions were scored for the presence of band (1) or its absence (0). Only those RAPD bands that appeared distinct in both of the replicate PCR reactions were recorded. The Pop-Gene (ver. 32) software was used to estimate genetic similarity. The dendrogram was drawn based on UPGMA (unweighted pair group method with the arithmetic averages).

RESULTS

Morphological variation

The biometric values were significantly different among all the thyme accessions except Number days to 2 leaves as shown in Table 3. The highest value for the number of days to two, four, six and eight leaves was found to be in the accession 9, 8, 9 and 9, respectively. While, the lowest value for the number of days to two, four, six and eight leaves was observed in the accession 4, 4, 13 and 13, respectively.

The most and the least value for the height of the plant were observed in the accession 8 and 10, respectively. The accessions 4 and 12 appeared to have the maximum and minimum diameter crown area of the canopy, respectively. The most and the least values for the crown area of the canopy were found in the accession 4 and 6, respectively. The most and the least values for the number of the stem in each plant were observed in the accession 7 and 1, respectively. For the traits such as a number of days to start flowering and 50% flowering, the accession 7 indicated the most and accession 1 showed the least values. The accession 3 and 13 showed the most and the least values for the germination percentage and speed, respectively. The dry weight value in the accession 7 was the most and in the accession 13 was the least value.

Table 3 Comparison of mean value of morphological characteristics in the 13 thyme accessions

Accessions	1	2	3	4	5	6	7	8	9	10	11	12	13
Number days to 2 leaves	10.33a	9.84 a	10.57 a	9.94a	11a	11.15a	10a	11.42a	12.11a	11.15a	9.8a	11.82a	11.4a
Number days to 4 leaves	15.11bc	15.15 bc	15c	15.27 bc	16.14a bc	17.5ab	15.14 bc	18.36a	18.33a	16abc	14.6c	17.52a b	17.7a
Number days to 6 leaves	19.55e	22bcd e	23.57 abc	21.16 cde	22.07 bcde	23.1ab cd	20.57 de	23.36a bc	25.33a	22.53 bcd	21cde	22.78a bcd	17.7a b
Number days to 8 leaves	24.22e	26.23c de	28abc	25.11 de	26.42c de	27.2cd e	25de	28.26a bc	30.11a	26.92 bcde	25.1d e	27.65a bcd	29.6a b
Height of stem	15.11cd	15.67c d	15.57 cd	11.22 d	11.92 d	21.15a bc	16.71 bcd	23.26a	17.66a bcd	115.15d	23ab	22.69a b	14.6c d
Maximum diameter of canopy	23.88ab cd	26.23a bcd	21.14 bcd	18.6d	21.5ab cd	31.25a b	25.57a bcd	28.78a bcd	28.11a bcd	19.15c d	31.7a	29.26a bc	21.5a bcd
Minimum diameter	14.66c	20.30a bc	17.57 abc	13.11 c	16.5bc	25.3a	21.42a bc	19.42a bc	21abc	15.38 bc	21.7a bc	23.73a b	13.8c

of canopy													
Canopy area	380.7abc	612.5abc	483.6abc	291.1c	417.1abc	829.2ab	619.3abc	694.2abc	702.7abc	340.6bc	850.4a	733.5abc	282.1c
Number stem of each plant	15.66c	23.69abc	18.71bc	15.55c	17.42bc	26.65abc	22abc	27.57ab	22.22abc	18.15bc	25.4abc	30a	16.7bc
Germination percentage	50f	83.33bcd	96.66a	56.66f	86.66abc	83.33bcd	83.33bcd	90ab	70e	86.66abc	73.33de	76.66cde	83.33bcd
Germination speed	9.12b	17.62ab	18.6a	10.7fg	17.04ab	14.71cd	11.25efg	17.22ab	10.31g	17.64ab	12.66def	13.2de	15.73bc
Fresh yield	90.8b	97.2b	92.5b	76.9b	76.3b	339.9ab	123.6b	385.9ab	126b	109.8b	144b	595a	82.9a
Dry yield	26.27b	34.8b	30.11b	26.67b	28.33b	127.37ab	47b	145.47ab	48.74b	33.97b	59.12b	214.82a	27.87b
Number days to starting of flowering	163.22bc	161.25bc	155bc	134.11d	154.5bc	148.77bcd	164.33b		161.16b	160bc	145.37cd	196a	144.77cd
Number days to 50% of flowering	181.11b	177.25b	171bc	147.44c	169.17bc	153.5bc	178.5b		176.33b	174bc	164.38bc	209.2a	160bc

