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Deliverable title	D3.4 Report with elaborated data overall collected within WP3
Deliverable Lead:	INRGREF
Related Work Package:	WP3
Related Task:	Task 3.1 (RES). Sampling and morphological characterization of spontaneously growing sea fennel populations Task 3.2 (RES) Chemical analysis of dried sea fennel aerial parts/seeds Task 3.3 (RES). Molecular analysis of sea fennel aerial parts Task 3.4 (RES) Elaboration of morphological, chemical, and genetic data for potential identification of different ecotypes
Author(s)	Prof Abdelhamid Khaldi
Dissemination level	PU
Due Submission Date:	MONTH 10 (30.05.2023)
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Summary of Deliverable D3.4 – Integrated Analysis of Sea Fennel Populations	Dozens of traits on leaves, umbels, fruits, and seeds, documenting variations in size, shape, and structure across coastal ecotypes were measured. In parallel, advanced chemical analyses revealed differences in carotenoids, tocopherols, phenolic compounds, fatty acids, and volatile organic compounds, pointing to populations with particularly high nutritional and antioxidant potential. DNA was extracted from leaves and subjected to Next Generation Sequencing, allowing genotyping of hundreds of individuals and the identification of genetic clusters. To interpret this wealth of data, the consortium employed sophisticated statistical tools such as PCA, PLS-DA, and UPGMA clustering, which helped to group ecotypes according to their morphology, chemistry, and genetics. These analyses showed that while environmental conditions strongly influence chemical composition, genetic factors also play a key role in shaping population differences. Importantly, the integration of datasets

	highlighted ecotypes with promising traits for cultivation, food innovation, and resilience to climate stress.

Versioning and Contribution History

Version	Date	Modified by	Modification reason
v1.0	20/03/2023	Abdelhamid Khaldi	First version
v2.0	30/03/2023	Abdelhamid Khaldi	Comments after peer reviewing process

Table of Contents

1 Italian sea fennel populations

Flowers, leaves and seeds from the 9 wild sea fennel populations sampled across different Italian regions and Corsica listed below have been subjected to morphometric, chemical and molecular analyses. Data were then statistically elaborated.

REGION	LOCALITY	COORDINATES	ALTITUDE (m a.s.l.)	EXPOSURE	ROCK	SAMPLING DATE
Calabria	Vasche di Cassiodoro – Copanello (CZ)	38°45'33.13"N - 16°34'16.03"E	5	E-SE	granodiorite	11/09/2022
Corsica (France)	Bussaglia	42°16'49,00"N - 8°41'19.00"E	5	W	granite	11-12/10/2023
Liguria	Bergeggi (SV)	44°24'23.42"N - 8°44'53.56"E	10	NE	dolomite	11-12/09/2023
Marche	Porto Potenza Picena (MC)	43°20'42.71"N - 13°42'18.53"E	0	E	sand	15/09/2022
Marche	Baia delle Due Sorelle, Mount Conero, Sirolo (AN)	43°32'56.82"N - 13°37'34.23"E	4	E-NE	limestone	05/10/2022
Puglia	Calalunga, Peschici – Gargano Peninsula (FG)	41°56'48.22"N - 16° 3'34.26"E	5	W-NW	limestone	31/08/2022-01/09/2022
Sardinia	La Licciola, Valle dell'Erica – Santa Teresa di Gallura (OT)	41°13'56.78"N - 9°16'23.16"E	0	E-NE	granite	24-25/08/2022
Sicily	Terrasini,Oriented Reserve of Capo Rama (PA)	38° 8'15.68"N - 13° 3'10.16"E	26	N-NW	dolomite	3-4 /08/2022
Tuscany	Calafuria (LI)	43°28'28.20"N - 10°19'50.41"E	15	W	sandstones	21-22/09/2022

