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Mentha aquatica L. Populations from the Hyrcanian Hotspot: Volatile Oil Profiles and Morphological Diversity

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Abstract: *Mentha aquatica* L. (Lamiaceae) is found in different parts of Iran. Its essential oil and preparations regulate bile function and are used as a stomach tonic and disinfectant. This study investigates the morphological and essential oil diversity of *M. aquatica* populations from the Hyrcanian hotspot of Iran. Plant samples were collected from Gilan, Golestan, and Mazandaran provinces in the Caspian Region for analysis. The results showed significant differences among the studied ecotypes for the stem diameter, collar diameter, number of inflorescences, length and width of inflorescence, sepal diameter, sepal length, and secondary stem length number. Principal component analysis showed that the first seven principal components explained 90.6% of the total variation. Moreover, essential oil concentration varied widely from 1.13% for a sample from Behshahr-Mazandaran, down to 0.27% for one from Abbas abad-Mazandaran. GC–MS analysis identified 29 constituents that accounted for 91% of the total essential oil. The main components of the essential oil were menthofuran (13.21–52.46%), 1,8-cineole (12.42–25.55%), (E)-caryophyllene (3.18–15.43%), viridiflorol (1.04–11.16%), germacrene D (1.70–8.29%), caryophyllene oxide (0.51–4.96%), neryl acetate (1.11–4.95%), p-cymene (1.55–4.77%), and β-pinene (1.7–3.45%). Overall, meaningful diversity was recorded among the populations; Rahimabad-Gilan and Behshahr-Mazandaran would be reliable selections for the food and pharmaceutical industries due to their higher yields and content of α-pinene, 1, 8-cineole, menthofuran, viridiflorol, and β-caryophyllene. Further evaluation of populations from diverse habitats is needed to guide future breeding programs.

Keywords: water mint; principal component analysis; essential oil content; menthofuran

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1. Introduction

Medicinal and aromatic plants are recognized as key sources of raw materials for human health, cosmetics, and pharmaceutical industries. Medicinal plants are essential sources of therapeutics, being the basis of traditional or indigenous healing systems, still widely used in many countries [1]. Recently, the ethnopharmacological potential of these materials has received much attention from scientists and the pharmaceutical industry towards complementing or even replacing conventional pharmacotherapies [2]. Moreover, many of these plants have also been highlighted for their value-added food characteristics, providing a dual role as food flavorings and bioactive compounds [3].

Mentha aquatica L. (water mint) is a perennial medicinal and aromatic herb, originating from the European continent, which produces a diverse group of terpenoid constituents [4]. Moreover, the species is found in moist places and cultivated worldwide [5]. Preparations from its aboveground parts are widely used for gastrointestinal disorders, pulmonary

diseases, and mental conditions such as epilepsy and depression [6]. The major essential oil chemical components of *M. aquatica* L. are piperitenone oxide, β-caryophyllene, 1,8-cineole, α-pinene, β-pinene, viridiflorol, carvone, thymol, germacrene D, borneol, pulegone, caryophyllene oxide, and α-humulene in differing amounts depending on local conditions and genetics [7].

Iran harbors many plant species due to its climatic diversity. Morphological and genetic diversity are the basis for the characterization and description of plant germplasm. This diversity supports the durability of both natural and agroecosystems against extreme environmental conditions, especially climate change. In the face of genetic resource erosion, knowledge of genetic diversity is essential to cope with environmental fluctuations and exploit promising plant species [8,9]. The genetic diversity of medicinal plants is declining in natural habitats, in the main part due to climatic variation and human activities [10,11]. Grazing and drought stress have put enormous pressure on endemic medicinal plants in their natural habitats. Therefore, careful phenotypic, phytochemical, and genetic studies aid in selecting potent ecotypes as an important step in their domestication [9,12,13].

Germplasm collection from diverse habitats, its characterization, and an evaluation of morphological, biochemical, and genetic diversity are the initial steps in breeding and crop-development programs. Afkar [14], in a study on the ecotypes of *M. longifolia* from the Lorestan province, found that Iran reported a reasonable diversity for morphological and biochemical traits. Also, Esmaeili et al. [15] performed a study on water mint samples collected from Amol, in the Mazandaran province, in which the major essential oil compounds from the stem tissue were viridiflorol (11.3%), β-caryophyllene (22.4%), germacrene D (7.7%), limonene (5.5%), and carvone (5.7%). In comparison, the leaf essential oil components were piperitenone oxide (25.7%), β-caryophyllene (12.0%), 1,8-cineole (10.3%), viridiflorol (7.5%), carvone (6.6%), and thymol (3.5%).

Mentha aquatica is commonly used in traditional Iranian medicine, and characterizing its morphological and essential oil diversity is crucial for future breeding programs. There has not been a detailed study to evaluate *M. aquatica* populations from the Hyrcanian biodiversity hotspot of Iran. The current study evaluated the morphological and essential oil compositional diversity of *M. aquatica* populations from the Hyrcanian hotspot of Iran, with hopes that these data will benefit germplasm identification and preservation, domestication, and breeding programs.

2. Materials and Methods

2.1. Plant Material

Habitats of *M. aquatica* were identified by using Flora Iranica, the available literature, and local information [16], and plant samples were collected from the identified areas. Sampling was conducted in 15 areas in the Gilan (Fouman, Rahim abad, Niloo, and Rudsar), Mazandaran (Abbas abad, Chalous, Amol, Kelardasht, Behshahr, Ramsar, and Savadkuh), and Golestan (Aliabad Katol) provinces. The geographical coordinates of the sampling sites were recorded by a GPS device (Table 1 and Figure 1). Additionally, geo-climatic information was obtained from the synoptic station closest to each site (Table 1).

2.2. Morphological Trait Analysis

Fifteen plant samples were taken from each habitat considering the local distribution at suitable spacings to generate a homogenous final sample. Attempts were taken to prevent the harvesting of close relatives. Morphological assays were performed on plants at the full-bloom stage. A taxonomist revisited and verified the plant samples, and the herbarium specimens were then stored in the Horticulture Department of the University of Maragheh, Iran.

Table 1. Geographical information of sampling sites for *M. aquatica* plants from the Hyrcanian hotspot of Iran.

No	Region	Sample Code	Longitude (N)	Latitude (E)	Elevation (m asl)	Average Annual Rainfall (mm)	Average Annual Temperature (°C)	Average Annual Relative Humidity
1	Abbas abad-Mazandaran	AA.M	51°05′43.2″	36°43′51.8″	−14	1125	18	80
2	Savadkuh-Mazandaran	AS.M	52°54′22.30″	36°05′10.0″	934	532	11.1	67
3	Behshahr-Mazandaran	ABh.M	53°35′43.95″	36°39′52.55″	370	630	17.5	75
4	Chalous-Mazandaran	CH.M	51°23′00.40″	36°36′16.00″	130	630	17.67	78
5	Aliabad Katol-Golestan	FAA.G	54°48′57.9″	36°44′59.7″	855	714	17.7	75
6	Fouman 1-Gilan	FM.G1	49°14′06.6″	37°12′34.79″	117	1023	15.91	74
7	Fouman 2-Gilan	FM.G2	49°11′54.1″	37°11′42.8″	133	1023	15.8	78
8	Fouman 3-Gilan	FM.G3	49°10′53.7″	37°11′09.8″	169	1030	15.6	79
9	Fouman 4-Gilan	FM.G4	49°09′01.8″	37°10′41.8″	209	1040	15.2	80
10	Ramsar-Mazandaran	JR.M	50°39′33.6″	36°49′8.2″	741	775	17.3	80
11	Kelardasht-Mazandaran	KD.M	51°09′30.5″	36°34′26.50″	1053	589	13.4	55
12	Niloo-Gilan	NR.G	50°14′40.3″	36°53′49.00″	616	926	15.1	73
13	Rahim abad-Gilan	RA.G	50°18′07.7″	37°00′12.00″	102	870	15.8	80
14	Rudsar-Gilan	RS.G	50°18′32.6″	37°07′21.8″	−14	823	16.6	80
15	Amol-Mazandaran	ZA.M	52°21′34.5″	36°19′46.30″	311	671	17.5	78

Figure 1. Geographic map (marked location) of sampling sites for *M. aquatica* plants from the Hyrcanian hotspot of Iran.