Clustering analysis of morphological traits

The clustering analysis was carried out using 15 traits. The dendrogram was designed using SPSS software. Cluster analysis allowed separating thyme in two groups. Group 1 was divided into two subgroups. The first subgroup was consisted of accessions of 2, 10, 3 and 6. The other subgroup was formed by the accessions of 5, 11, 4, 13 and 1. The group 2 consisted of 12, 7, 9 and 8 which the accession 8 was placed in a separate subgroup (Figure 1).

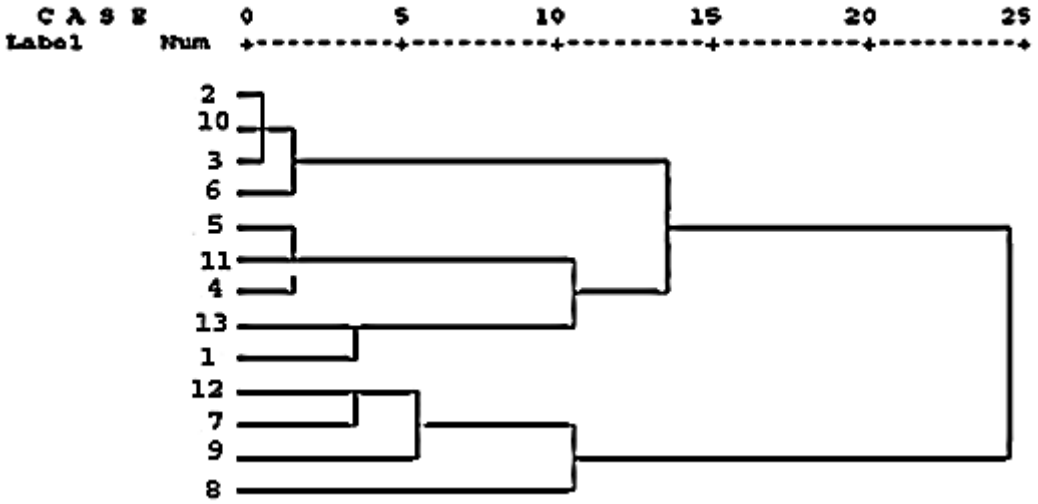

Figure 1The dendrogram constructed using morphological traits

Essential oil content of the thyme accessions

The content of the essential oils was significantly ($p<0.05$) different among all the accessions (Table 4). The accession 8 showed the highest volume and the weight of essential oil. The accession 11 showed the highest and accession 3 showed the lowest values in the percentage of the essential oil production as shown in Table 4.

Table 4 Essential oil content of the thyme accessions

Accessions	Weight (mg)	Volume (cc)	Percentage (%)
1	0.4615abc	0.65 bc	55bc
2	0. 3270bc	0.4 c	64.11bc

3	0.1635c	0.2c	32.05c
4	0.3226bc	0.565bc	55.29bc
5	0.3838bc	0.5bc	39.56c
6	0.6822abc	0.8abc	85.27bc
7	0.7565ab	0.9667ab	87.49bc
8	0.8868a	1.667a	108.01ab
9	0.7063abc	0.86abc	108.66ab
10	0.8493a	0.9ab	78.63bc
11	0.6766abc	0.7abc	153.77a
12	0.65abc	0.75abc	87.84bc
13	0.2798c	0.4c	41.76c

Clustering analysis based on essential oil content

Clustering analysis allowed separating thyme in two groups. Group 1 could be separated into two subgroups. The first subgroup was formed by accession 11 and the second was formed by accessions of 8, 10, 7, 9, 12 and 6. Group 2 could be separated into two subgroups. The first was formed by 3 and the other was formed by accessions of 2, 13, 4, 5 and 1 (Figure 2).