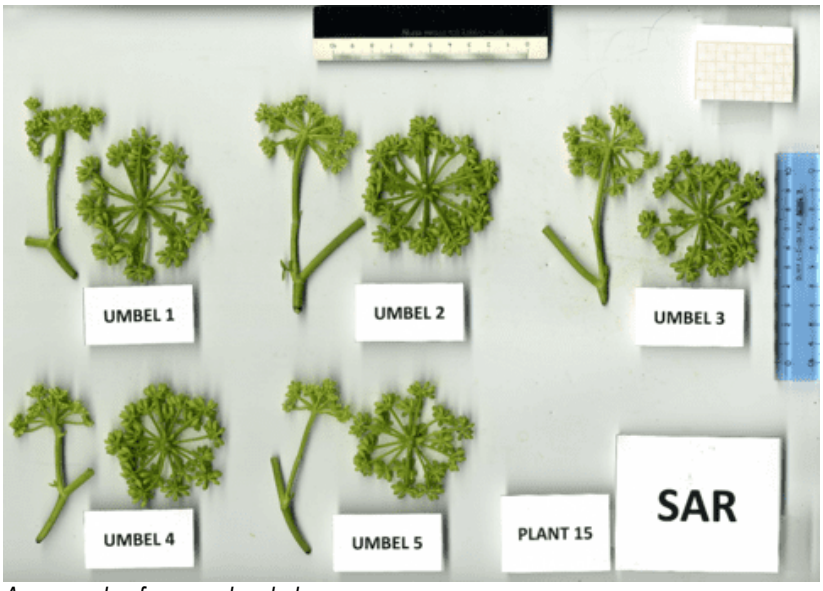
1.1 Morphological characterization

Materials and methods

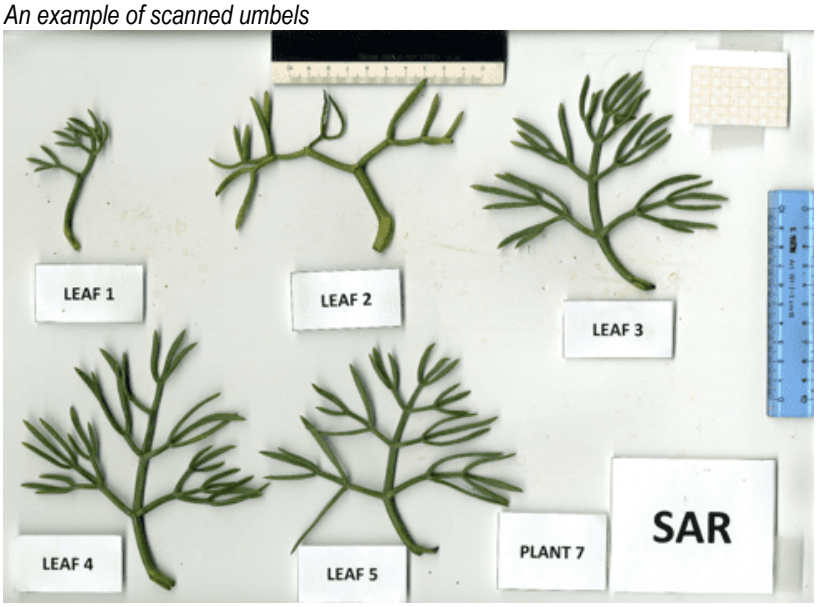
The morphometric characterization of the 9 Italian wild sea fennel populations was done according to the shared procedure among partners detailed in Annex I (see the end of this deliverable).

Measurements of the whole plant (length, width, height and diameter of the main stem), as well as counting the number of branches and number of umbels were performed in the field. A plastic label with the sampling number was attached to each sampled plant for fruit/seed collection, which in most cases was done 2-3 months later. For each of the 20 plants sampled in each location, 5 main umbels and 5 basal leaves were collected for measurements of some traits performed in the laboratory. In total, 100 umbels and 100 leaves were collected for each sampling location.

The samples were transported in refrigerated bags with blue ice to the laboratory where all the umbels and leaves were scanned with a 600dpi scanner.



An example of scanned umbels



An example of scanned leaves

The diameter of the umbels, the length of the umbel peduncle and its diameter were measured with the precision caliper.