The evaluated traits were: the petal length, stem diameter, collar diameter, sepal width, sepal length, leaf width, leaf length of the lateral branches, leaf length of the main branch, the distance between the internodes, petiole diameter, petiole length, inflorescence length, branch length, plant height, number of flowering branches, and the number of flowers per inflorescence, all measured by using a digital caliper and ruler. The aerial parts were dried under shade conditions at ambient laboratory temperatures until they reached a constant weight.

2.3. Phytochemical Analysis

2.3.1. Essential Oil (EO) Extraction

To extract *M. aquatica* essential oils, 50 g of shade-dried aerial parts were ground and subjected to hydro-distillation for three hours in a Clevenger-type apparatus (British Pharmacopoeia model). To remove possible water droplets in the essential oils, the samples were dehydrated over anhydrous sodium sulfate (0.5–1 g) and then kept at 4 °C until the gas chromatography–mass spectrometry (GC–MS). The essential oil concentration (EO %) was calculated considering the dry mass as in the following equation:

$$\text{EO (\%)} = (\text{essential oil (g)}/40\ \text{g}) \times 100. \quad (1)$$

2.3.2. GC–MS Analysis

Essential oils were analyzed by using GC–FID and GC–MS. The GC–MS analysis was conducted on an Agilent 7990 B gas chromatograph equipped with a 5988A mass spectrometer and a HP-5MS (0.25 mm i.d., 30 m, 0.25 µm f.t., 5% phenyl methylpolysiloxane). The following oven temperature was used: 5 min at 60 °C, then up to 240 °C rising by 3 °C/min, held for 10 min. The helium (carrier gas) flow rate was 1 mL/min; the injector split ratio was 1:30; and the mass range and electron impact (EI) was 40–400 m/z and 70 eV, respectively. The identification of constituents was performed using the procedures explained by Morshedloo et al. [17] based on an interactive combination of linear retention indices (RIs), calculated against a homologous series of n-alkanes (C8–C40, Supelco, Bellefonte, CA, USA) and mass spectrum (MS) matching with commercial libraries (ADAMS, WILEY 275 and NIST 17). The GC–FID analysis was performed on an Agilent 7990 B gas chromatograph equipped with a flame ionization detector (FID), capillary column VF 5MS (30 m, 0.25 mm i.d., 0.50 µm f.t., 5% phenyl methylpolysiloxane). The same oven temperature reported for GC–MS was used. The injection volume was 1µl of an essential oil sample in n-hexane (1:100). The quantification of the constituents was based on peak area normalization without using correction factors [18].

2.4. Statistical Analysis

All data were tested for normality with the Anderson–Darling method, and the homoscedasticity of data was checked with a Levene test and data were standardized by means of a Z-value. ANOVAs were performed with MSTAT-C ver. 2.1 software (USA). Mean data comparisons were analyzed by using the least significant difference (LSD) test at 1% and 5% probability levels. Pearson's correlation coefficients and cluster analysis dendrograms were drawn by XLSTAT software based on Ward's method and Euclidean square distance. Pearson correlation and cluster dendrogram heat maps for the evaluated morphological traits among 15 genotypes were performed in R software for statistical computing (R foundation for statistical computing (version 4.1.2), Iran (2021) URL https://cran.um.ac.ir/, accessed on 10 June 2022). R packages of 'corrplot' (Visualization of a Correlation Matrix, version 0.91; https://github.com/taiyun/corrplot, accessed on 10 June 2022) and 'gplots' (Various R Programming Tools for Plotting Data, version 3.1.1; https://github.com/talgalili/gplots/issues, accessed on 10 June 2022) were employed as well. Owing to a large number of data, principal component analysis (PCA) was done by visualization of the assessed growth parameters and EO components separately, aiming to the distinction of the important traits and components by using Minitab 18 statistical software (Minitab LLC, State College, PA, USA).

3. Results

3.1. Morphological Trait Diversity

The analysis showed significant differences among the ecotypes for the evaluated morphological traits. As shown in Table 2, local environmental conditions affect the plant height, height-to-width ratio, and internode length in the different ecotypes of water mint. The largest plant heights and widths, height-to-width ratios, and internode lengths (118.60, 69.30, 2.91, and 7.66 cm, respectively) belonged to the ecotypes from Niloo-Gilan, Amol-Mazandaran, Kelardasht-Mazandaran, and Fouman 2-Gilan. In contrast, their lowest values (55.30, 19, 1.38, and 4.36 cm) were observed in Kelardasht-Mazandaran and Aliabad Katol-Golestan ecotypes, which in comparison to the first group showed a decrease of 114, 264, 58.69, and 68.75%, respectively (Figure 2a–d).

Table 2. ANOVAs for morphological traits of *M. aquatica* populations from the Hyrcanian hotspot of Iran.

Trait	Ecotypes	Error	% CV
Plant height	543.00 *	167.00	18.47
Plant width	408.00 *	69.00	21.27
Plant length/width ratio	0.62 *	0.19	18.17
Stem diameter	0.01 *	0.02	16.48
Internode length	1.64 **	1.35	18.38
Branches number	25.42 *	4.90	21.49
Lateral branch length	613.64 *	82.41	27.74
Collar diameter	2.16 **	0.11	6.32
Leaf length	0.47 *	0.83	19.76
Leaf width	0.003 *	0.001	10.05
Inflorescence length	0.04 **	0.005	12.67
Inflorescence width	0.08 **	0.002	14.47
Flowers number in inflorescence	0.04 *	0.019	9.46
Corolla length	0.83 *	0.39	12.47
Corolla width	0.40 *	0.12	13.34
Calyx width	1.61 **	0.09	10.44
Calyx length	0.08 *	0.034	18.25
Leaf length/width ratio	0.06 *	0.132	21.98
Leaf width in lateral branches	0.15 *	0.108	12.41
Leaf length in lateral branches	0.08 *	0.032	12.26
Inflorescences number	352.00 **	6.50	16.33
Plant dry weight	342.00 **	27.50	12.45

* and ** indicate significant at 5% probability level, and significant at 1% probability level, respectively.