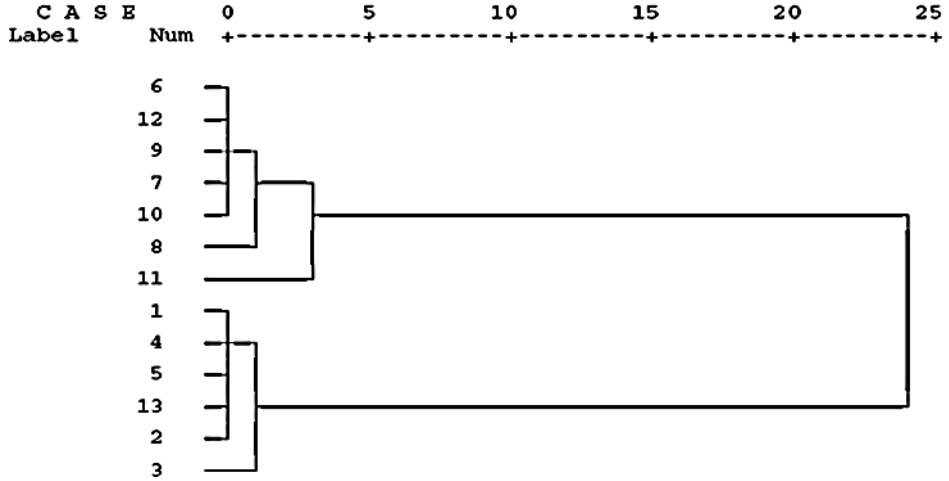

Fig 2The dendrogram constructed based on essential oil content

Assessment of genetic variability

Twenty RAPD primers were used in the PCR reactions. Only eight primers produced clear bands upon gel electrophoresis analysis. For the examined thyme accessions, 149 amplicons were scored which 83.2% of them were polymorphic. The mean of amplicon for each primer was 16.5 and the mean of polymorphic bands for each primer was 13.7. The number of amplification products per primer varied from 12(OPC 8) to 23(OPA9) (Figure 3 and 4). Mean of an effective allele (Ne) per loci was 16 which were similar to the real allele (Figure 2). Based on RAPD data, genetic distance ranged from 0.2559 to 0.6931. Accessions 5 and 3 were in the lowest genetic distance and accessions 1 and 8 were in the most genetic distance (Table 5). Nei›s [16] gene variation average was 0.3560 and Shannon's index (1948) was 0.5322.

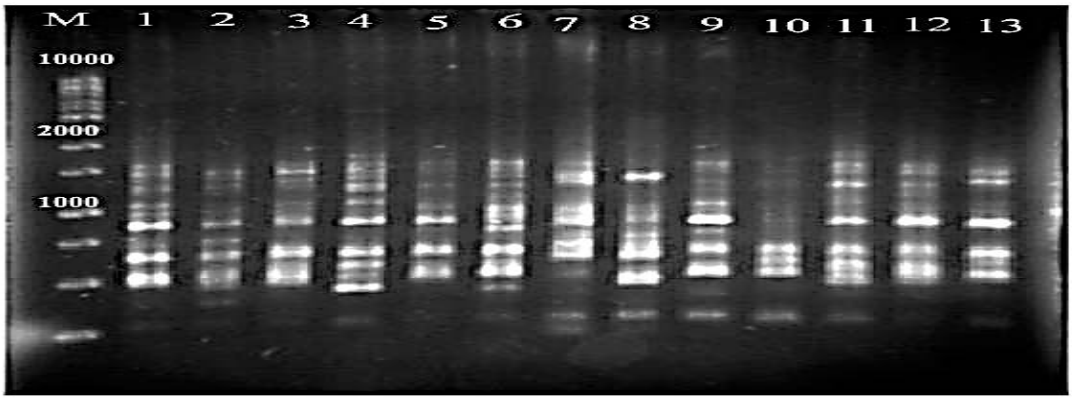

Figure 3 RAPD profiles of the thyme accessions amplified using OPA9 primer on 1.2% agarose gel. M: DNA ladder (Lambda DNA digested with ECORI), number 1-13 are accessions of table 1.