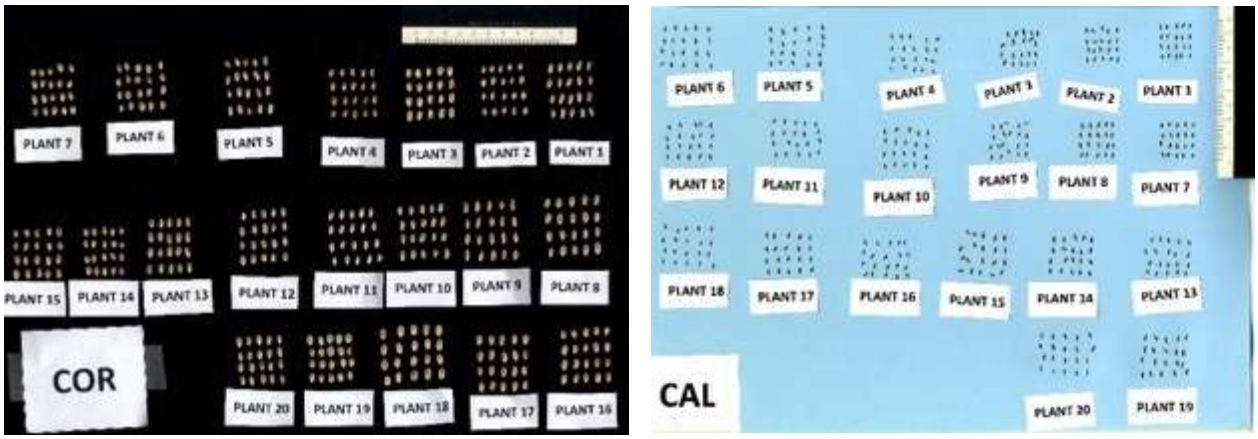
Measurement of the diameter of the umbels; measurement of the length of the pedicel; measurement of the diameter of the pedicel

The measurements of the characters indicated in the table below were performed on the scanned images using the ImageJ software.

Morphological traits measured/counted on scanned images with the software ImageJ

Kind of parameter	Descriptive parameters	Unit of measure
Leaves	length	mm
	width	mm
	length petiol	mm
	Number lobes	unit
	Area	mm^2
	Perimeter	mm
Umbels	Number rays/peduncle	unit
	thickness of the ray	mm
	length of the ray	mm
	Number of the bract	unit
	Length of the bract	mm
	Width of the bract	mm

	Shape of the bract	mm
	Diameter of the umbellet	mm
	color	Visual rating
	Length of the bracteoles (average)	mm
	Width of bracteoles (average)	mm
	Shape of bracteoles (average)	mm
	Number of bracteoles (average)	mm
	Number of pedicels/fruits	
Fruits	Length	mm
	width	mm
	Number of fruits/umbellet	unit
Seeds	Length	mm
	Width	mm
	Area	mm^2
	perimeter	mm
	number of seeds/ fruit	unit
	form	Visual rating
	weight 100 seeds	gr



Examples of scanned fruits (on the left) and seeds (on the right)

Results

In the following tables, the number of samples counted and measured for each character is reported.

	WHOLE PLANT						
	n. of indivic	Length	width	Heigth	Diamet	N°of brancl	N°of umb
Calabria	20	20	20	20	20	20	20
Conero	20	20	20	20	20	20	20
corsica	20	20	20	20	20	20	20
Liguria	20	20	20	20	20	20	20
Marche	20	20	20	20	20	20	20
Puglia	20	20	20	20	20	20	20
Sardegna	20	20	20	20	20	20	20
Sicilia	20	20	20	20	20	20	20
Toscana	20	20	20	20	20	20	20