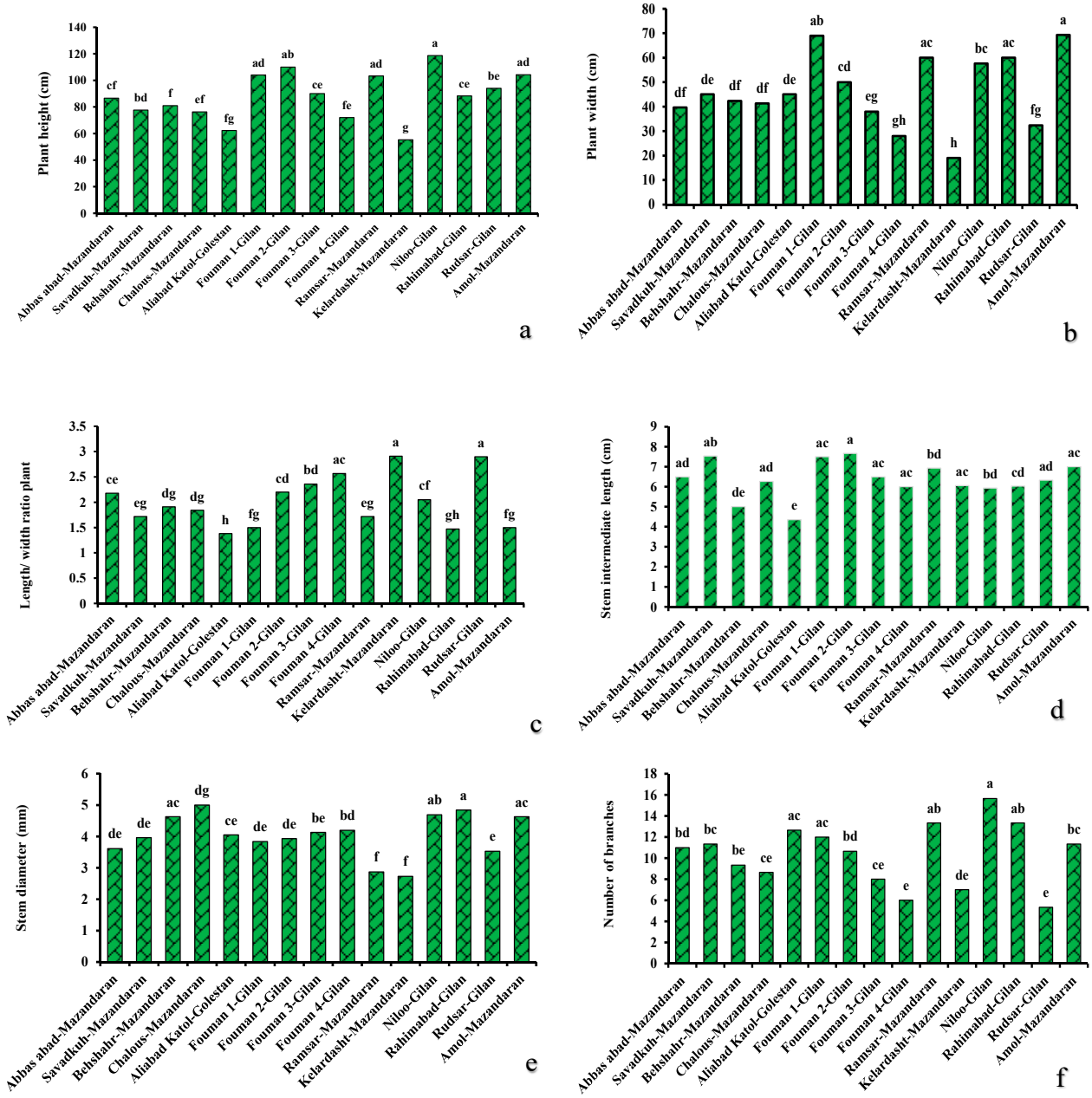

Figure 2. Mean comparisons for plant height (**a**), plant width (**b**), height-to-width ratio (**c**), internode length (**d**), stem diameter (**e**), and lateral branch number (**f**) of 15 different ecotypes of *M. aquatica* collected from the Hyrcanian hotspot of Iran. Different letters indicate significant differences according to the LSD test at $p < 0.05$.

The results also showed that the main stem diameter and lateral branch number were significantly related to the ecotypes (Table 2). The largest main stem diameters and lateral branch numbers (5 and 15.66) were observed in Rahimabad-Gilan and Niloo-Gilan ecotypes, respectively. The lowest values (2.73 and 5.3) for these traits were seen in Kelardasht-Mazandaran and Fouman 4-Gilan ecotypes, respectively, exhibiting up to 83.15 and 195.00% decreases compared to the two largest ecotypes (Figure 2e,f).

The results revealed that locality influences the branch length, collar diameter, leaf length, and leaf width in the different ecotypes of water mint (Table 2). The longest lateral branches (53.53 and 36.00 cm) were measured in Ramsar-Mazandaran and Fouman 2, while the shortest (9.83 and 8.00 cm) were from Kelardasht-Mazandaran and Rudsar-Gilan ecotypes, respectively (Figure 3a). Furthermore, the widest collar diameters (7.66–7.29 mm)

were noted in Savadkuh-Mazandaran and Behshahr-Mazandaran ecotypes, and the thinnest (4.06–4.19 and 4.20 mm) were from Kelardasht-Mazandaran, Aliabad Katol-Golestan, and Fouman 1-Gilan, respectively (Figure 3b). The longest and widest leaves (5.83 and 3.38 cm) were found in Amol-Mazandaran and Ramsar-Mazandaran ecotypes. In contrast, the smallest (3.93 and 2.46 cm) were observed in the Fouman 2-Gilan and Fouman 4-Gilan ecotypes (Figure 3c,d).

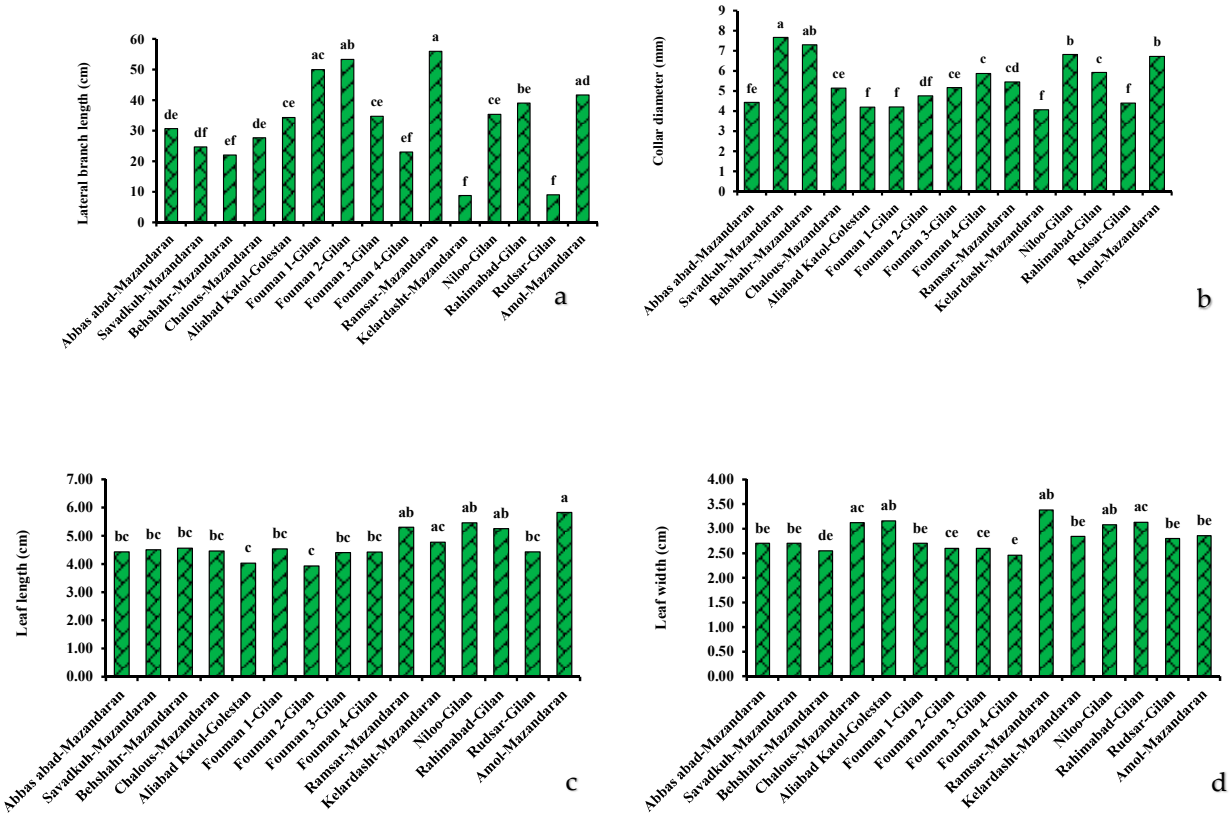

Figure 3. Mean comparison for lateral branch length (**a**), collar diameter (**b**), leaf length (**c**), and leaf width (**d**) of different ecotypes of *M. aquatica* collected from the Hyrcanian hotspot of Iran. Different letters indicate significant differences according to the LSD test at $p < 0.05$.

The water mint ecotypes collected from dissimilar ecological conditions showed significant differences in their leaf length-to-width ratio, and leaf length and width along the lateral branches (Table 2). The greatest leaf length-to-width ratio and leaf length and width along the lateral branches (2.04, 3.26, and 1.91 cm) were observed in Amol-Mazandaran, Abbas abad-Mazandaran, and Amol-Mazandaran ecotypes, respectively. The corresponding lowest values (1.27, 2.3, and 1.20 cm) were noted in the Aliabad Katol-Golestan, Chalous-Mazandaran, and Fouman 4-Gilan ecotypes where reductions of 60.69, 41.73, and 59.16% were recorded compared to the ecotypes with the highest values (Figure 4a–c). The results also revealed that the inflorescence length and width, corolla length, and calyx length in the different ecotypes were significantly influenced by geographical variation (Table 1). The longest and widest inflorescences (2.52 and 2.30 cm) were noted in the Niloo-Gilan ecotype, whereas the other extreme (0.40 and 0.30 cm) was observed in Kelardasht-Mazandaran (Figure 4d,e). Additionally, the greatest corolla and calyx lengths (7.87 and 6.85 cm) were measured in the Chalous-Mazandaran ecotype, and the least (3.55 and 2.01 cm) were found in the Savadkuh-Mazandaran and Fouman 1-Gilan ecotypes, respectively (Figure 4f,g).