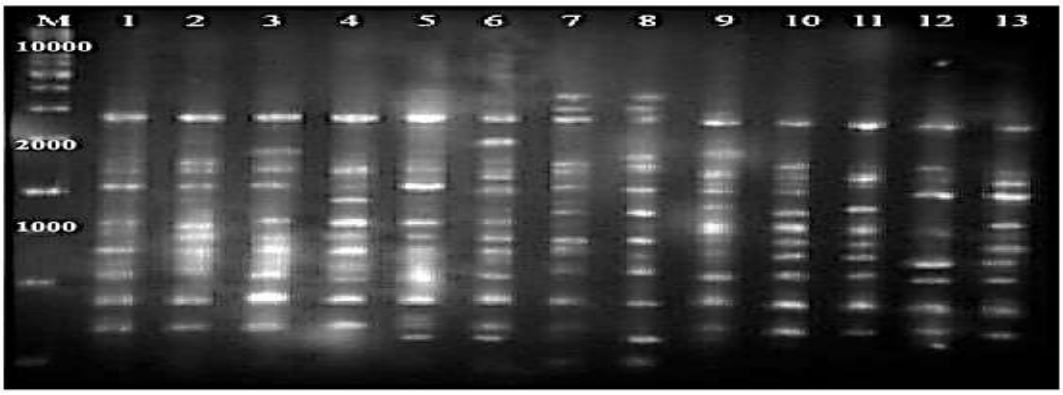

Figure 4 RAPD profiles of the thyme accessions amplified using OPF5 primer on 1.2% agarose gel. M: DNA ladder (Lambda DNA digested with ECORI), number 1-13 are accessions of table 1.

Table 5 Genetic distance matrix for 13 thyme accessions.

POP ID	1	2	3	4	5	6	7	8	9	10	11	12
1	0											
2	0.4258	0										
3	0.4136	0.4765	0									
4	0.5436	0.4258	0.3895	0								
5	0.4636	0.5298	0.2559	0.3895	0							
6	0.6459	0.5162	0.3776	0.4765	0.3544	0						
7	0.6614	0.5298	0.5436	0.5436	0.5436	0.5298	0					
8	0.6931	0.6459	0.6008	0.6614	0.5718	0.4258	0.3429	0				
9	0.6008	0.5298	0.4895	0.5162	0.4136	0.4765	0.6306	0.5718	0			
10	0.5576	0.6306	0.3776	0.5028	0.4014	0.5162	0.5862	0.5576	0.4014	0		
11	0.5436	0.5576	0.3659	0.5162	0.4383	0.5028	0.6614	0.6931	0.5162	0.4765	0	
12	0.6771	0.6614	0.4014	0.4508	0.3776	0.3895	0.5862	0.5298	0.4258	0.3429	0.3544	0
13	0.5028	0.4636	0.4258	0.5028	0.4014	0.3895	0.5298	0.4765	0.4014	0.3659	0.4258	0.2985

Clustering analysis based on RAPD markers

The clustering based on RAPD markers was used to determinethe genetic distance and the relationship among all the thyme accessions. The dendrogram divided the thymes into two groups. Group 1 was formed by accession 7 and 8. Group 2 was separated into various subgroups(Figure 5).