	UMBELS																
els	n. of individu	Length pedun	Diameter p	Number ra	thickness r	length ray	Number br	Length bra	Width brac	shape brac	Diameter f	color		Length of b	Width of br	Shape of bi	N° of bract
Calabria	20	100	100	100	100	100	100	100	100	100	100	100		100	100	100	100
Conero	20	101	101	101	98	101	101	99	98	98	101	101		97	97	97	101
corsica	20	107	107	107	107	107	107	107	107	107	107	107		107	107	107	107
Liguria	20	102	102	102	102	102	102	100	100	100	102	102		102	102	102	102
Marche	20	102	102	102	102	102	102	100	99	99	102	102		102	102	102	102
Puglia	20	105	105	105	105	105	105	105	105	105	105	105		105	105	105	105
Sardegna	20	101	101	101	101	101	101	101	101	100	101	101		101	101	101	101
Sicilia	20	101	100	100	100	100	101	100	100	100	101	101		100	100	100	100
Toscana	20	102	102	102	102	102	102	102	102	102	102	102		102	102	102	102

	UMBELLETS			
eoles	n. of indivic	Number pe	Number flc	DIAMETER
Calabria	20	100	100	100
Conero	20	100	100	100
corsica	20	102	100	101
Liguria	20	100	100	100
Marche	20	100	100	99
Puglia	20	100	100	100
Sardegna	20	100	100	100
Sicilia	20	99	100	99
Toscana	20	100	100	100

	FRUIT				
OF UMBELL	n. of indivic	n.fruits	length	width	number fru
Calabria	20	400	400	400	100
Conero	19	380	380	380	100
corsica	20	400	400	400	107
Liguria	11	313	313	313	102
Marche	20	400	400	400	100
Puglia	20	400	400	400	101
Sardegna	20	400	323	323	101
Sicilia	20	400	381	381	99
Toscana	20	400	400	400	100

	SEEDS							
its/umbell	n. of indivic	n. of seeds	Length	Width	Area	perimeter	number of	form
Calabria	20	400	400	400	400	400	400	400
Conero	19	380	380	380	380	380	380	380
corsica	20	400	400	400	400	400	400	400
Liguria	11	172	172	172	172	172	172	172
Marche	20	400	400	400	400	400	400	400
Puglia	20	400	400	400	400	400	400	400
Sardegna	14	271	271	271	271	271	271	271
Sicilia	19	380	380	380	380	380	380	380
Toscana	20	405	405	405	405	405	405	405

	LEAVES							
	n. of indivic	rep	length	width	length peti	Number lo	Area	Perimeter
Calabria	20	5	100	100	100	100	100	100
Conero	20	5	100	100	100	100	100	100
corsica	20	5	100	100	100	100	100	100
Liguria	20	5	100	100	100	100	100	100
Marche	20	5	100	100	100	100	100	100
Puglia	20	5	100	100	100	100	100	100
Sardegna	20	5	100	100	100	100	100	100
Sicilia	20	5	100	100	100	100	100	100
Toscana	20	5	101	101	101	101	101	101

	WEIGHT OF 100 SEEDS		
	n. of individuals	rep	weight 100 seeds
Calabria	20	1	2000
Conero	19	1	1900
corsica	20	1	2000
Liguria	13	1	1300
Marche	20	1	2000
Puglia	20	1	2000
Sardegna	14	1	1400
Sicilia	19	1	1900
Toscana	20	1	2000

The results of the elaboration are discussed at the pages 41-52.

References

Schneider CA, Rasband WS, Eliceiri KW, et al. Nih image to imagej: 25 years of image analysis. Nat Methods. 2012;9(7):671–5

1.2 Chemical analyses

Flowers, leaves and seeds from the Italian wild sea fennel populations have been subjected to the chemical analyses as detailed below. Samples from Liguria and Corsica were excluded as they lacked triplicate analysis, and results were based only on the best-performing samples.