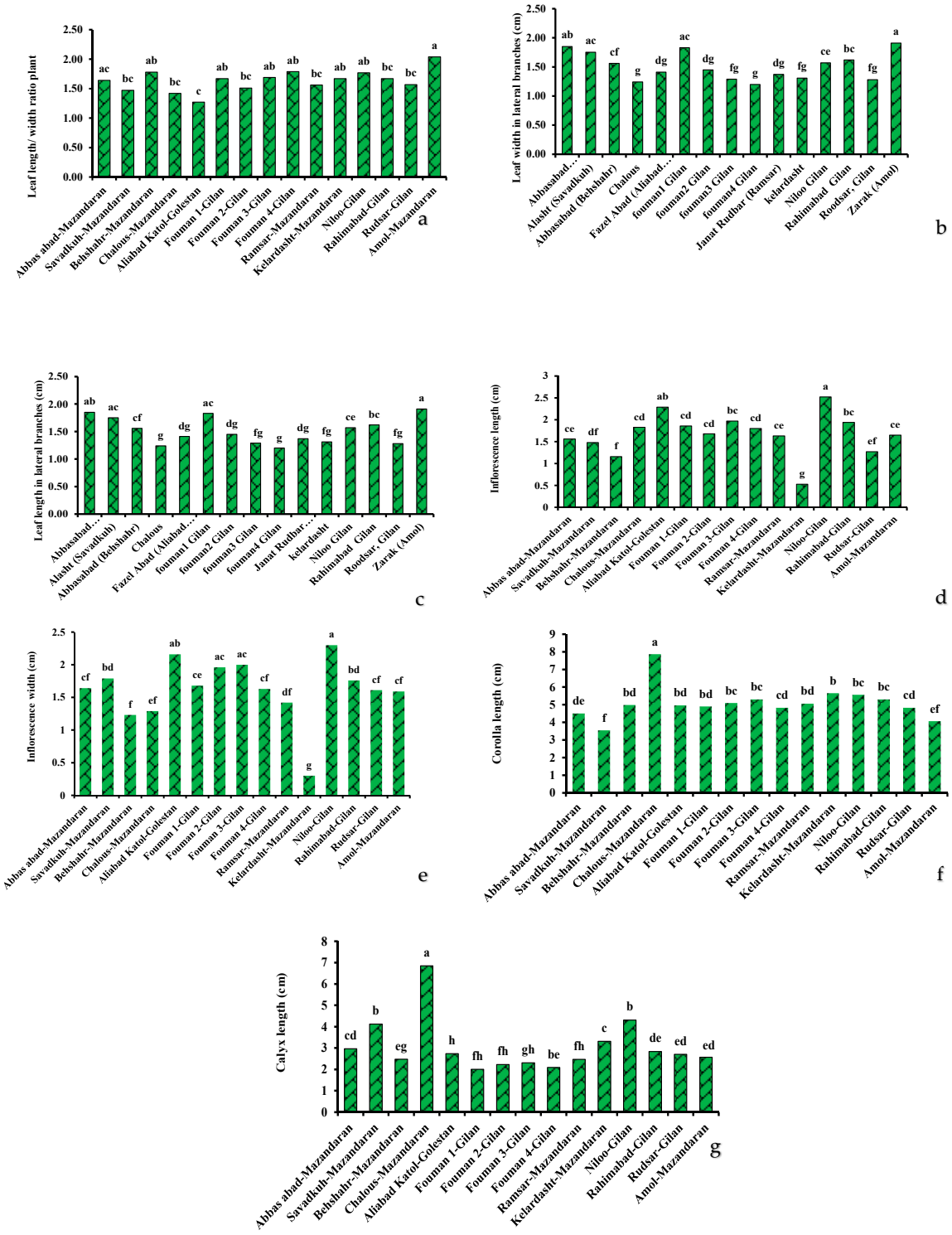

Figure 4. Mean comparison for leaf length-to-width ratio (**a**), leaf width in lateral branches (**b**), leaf length in lateral branches (**c**), inflorescence length (**d**), inflorescence width (**e**), corolla length (**f**), and calyx length (**g**) in different ecotypes of *M. aquatica* collected from the Hyrcanian hotspot of Iran. Different letters indicate significant differences according to the LSD test at $p < 0.05$.

Our findings also showed that these water mint ecotypes had significant differences in the inflorescence number, flower number per inflorescence, and corolla and calyx diameters (Table 2). The highest inflorescence number and flower number in inflorescences (44 and 55) was obtained in the Niloo-Gilan and Fouman 3-Gilan ecotypes, whereas the lowest

(15.00 and 5.66) was recorded for the Chalous-Mazandaran and Kelardasht-Mazandaran ecotypes (Figure 5a,b). Moreover, the widest corollas and calyces (3.36 and 1.47 mm) were observed in the Niloo-Gilan ecotype, with the narrowest (0.85 and 1.32 mm) noted in the Fouman 2-Gilan and Abbas abad-Mazandaran ecotypes (Figure 5c,d).

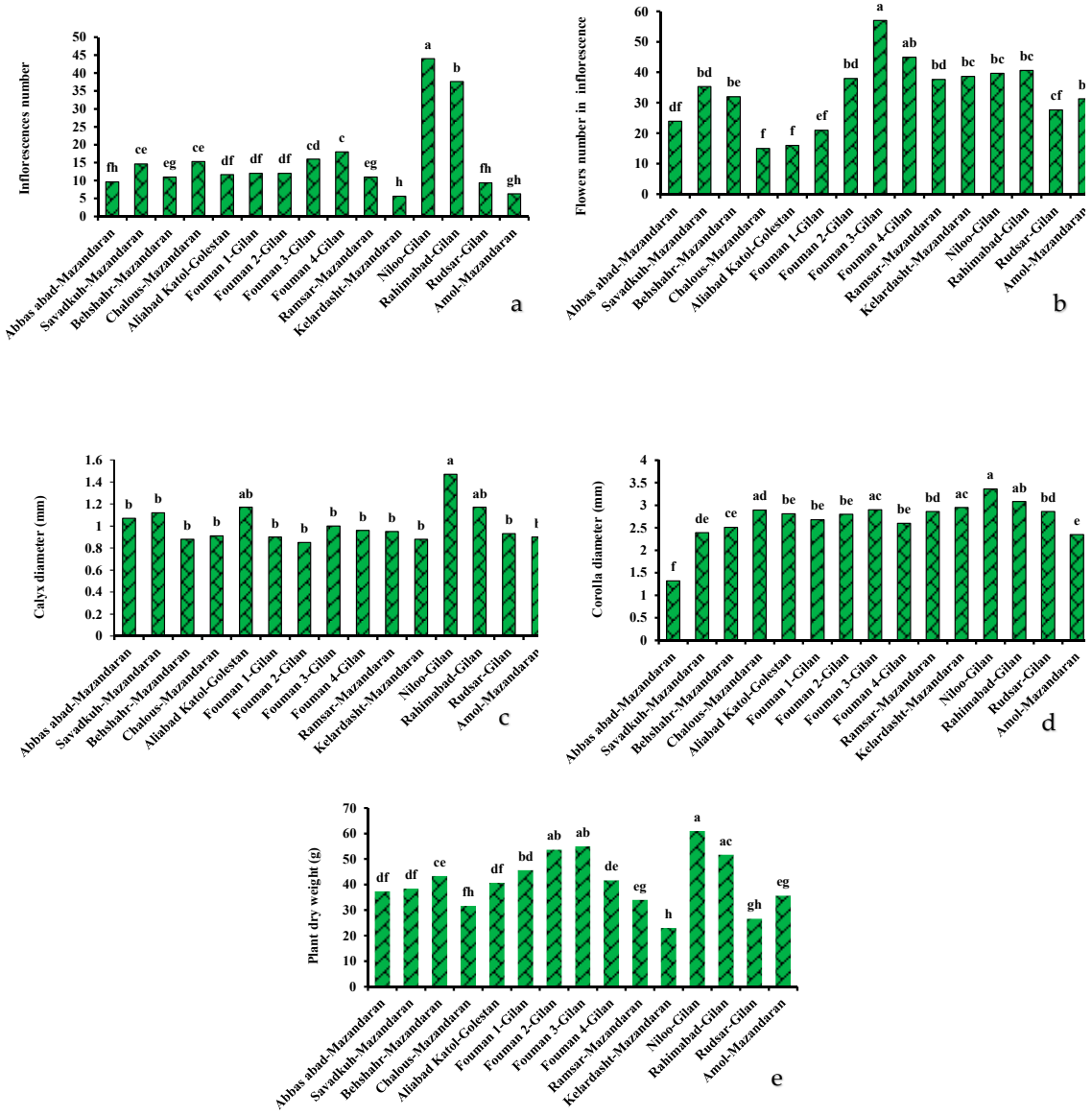

Figure 5. Mean comparison for inflorescence number (**a**), flower number in inflorescence (**b**), corolla diameter (**c**), calyx length (**d**), and plant dry weight (**e**) in different ecotypes of *M. aquatica* collected from the Hyrcanain hotspot of Iran. Different letters indicate significant differences according to the LSD test at $p < 0.05$.