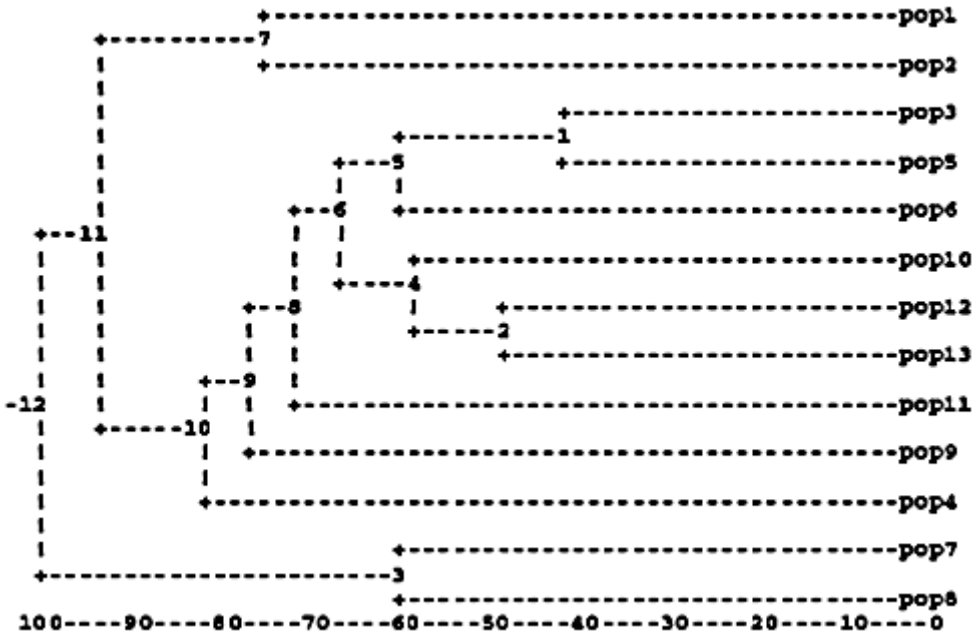

Figure 5 UPGMA dendrogram constructed using RAPD data

Correlation

Correlation coefficients between morphological and molecular matrices were statistically significant (R^2=0.166, p=0.03).The correlation coefficient between distance matrices of molecular and essential oil content (R^2=0.0045, p=0.28) and also correlation coefficient between distance matrices of morphological and essential oil content (R^2=0.0125, p=0.26) was very low and not significant (Figure 6).