Area of the sample	Abbreviation
Calabria	CAL
Marche, Conero regional park	CON
Marche, Porto Potenza Picena	MAR
Apulia	PUG
Sardinia	SAR
Sicily	SIC

Tuscany	TOS

1.2.1 Carotenoids

Materials and methods

Carotenoids were extracted and analyzed according to Nartea et al. (2023). Briefly, freeze-dried samples of plant material (100 mg) were extracted in acetone (5 mL, 4 °C) and the mixtures were kept at 4 ± 1 °C (15 min), vortexed (5 min), and centrifuged (1370 rpm, 10 min, 4 °C), repeating the acetone extraction a second time. The supernatant was filtered (0.45 µm, Sartorius Regenerated Cellulose Membrane), dried, resuspended in 0.5 mL acetone and injected in an Acquity Ultra Pressure Liquid Chromatographic H-class system (Waters Corporation, Milford, US), equipped with PDA and an Ascentis column UPLC C18 (2.1 mm × 100 mm, 1.7 µm). The mobile phase was composed of phase A consisting of acetonitrile (75%), dichloromethane (10%), and methanol (15%), and phase B consisting of acetate ammonium in water (0.05 M). Gradient started at 75% A, held for 10 min, up to 98% in 1 min and held in isocratic mode till 20 min. The flow rate was 0.4 mL/min, column oven was set at 35 °C and sample loading was carried out at 20 °C. PDA analysis was performed at 450 nm wavelength upon a spectrum scanning in the 210–500 nm range. Carotenoids were identified by comparison of retention time and absorbance spectrum with pure standards. Their quantification was performed by external calibration. Good correlation coefficients (R^2) of 0.999 were obtained in the range of 1–100 µg/mL for lutein and 0.05–100 µg/mL for β-carotene.

Results

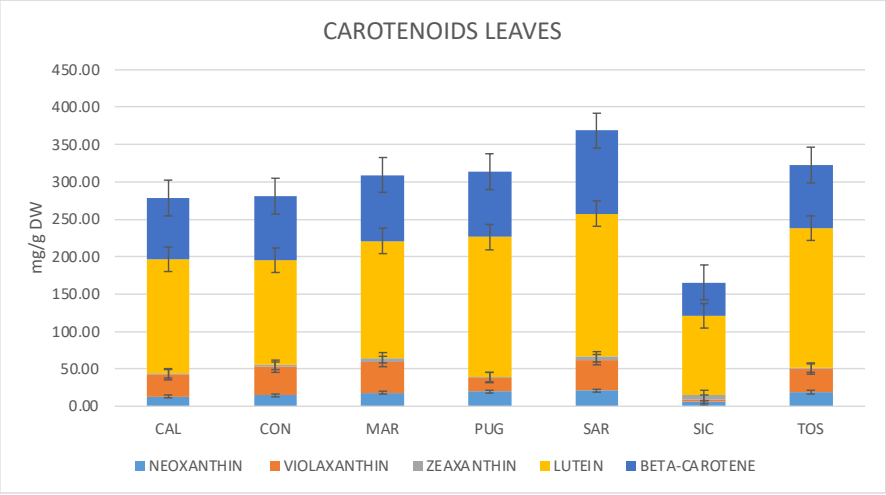
According to the analysis, carotenoid concentrations in leaves are typically higher than in flowers, especially in lutein and beta-carotene. SAR leaves had the highest levels of beta-carotene (111.05 mg/kg) and lutein (190.89 mg/kg) among the samples that were examined, while SIC leaves had the lowest levels of most carotenoids. Similarly, CON had the highest levels of beta-carotene (104.26 mg/kg) and lutein (190.88 mg/kg) in flowers, while TOS had the lowest levels, especially for beta-carotene (16.97 mg/kg) and neoxanthin (1.59 mg/kg). The CON sample continuously had significant carotenoid contents in both leaves and flowers, particularly for beta-carotene and lutein, indicating a noteworthy trend.