As shown in Table 1, the plant dry weights were significantly different in diverse ecotypes. The heaviest were from Niloo-Gilan and Fouman 2-Gilan, whereas the lightest was recorded from the Kelardasht-Mazandaran ecotype (Figure 5e).

3.2. Essential Oil Concentration (EOC)

Differing environmental conditions significantly influenced the EOC of water mint (Table 2). The highest essential oil concentrations were obtained from Behshahr-Mazandaran (1.13%) and Fouman 3-Gilan (1.00%), while the lowest EOCs were observed in Abbas abad-Mazandaran (0.27%) and Fouman 4-Gilan (0.30%) (Figure 6).

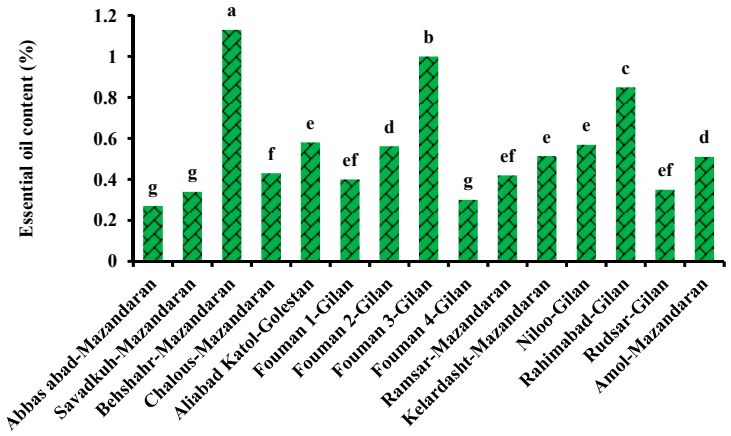

Figure 6. Essential oil concentrations of *M. aquatica* populations from the Hyrcanian hotspot of Iran. Different letters indicate a significant difference according to the LSD test at $p < 0.05$.

3.3. Correlations between Morphological Traits

Results from our Pearson's correlation coefficient testing identified significant positive correlations between selected traits. Plant height showed positive correlations with plant width (r = 0.65) and the number of lateral branches (r = 0.97). Plant width was positively correlated with the lateral branch length (0.67), number of inflorescences (r = 0.57), and leaf width on the main stem (r = 0.69). The plant length-to-width ratio had a significant negative correlation with the number of lateral branches (r = −0.69) and their length (r = −0.75). The lateral branch diameter had significant positive correlations with leaf length and width on those branches. Moreover, the crown diameter significantly correlated with the number of inflorescences (r = 0.72). Leaf length positively correlated with leaf width on the main stem, inflorescence length and width, and corolla length and diameter (Figure 7).

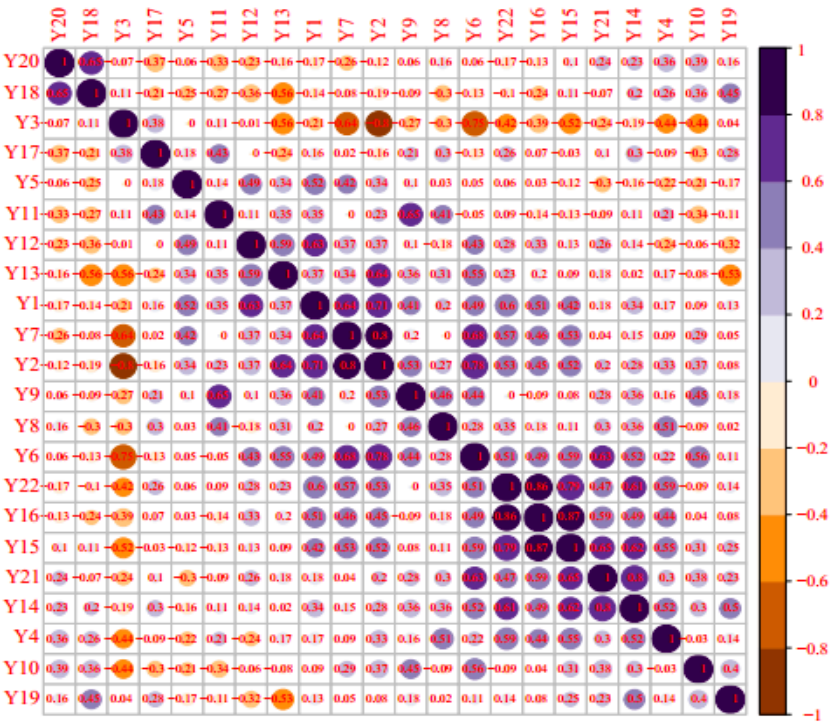

Figure 7. Heat map of Pearson's correlation analysis for morphological traits in different populations of M. aquatia in the Hyrcanian hotspot of Iran. Heat map representing Y1: plant height, Y2: plant width, Y3: plant length /width ratio, Y4: main stem diameter, Y5: tnternode length, Y6: number of lateral branches, Y7: lateral branch length, Y8: collar diameter, Y9: leaf length, Y10: leaf width, Y11:

leaf length-to-width ratio, Y12: leaf length on lateral branches, Y13: leaf width on lateral branches, Y14: inflorescence number, Y15: inflorescence length, Y16: inflorescence width, Y17: flower number in inflorescence, Y18: corolla length, Y19: corolla diameter, Y20: calyx length, Y21: calyx diameter, and Y22: plant dry weight.

3.4. Essential Oil Constituents

The chemical composition of EO obtained from *M. aquatica* populations was investigated by GC–MS. As presented in Table 3, 29 components were identified, which comprised up to 91.33% of the total EO. The dominant EO component was menthofuran (13.21–52.46%). In addition, 1,8-cineole (12.42–25.55%), €-caryophyllene (3.18–15.43%), viridiflorol (1.04–11.16%), germacrene D (1.70–8.29%), *p*-cymene (1.55–4.77%), neryl acetate (1.11–4.95%), *β*-pinene (1.70–3.45%), and caryophyllene oxide (0.51–4.96%) were the other major components (Table 3).

3.5. Cluster Analysis for Morphological Traits

A cluster analysis for the morphological traits was performed based on the Ward method. It separated the 15 ecotypes into three main groups (Figure 8). Cluster I included Savadkuh-Mazandaran, Behshahr-Mazandaran, Abbas abad-Mazandaran, Chalous-Mazandaran, Aliabad Katol-Golestan, Rudsar-Gilan, Fouman 3-Gilan, and Fouman 4-Gilan. This cluster mostly consisted of relatively similar ecotypes in terms of the lowest mean values for such traits as the plant height and width, internode and branch length, leaf width, number of inflorescences, and calyx length. Cluster II contained Fouman 1-Gilan, Amol-Mazandaran, Ramsar-Mazandaran, Fouman 2-Gilan, Niloo-Gilan, and Rahim abad-Gilan. These ecotypes were similar in trait values for the plant height and width, main stem diameter, number and length of secondary stems, number of flowers and inflorescences, and dry weight.

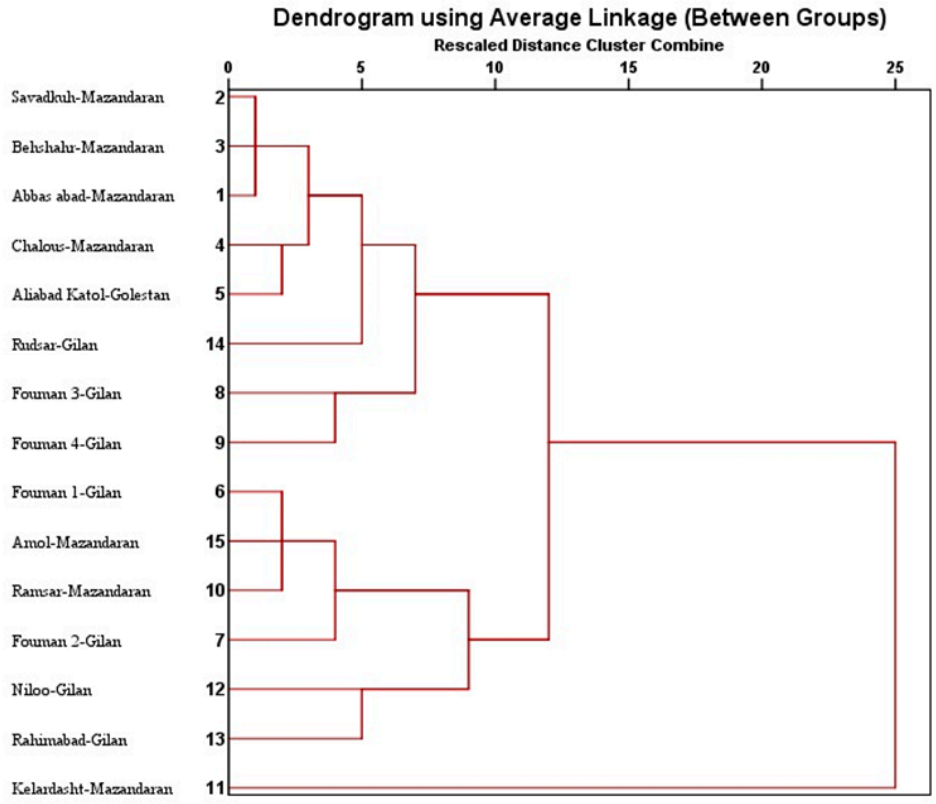

Figure 8. Dendrogram of cluster analysis for morphological traits of *M. aquatica* populations from the Hyrcanian hotspot of Iran.