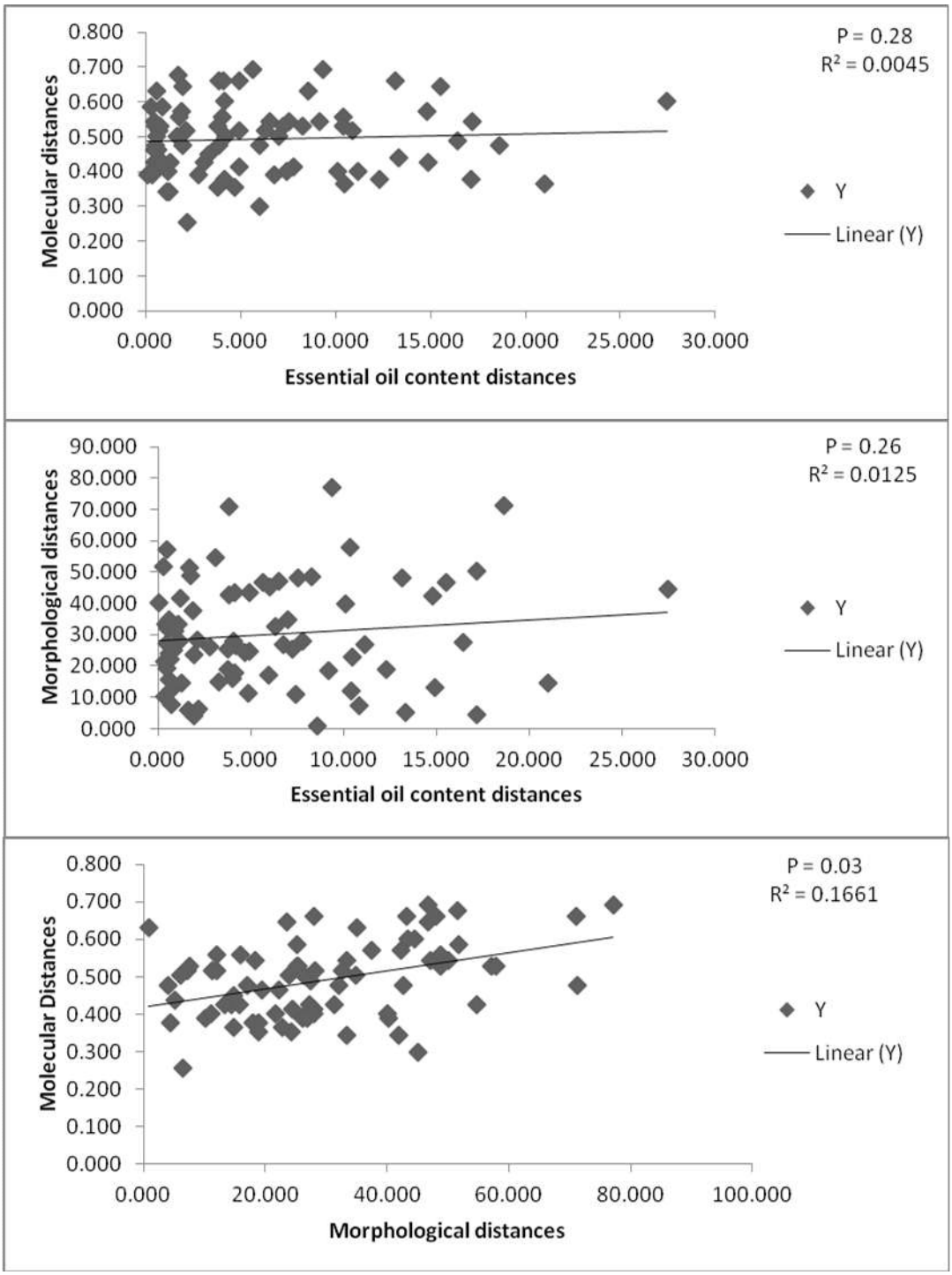

Figure 6The correlation between molecular distances, morphological and essential oil content

DISCUSSION

DNA analysis

PCR analysis indicated that 83.22% of the bands were polymorphic. Alamdari *et al* [17, 18] reported that 61.86% polymorphism was observed in thyme. Percentage of polymorphism was reported 92% in thyme

by Bagherzadeh [19]. Also, Echeverrigaray [20] showed 63.8% polymorphism in *Thymus vulgaris*. As a result, 35% variation was observed between accessions. In a study on two species of *Cuminum* 0.15% and 0.084% variation was observed [21]. Alamdari *et al* [17] reported there was 22% variation between thyme and it is due to one province dispersal of thyme in Iran. Khadivi-khub *et al* [22], showed 63.8% polymorphism in *Satureja bachtiarica*. Hadian *et al* [23] detected 83% polymorphism in *S. hortensis* by RAPD markers so when variation decreases, disease increases and quality of essential oil decreases [24]. In a study on *Bunium* reported an investigation of genetic variation in this species indicated that RAPD marker is a suitable approach to determine the polymorphic loci and to estimate the genetic distance between the populations of the species [26, 27].

Morphological traits and Essential oil content

ANOVA showed that there was a significant difference between thyme accessions for morphological traits. It is important for plant breeding which high variation increases the selection of desirable traits for breeding. In order to crossing and hybridization, parents must be genetically distant to gain the most variation. In this study relationship between thyme accessions using morphological, phytochemical and molecular traits was investigated so plant breeder can use it for production desirable hybrid.

Investigation the morphological traits in this study showed a significant difference between thyme accessions. As a result, the most yield was observed in accession 7 (*T.pubescens*) from East Azerbaijan-Maragheh, 8 (*T. vulgaris*) from Khorasan Razavi and 12 (*T. vulgaris*) from Markazi, so they were supposed to large cultivation. Accession 1 (*T. kotschyanus*) from Zanjan and 8 (*T. vulgaris*) from Khorasan Razavi were placed in separate groups in the morphological and phytochemical dendrogram that confirm with molecular dendrogram. So they can use for desirable hybridization. Accession 3 (*T. kotschyanus*) from West Azerbaijan and 8 (*T. vulgaris*) from Khorasan Razavi were in the lowest distance and they were in one group in the morphological and phytochemical dendrogram. So they don't suggest for hybridization.

In the chemical study, significant differences were observed between thyme samples. According to the available results, it is better at the time of selection of these samples for mass cultivation to be used of accessions with high essential oil content. The most percentage of essential oil was observed in accession 11 (*T.pubescens*) from Zanjan, 8 (*T. vulgaris*) from KhorasanRazavi and 9 (*T. lancifolius*) from Fars so they are suitable for cultivation.