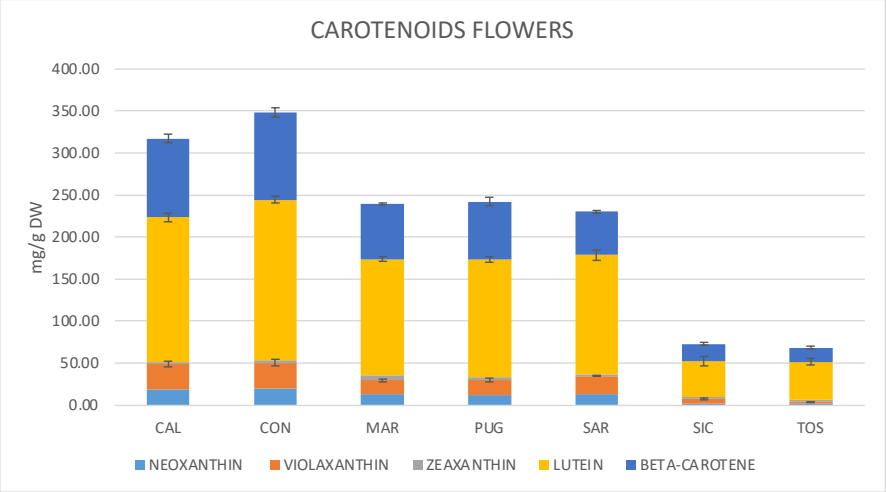
CAROTENOIDS LEAVES UPLC/PDA					
MEAN mg/kg DW	NEOXANTHIN	VIOLAXANTHIN	ZEAXANTHIN	LUTEIN	BETA-CAROTENE
CAL	12,86	29,55	1,88	152,10	82,37
CON	14,95	37,88	2,43	140,07	85,50
MAR	17,96	41,75	4,94	156,16	88,43
PUG	19,57	18,33	1,10	187,31	87,69
SAR	21,29	41,04	4,21	190,89	111,05
SIC	6,12	2,35	6,33	106,41	44,37
TOS	18,99	30,98	1,60	186,32	84,28
DEV.ST mg/kg DW	NEOXANTHIN	VIOLAXANTHIN	ZEAXANTHIN	LUTEIN	BETA-CAROTENE
CAL	11,86	24,65	1,00	57,38	33,88
CON	5,89	14,95	2,99	29,38	27,05
MAR	17,64	28,86	0,08	146,56	51,32
PUG	1,20	4,35	0,44	9,89	7,13
SAR	1,99	0,42	1,63	38,84	14,17

SIC	0,70	2,08	0,57	2,13	1,54
TOS	0,56	1,85	0,64	8,20	8,04
CAROTENOIDS FLOWERS UPLC/PDA					
MEAN mg/kg DW	NEOXANTHIN	VIOLAXANTHIN	ZEAXANTHIN	LUTEIN	BETA-CAROTENE
CAL	18,77	29,88	2,27	172,73	93,61
CON	19,62	30,74	3,15	190,88	104,26
MAR	13,08	16,45	5,70	138,62	65,22
PUG	12,21	17,87	3,12	140,22	68,52
SAR	13,33	21,54	2,06	141,83	51,54
SIC	2,09	4,93	2,14	42,74	20,83
TOS	1,59	2,74	1,96	45,22	16,97
DEV.ST mg/kg DW	NEOXANTHIN	VIOLAXANTHIN	ZEAXANTHIN	LUTEIN	BETA-CAROTENE
CAL	1,17	3,07	0,27	4,93	5,26
CON	1,19	4,25	0,26	4,12	5,44
MAR	1,11	1,64	0,10	2,51	1,20
PUG	0,32	1,96	0,21	2,98	4,89
SAR	0,72	0,09	0,37	6,22	1,61
SIC	0,89	1,19	0,41	5,76	1,47
TOS	0,94	0,25	0,98	3,94	1,23

Carotenoids, expressed as mg/g dry weight (DW), of leaves (L) and flowers (F) of sea fennel wild populations sampled in different Italian regions.

On the other hand, the total carotenoid levels were lowest in the SIC and TOS samples. Additionally, MAR leaves in Lutein had a considerably high standard deviation (146.56 mg/kg), suggesting a greater range of variance in this sample. Flowers, on the other hand, showed a lower standard deviation, indicating that their carotenoid contents were more stable than those of leaves.