Table 3. Essential oil composition of *M. aquatica* populations from the Hyrcanian hotspot of Iran.

NO	Compound	RI	Abbas abad-Mazandaran	Savadkuh-Mazandaran	Behshahr-Mazandaran	Chalous-Mazandaran	Aliabad Katol-Golestan	Fouman 1-Gilan	Fouman 2-Gilan	Fouman 3-Gilan	Fouman 4-Gilan	Ramsar-Mazandaran	Kelardasht-Mazandaran	Niloo-Gilan	Rahim abad-Gilan	Rudsar-Gilan	Amol-Mazandaran
1	α-Pinene	932	1.72	0.90	1.58	1.68	1.35	1.91	1.80	0.76	1.74	1.70	1.26	1.49	1.91	1.23	1.68
2	Sabinene	969	1.37	0.69	1.26	1.30	1.35	1.22	1.58	0.79	1.00	1.12	1.12	1.24	1.49	0.59	0.97
3	β-Pinene	974	3.06	1.70	2.91	3.42	2.54	2.89	2.89	1.85	3.03	2.87	2.45	2.77	3.45	1.70	2.59
4	β-Myrcene	988	0.99	0.23	1.50	1.04	-	-	0.95	0.79	1.18	0.78	1.05	0.96	0.84	0.41	1.00
5	*n*-Decane	1000	1.82	2.08	2.01	2.06	2.75	2.21	2.21	1.54	2.63	2.18	2.05	1.91	1.86	2.57	2.21
6	*p*-Cymene	1022	1.74	2.09	4.77	1.94	2.86	4.60	4.60	1.55	3.11	2.98	3.16	3.17	2.16	2.49	3.83
7	1,8-Cineole	1026	22.55	13.85	23.09	25.69	17.64	21.11	21.11	14.86	21.19	21.42	18.12	19.8	26.1	12.42	18.57
8	(Z)-β-Ocimene	1032	-	-	0.6	-	-	-	-	-	-	-	-	-	-	-	-
9	cis-Sabinene hydrate	1065	-	-	-	0.22	-	0.18	0.18	0.19	0.25	-	-	0.16	0.18	0.29	0.31
10	Terpinolene	1086	0.31	0.55	0.53	0.34	0.5	0.43	0.43	0.23	0.47	0.48	0.38	0.35	0.38	0.68	0.72
11	Linalool	1095	0.46	0	0.16	0.22	-	-	-	0.23	-	0.32	0.32	0.4	-	0.56	0.29
12	Menthofuran	1159	19.88	52.46	39.85	21.81	42.38	28.14	28.14	13.21	33.25	33.87	46.34	43.54	36.9	18.61	38.93
13	Menthol	1167	2.80	0	0	1.73	0.48	1.4	1.40	0.97	1.04	0.57	0.64	0.83	0.23	1.95	0.61
14	Terpinen-4-ol	1174	0.39	0.23	0.26	0.42	0	0.38	0.38	0.36	0.32	0.43	0.35	0.32	0.24	0.26	0.28
15	α-Terpineol	1186	-	0.20	0.25	-	-	-	-	0.25	-	0	0.22	-	-	-	-
16	Nerol	1227	-	0.34	-	-	-	-	-	0.93	-	0.68	0.30	-	0.27	-	0.31
17	Carvone	1239	-	-	-	-	-	-	-	-	-	0.17	-	-	-	-	-
18	Linalyl acetate	1258	-	-	-	-	-	-	-	0.16	-	-	-	-	-	-	-
19	Lavandulyl acetate	1288	2.18	-	0.28	0.86	-	-	-	1.55	0.46	0.56	0.31	0.44	0.57	3.06	0.66
20	Menthyl acetate	1294	1.20	-	-	-	-	-	-	0.44	-	0.21	0.18	0.51	-	1.54	0.23
21	Neryl acetate	1361	1.01	4.52	1.12	2.21	2.5	0.65	0.65	4.95	2.17	2.58	2.71	1.78	1.49	1.11	2.3
22	Geranyl acetate	1381	0.89	0.31	0.25	0.59	0.39	0.49	0.49	0.70	0.37	0.27	0.2	0.28	0.18	0.80	-
23	(E)-Caryophyllene	1417	7.72	5.12	5.35	9.75	8.33	8.46	8.46	13.46	8.26	4.29	3.18	5.09	4.87	15.43	9.03
24	α-Humulene	1452	0.85	0.36	-	1.29	0.67	0.93	0.93	2.06	1.17	0.63	0.36	0.53	0.40	2.41	0.61
25	trans-β-Farnesene	1456	0.22	-	-	-	-	-	-	0.23	-	-	-	-	-	-	-
26	Germacrene D	1484	8.29	2.02	3.67	5.23	3.29	7.67	7.67	4.41	4.20	1.81	1.78	2.44	2.12	7.76	1.7
27	Elemol	1548	-	0.84	-	-	-	0.36	-	1.85	-	-	0.41	-	-	0.32	0.42
28	Caryophyllene oxide	1582	1.02	1.33	0.51	1.62	1.50	0.53	0.53	4.96	1.18	1.86	0.98	0.63	0.78	2.31	2.08
29	Viridiflorol	1592	8.90	1.04	2.05	6.81	2.80	2.45	2.45	11.16	2.16	3.8	1.98	2.16	2.47	7.66	0.79
	Total		89.37	90.86	92	90.23	91.33	86.01	86.85	84.44	89.18	85.58	89.85	90.8	88.89	86.16	90.12

Moreover, the local climatic conditions for the Cluster II sites were very similar. Cluster III had a single member, Kelardasht-Mazandaran, with the lowest values for all 22 traits. Major climatic differences from the other populations confirmed the position of Kelardasht-Mazandaran in this unique cluster, with this ecotype adapted to the lowest average annual temperature (13.4 °C), lowest relative humidity (55%), and highest elevation (1053 m) of all the collection sites. As expected at such a high elevation, this location had lower temperatures and relative humidity, and a high light intensity.

3.6. Cluster Analysis for Essential Oil Constituents

The populations of water mint were divided into four main clusters by the Ward method (Figure 9). Cluster I included Fouman 1-Gilan, Fouman 2-Gilan, Abbas abad-Mazandaran, and Chalous-Mazandaran. The populations from Fouman 3-Gilan and Rudsar-Gilan were placed in Cluster II. Cluster III contained populations from Kelardasht-Mazandaran, Niloo-Gilan, Aliabad Katol-Golestan, Amol-Mazandaran, Fouman 4-Gilan, Ramsar-Mazandaran, Behshahr-Mazandaran, and Rahim abad-Gilan. Savadkuh-Mazandaran was the sole member of Cluster IV; its essential oil components differed significantly from the other groups and it had the highest proportion of menthofuran (52.46%). These results can be used to assist in the selection of plants with desirable traits for future breeding and cultivar development.