Correlations between morphological, phytochemical and molecular traits

Correlation coefficients between morphological, molecular and essential oil content matrices were very low. Mantel test results showed the average correlation (r=0.166) among morphological and molecular marker. This low correlation may be due to affecting the molecular differentiation by mutations, genetic drift and gene flow. But the difference in morphological traits is more dependent on natural selection and influenced by environmental factors. Also, it has been reported differences between RAPD data and morphological based on grouping by Harrison *et al*., [33]; Persson *et al*., [43]; Samal *et al*., [29] that it is matched to our results. Persson *et al* [44] to identify12varieties of rhubarb studied 12 morphological traits and 47 RAPD markers.

In a study on *Satureja bachtiarica*, the results showed that grouping based on molecular markers and morphological traits were different so these two systems could not discriminate individuals as the same way. The genetic relatedness among the studied individuals could provide useful information for conservation and selection of cross-parents in breeding [22]. Incongruence reported between genotype and phenotype suggests that parental phenotypes are affected by introgression, and intermediate hybrid phenotypes can be genetically closer to one of the parents. Thus, it is evident that morphology, when used alone, can be misleading for interpreting hybridization, and critical evaluation of another data is needed [35].

Which only small portions are the coding regions [39, 42]. According to Persson and Gustavsson [44], the relationship between molecular markers and phenotypic traits could be significant if the markers were linked toselected loci.Some authors have reported an association between volatile compounds and molecular diversity. Some authors have reported an association between volatile compounds and molecular diversity. In a study on *Ophrys lupercalis, Ophrys iricolor* (Family Orchidaceae) and their hybrids, no correlation was found between scent compounds and AFLP data using the Mantel test [48]. On the other hand, the Mantel test showed a correlation between scent compounds in Sorbus species (family Rosaceae)and the genetic variability revealed by AFLP [32]. A weak correlation was also found between AFLP and theessential oil profile of *C. sativum* L. fruits from different populations[40].

Azizi *et al* [30] in a study of 42 accessions of *Origanum vulgare* L., mostly originating from Europe, were evaluated, to detect molecular, quantitative morphological, and chemotype polymorphisms and to discover possible correlations between them. A relatively high correlation between chemotypic patterns and genetic markers was identified, while a lower correlation was found between the morphological and genetic matrices.

In a study on Tansy (*Tanacetum vulgare*), Keskitalo *et al* [34] observed a high correlation (r^2=0.407) between their genetic distance matrix, based on random amplified polymorphic DNA (RAPD) and their chemical distance matrix, suggesting that differences in terpenoid composition could be related to the differential activation of specific enzymes and indirectly to molecular-marker polymorphisms.

In a report on *Teucrium arduini* L. the Mantel test showed a very weak correlation between the AFLP data and morphological traits(r= 0.19). A weak correlation was found between the morphological traits and geographical position of the populations. There was no correlation between the AFLP data and essential oil profile [37].

In another study, to ascertain whether there are chemical and genetic relationships among some *Thymus* species and also to determine a correlation between these two sets of data, the essential oil composition and genetic variability of six populations of *Thymus* were analyzed by GC and GC/MS, and also by randomly amplified polymorphic DNA (RAPD). RAPD Markers allowed a perfect distinction between the different species based on their distinctive genetic background. However, they did not show identical clustering with the volatile oil profiles [46].

Chemical and genetic differences of twenty taxa belonging to four *Thymus* species were studied in order to determine whether molecular characters and essential oil components could be used as taxonomic markers and to examine the correlation between them. Partial correlation has been found between molecular and chemical assessments [46].

In another paper, Labra *et al* [38], experiment the usefulness of molecular markers of DNA polymorphism, based on AFLP analysis, to unravel disputed attributions. They conclude that the combined analysis of morphological traits, volatile oil composition and molecular markers represents the optimal approach to verify taxonomy and to correlate it with agronomic traits.

CONCLUSION

Morphological, molecular and phytochemical analyses showed a good genetic diversity between different accessions. These achievements are important for germplasm management and also will help breeders in selection programs to obtain a desirable cultivar. So, it is expected that this collection could provide a sufficient genetic variation and good sample set for choosing highly polymorphic markers.

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