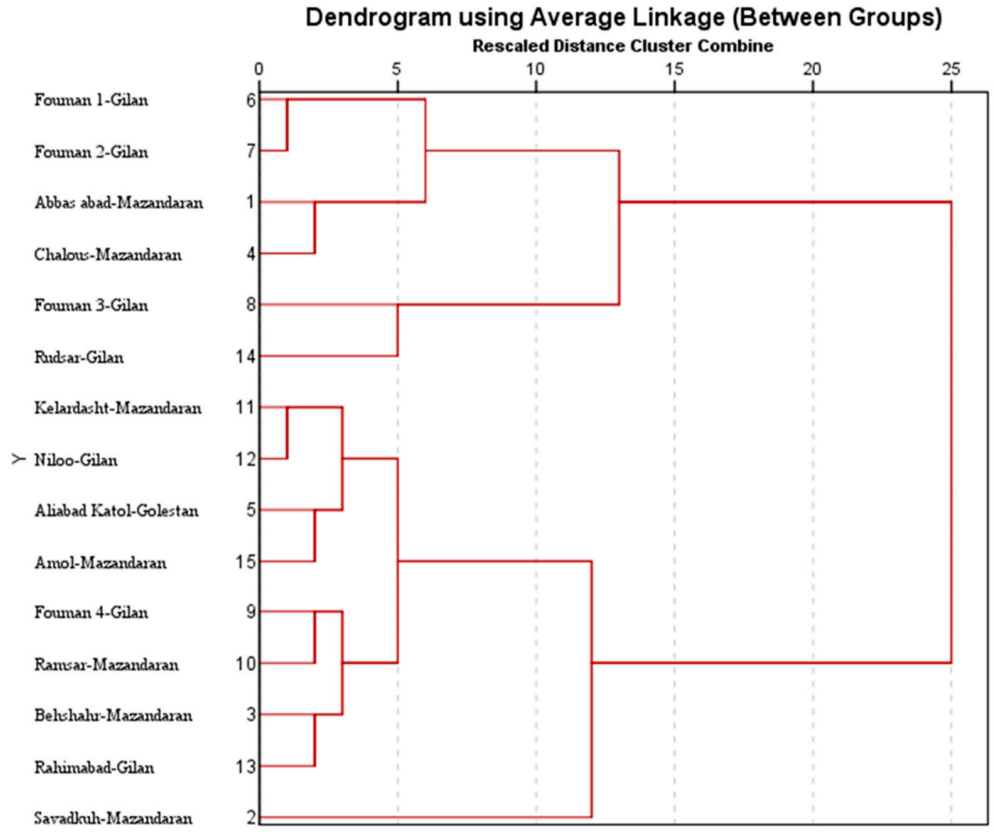

Figure 9. Dendrogram of cluster analysis for essential oil components of *M. aquatica* populations from the Hyrcanian hotspot of Iran.

3.7. Principal Component Analysis (PCA) of Morphological Traits

PCA was performed to determine the role of each trait in the diversity observed across populations (Tables 4 and 5). Our aim was to identify the most influential factors and reduce the total number of traits needed to distinguish populations. The first seven principal components (PC) explained 90.60% of the total variation. Among these, PC1, PC2, and PC3, respectively, explained 31.31%, 17.06%, and 11.73% of the total ecotypic variance. Coefficients of specific vectors in PC1 showed that the number of lateral branches, plant width, plant dry weight, and leaf length played the most critical roles (Table 5). In PC2, the

calyx diameter, leaf length, and leaf width were the most important traits. Traits that are major yield components played a dominant role in the formation of both PC1 and PC2.

Table 4. Eigenvalues, percentage of changes, and cumulative percentage of variance explained morphological traits of *M. aquatica* populations from the Hyrcanian hotspot of Iran.

Factor	Eigenvalues	Changes (%)	Cumulative Percentage of Changes
1	6.88	31.31	31.31
2	3.75	17.06	48.37
3	2.58	11.73	60.11
4	2.19	9.96	70.08
5	1.85	8.44	78.52
6	1.44	6.55	85.07
7	1.21	5.52	90.60

Table 5. PCA analysis for morphological traits of *M. aquatica* populations from the Hyrcanian hotspot of Iran.

	Component						
	PCA 1	**PCA 2**	**PCA 3**	**PCA 4**	**PCA 5**	**PCA 6**	**PCA 7**
Plant height	0.713	−0.333	0.91	−0.065	0.395	0.167	0.254
Plant width	0.860	−0.220	−0.261	0.223	0.089	0.229	−0.109
Plant length/width ratio	−0.703	−0.066	0.443	−0.220	0.309	−0.202	0.290
Stem diameter	0.499	0.400	0.281	0. 108	−0.448	0.410	0.197
Internode length	0.174	−0.608	−0.100	−0.016	0.358	0.262	0.418
Number of branches	0.865	0.053	−0.285	0.139	0.050	−0.250	−0.108
Lateral branch length	0.711	−0.232	−0.327	−0.140	0.303	0.335	−0.201
Collar diameter	0.410	0.018	0.514	0.422	−0.377	0.074	0.009
Leaf length in sub-branch	0.457	−0.093	0.224	0.768	0.258	−0.137	−0.068
Leaf width in sub-branch	0.355	0.509	−0.464	0.362	0.370	−0.227	−0.187
Leaf length/width ratio	0.165	−0.465	0.601	0.460	0.014	0.116	0.008
Leaf length in lateral branches	0.423	−0.552	−0.177	−0.243	0.228	−0.401	0.410
Leaf width in lateral branches	0.540	−0.588	−0.219	0.276	−0.373	−0.164	0.142
Inflorescence number	0.640	0.473	0.420	−0.012	0.118	−0.231	0.155
Inflorescence length	0.782	0.385	−0.004	−0.358	−0.063	0.056	−0.041
Inflorescence width	0.742	0.096	0.087	−0.571	−0.162	−0.030	0.013
Flower number in inflorescence	0.038	−0.166	0.798	−0.114	0.363	−0.031	−0.168
Corolla length	−0.169	0.727	−0.148	0.092	0.291	0.341	0.316
Corolla diameter	0.142	0.598	0.259	0.030	0.557	0.133	−0.189
Calyx length	−0.018	0.635	−0.198	0.329	−0.098	0.035	0.605
Calyx diameter	0.609	0.408	0.186	−0.081	−0.097	−0.628	0.087
Plant dry weight	0.795	0.055	0.289	−0.448	−0.122	0.206	0.013

3.8. PCA for Essential Oil Constituents

To examine the patterns and relationships among major essential oil components, we performed a PCA (Table 6). The first eight PCs explained 92.66% of the total recorded variance. Meanwhile, PC1 explained 30.94% of the variance with strong weightings given to viridiflorol (0.88%), caryophyllene oxide (0.83%), and α-humulene (0.83%). Viridiflorol was the main essential oil constituent. The second PC2 accounted for 19.84%, dominated by neryl acetate (−0.829) and menthol (0.81). PC3 accounted for 13.3% of the variance in which terpinolene (−0.826) was the most heavily weighted component.

Table 6. PCA for essential oil constituents of *M. aquatica* populations from the Hyrcanian hotspot of Iran.

	Component							
	PCA1	**PCA2**	**PCA3**	**PCA4**	**PCA5**	**PCA6**	**PCA7**	**PCA8**
a-pinene	−0.625	0.678	0.131	0.268	0.086	−0.032	0.074	0.103
sabinene	−0.590	0.446	0.494	−0.013	−0.281	0.022	0.006	0.047
β-pinene	−0.597	0.545	0.479	0.262	0.006	−0.020	−0.045	0.018
β-myrcene	−0.165	0.135	0.460	0.075	0.662	0.343	−0.224	−0.123
n-decane	−0.274	0.165	−0.811	0.042	−0.124	−0.091	0.160	0.085
p-cymene	−0.599	0.103	−0.229	−0.083	0.165	0.499	0.384	0.161
1,8-cineole	−0.568	0.473	0.588	0.212	0.024	0.027	−0.069	0.091
(Z)-β-ocimene	0.241	0.334	0	0.710	0.114	0.258	0	0
cis-sabinene hydrate	0	0	0	0.001	0.332	0.016	0	0.224
terpinolene	−0.219	−0.101	−0.826	0.001	0.332	0.016	−0.068	0.224
linalool	0.488	0.259	−0.041	−0.318	0.622	−0.281	−0.165	−0.076
menthofuran	−0.709	−0.576	−0.180	−0.199	0.014	−0.149	−0.131	−0.220
menthol	0.489	0.811	0.010	−0.093	−0.020	−0.059	0.109	−0.257
terpinene-4-ol	0.196	0.343	0.489	0.154	0.432	0.010	0.441	−0.200
α-terpineol	0.196	0.234	0.489	0.154	0.432	0.010	0.441	0
nerol	0.441	−0.630	0.359	0.293	0.185	−0.227	0.175	0.205
carvone	0	0	0.167	0.046	0.392	0	0.510	0.391
linalyl acetate	0.735	−0.366	0.354	0.237	−0.107	0.263	0.062	0.115
lavandulyl acetate	0.764	0.407	−0.120	−0.122	0.273	−0.143	−0.321	0.118
menthyl acetate	0.651	0.410	−0.221	−0.340	0.274	−0.229	−0.263	−0.013
neryl acetate	0.417	−0.819	0.112	0.165	−0.060	−0.134	0.037	−0.177
geranyl acetate	0.729	0.524	0.056	−0.272	−0.259	0.027	0.096	−0.003
(E)-caryophyllene	0.757	0.297	−0.389	0.225	−0.111	0.266	−0.010	0.183
a-humulene	0.836	0.293	−0.270	0.243	−0.068	0.052	0.073	0.119
trans-β-farnesene	0.836	0.293	0	0.243	0	0.052	0.073	0.119
germacrene D	0.380	0.758	−0.093	−0.200	−0.257	0.274	0.219	0.025
elemol	0.711	−0.584	0.099	0.147	−0.105	0.220	0.075	−0.045
caryophyllene oxide	0.833	−0.349	0.033	0.315	0.074	0.006	0.035	0.167
viridiflorol	0.880	0.288	0.0294	−0.056	−0.038	−0.034	−0.037	0.122

4. Discussion

There were significant differences among populations based on traits, such as the plant width, stem diameter, collar diameter, inflorescence number and length, inflorescence width, and calyx length and diameter (Table 1). The existence of differences between populations in each habitat, in addition to inherent genetic variation, can be due to the adaptation to specific environmental conditions. The geo-climatological differences are the most critical parameters affecting the growth of plants [5]. Accordingly, parameters such as the climate, latitude, longitude, altitude, precipitation rate, temperature, and soil pH influence morphological traits and, consequently, the growth and yield of a given plant [19].

The Niloo-Gilan and Rahim abad-Gilan populations had the highest values in terms of important traits such as the plant height and width, leaf length and width, number of lateral branches, leaf width in lateral branches, inflorescence length and width, number of inflorescences, and the length and diameter of calyx (Figures 1–5). The lowest values for these traits were generally measured in the population from Kelardasht-Mazandaran with 1053 m asl, an average temperature of 13 °C, annual rainfall of 589 mm, and 55% mean relative humidity. These extreme conditions most likely have reduced the biological performance of the Kelardasht-Mazandaran ecotype. In agreement with our results, Abbaszadeh et al. [20] reported a significant difference between two water mint ecotypes collected from Gilan and Ardabil. Mazandarani et al. [21] studied *M. longifolia* from different elevations (1000 to 2250 m). They noted that the higher light and ultraviolet intensity and prolonged stomatal closure at high altitudes reduced the photosynthetic rate and plant growth. Andi et al. [22] reported significant differences in morphological traits, such as the

inflorescence length, number, and leaf length, between the subspecies of *Origanum vulgare*. Binava et al. [23] found significant differences between *Salvia mirzayanii* populations in the plant height, number of branches, leaf length and width, inflorescence length, number of flowers, fresh and dry biomass, and EOC.

Our data showed that the EOC of water mint varied significantly across the 15 different ecotypes (Table 3). Differences in the phytochemical content and composition depend on the environmental conditions where the plant grows along with other intrinsic factors, such as genetic variation and the biochemical pathway activity. The edaphic properties of the growing medium, including water, mineral, and nutrients availability, are also essential factors in the production and accumulation of secondary metabolites in medicinal plants [24]. In water mint plants, secretory glands are widely distributed on vegetative and reproductive organ surfaces. The populations of Aliabad Katol-Golestan (mean temperature of 17.17 °C) and Behshahr-Mazandaran (mean temperature of 17.50 °C) occurred in the warmest of the sampled habitats. The high light intensity and temperature most likely contributed to the high EOC levels in these ecotypes. In general, increasing the habitat temperatures intensifies the biosynthesis of the essentail oils in many plant taxa [25]. The water, nutrients, and minerals availability in the soil play a crucial role in influencing the chemical composition and quality of the plant's active ingredients. Our results were consistent with Abbaszadeh et al. [20] on *M. aquatica*, and Hassanpouraghdam et al. [26] on *Stachys lavandulifolia*, which the EOC impacted by the climate and geographical change.

It is clear from the present study that environmental factors had a significant impact on morphological traits, such as the plant height, length and number of the main and lateral stems, leaf length and width, inflorescence number and length, and calyx length and diameter, thus indirectly influencing the EO production across different populations (Table 1). Meanwhile, the quality of the medicinal plants reflects the impact of many environmental factors during plant growth and development. The natural populations' diversity is directly and indirectly affected by environmental cues, epigenetics, and genetic factors. Such variations may also be related to different growth stages, environmental conditions such as seasonal and geographical changes, and soil features [27]. Esmaeili et al. [15] distinguished the presence of β-caryophyllene (22.40%), viridiflorol (11.30%), and 1,8-cineol (10.90%) in the stem EO and piperitone oxide (25.70%) in the leaf EO of water mint collected from Amol city of Mazandaran. These constituents were also observed in our study. Bozin et al. [28] reported that the main constituents of EO of water mint in Serbia were menthofuran, 1,8-cineol, E-caryophyllene, and viridiflorol, which were entirely consistent with our results. In the present study, menthofuran (13.21–52.46%) was a major and ubiquitous constituent of water mint essential oil (Table 3). The populations from Savadkuh-Mazandaran, Kelardasht-Mazandaran, and Aliabad Katol-Golestan produced the highest proportions of this constituent (52.46, 46.34%, and 42.38%, respectively). Savadkuh-Mazandaran, with the higher proportions of neryl acetate, was differentiated from Kelardasht-Mazandaran and Aliabad Katol-Golestan. The three populations, as mentioned earlier, were otherwise quite similar in terms of the EOC. The similarity of EO constituents could reflect local similarities in elevation and light intensity, but may also reflect genetic relatedness among populations.

Correlation and cluster analyses indicated that flower number, along with the lateral branch length and number, are key factors related to biosynthetic potential and the accumulation of secondary metabolites in *M. aquatica.* Mohammadi et al. [29] on *Thymus* populations, Ebadi et al. [30] on *Hypericum perforatum,* and Karimi et al. [31] on *Satureja mutica,* all reported significant positive correlations between the plant height and flower length with essential oil yield.

The idea is that the heritability of the EO components in essential oil-bearing plants is a multifaced trait influenced by genetics, environmental cues, and agronomic practices. Thus, for the domestication and agricultural production of desired oil constituents, we need to sort out the various effects of environmental signals from those of genetic diversity.

Without such studies, the large-scale cultivation of superior populations is much less likely to succeed.

When plants are exposed to different ecological conditions, the quantity and quality of their active ingredients change to adapt to these environments. Therefore, populations of the same species grown in different environmental conditions are often diverse in their secondary metabolite profiles. Moreover, this diversity can produce differences in the scope and strength of their pharmacological and biological activities. The genetic flexibility of plant populations is gradually happening under the influence of the evolution in different geographical areas; ecotypes of plants are formed that are different in terms of their developmental, physiological, chemical, botanical, and, finally, genetic activities. When plants undergo any changes in their natural habitats, substantial variation in their physiological behavior may result, within the context of genes controlling their metabolic pathways. Therefore, to more fully exploit medicinal plants, in relation to wild harvesting, appropriate cultivation conditions, domestication, and/or breeding programs, we need to carefully document both the phenotypic and genetic diversity of these plants. After identifying the pharmaceutical potential of different populations, it may become necessary to produce them on a large scale, in agricultural systems, targeting the most desired secondary metabolites.

5. Conclusions

The results show significant morphological differences among ecotypes. Similarities in climatic conditions helped to explain morphological and EO compositional similarity among the ecotypes from Niloo-Gilan, Rahimabad-Gilan, Amol-Mazandaran, and Ramsar-Mazandaran. Behshahr-Mazandaran had the highest EOC (1.13%), and Savadkuh-Mazandaran with 52.48% of its EO as menthofuran was the ecotype of choice considering this high-valued constituent. Overall, habitat variation influenced the plants' morphological and chemical profiles. As we progress toward the sustainable agricultural production of chosen ecotypes, we must thoroughly survey plants' biochemical and genetic diversity from their natural habitats and then proceed to evaluate the best ecotypes in the appropriate production systems, with the ultimate goal of mass producing desired secondary metabolites.

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