References

- Nartea, A.; Fanesi, B.; Pacetti, D.; Lenti, L.; Fiorini, D.; Lucci, P.; Frega, N.G.; Falcone, P.M. Cauliflower By-Products as Functional Ingredient in Bakery Foods: Fortification of Pizza with Glucosinolates, Carotenoids and Phytosterols. Curr. Res. Food Sci. 2023, 6, 100437, doi:10.1016/J.CRFS.2023.100437.

1.2.2 Tocopherols

Material and methods

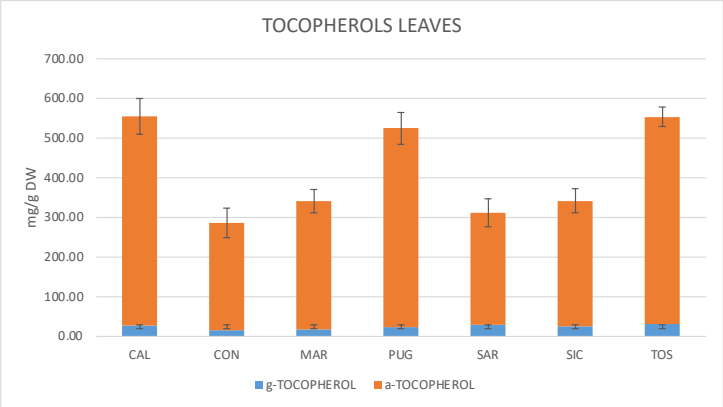
Tocopherols were determined simultaneously to carotenoids, thus in the same sample preparation and chromatographic separation, but a fluorimetric detector (FLD) was used. FLD was set with an excitation and emission wavelength of 290 and 330 nm, respectively. Tocopherols were identified by comparison of the retention time with pure standards and quantified with external calibration. Standard stock solutions of each tocopherol (α-, γ-, β- δ-tocopherol) were prepared in the range of 0–100 µg/mL, and good correlation coefficients were obtained for the calibration curves (R2 = 0.9836–0.9965).

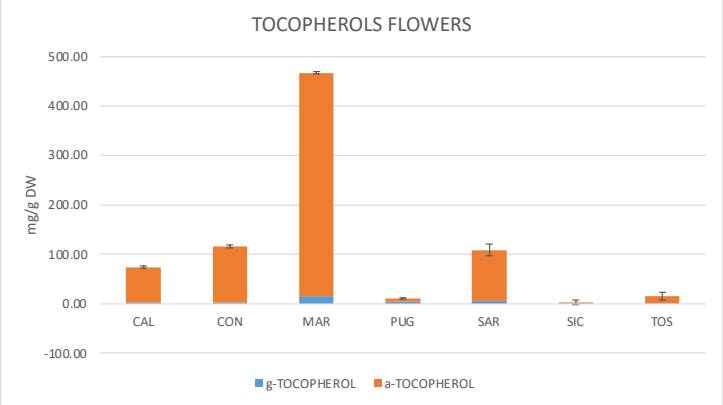
Results

TOCOPHEROLS LEAVES UPLC/FLR		
MEAN mg/kg DW	GAMMA-TOCOPHEROL	ALFA-TOCOPHEROL
CAL	26,20	528,12
CON	14,85	270,58
MAR	18,00	322,72
PUG	23,81	500,25
SAR	28,31	282,60
SIC	25,26	315,87
TOS	31,62	521,26
DEV.ST mg/kg DW	GAMMA-TOCOPHEROL	ALFA-TOCOPHEROL
CAL	2,41	45,43
CON	2,79	36,57
MAR	2,94	29,01

PUG	2,29	40,47
SAR	2,81	35,40
SIC	2,15	30,56
TOS	2,78	24,06
TOCOPHEROLS FLOWERS UPLC/FLR		
MEAN mg/kg DW	GAMMA-TOCOPHEROL	ALFA-TOCOPHEROL
CAL	3,45	70,96
CON	2,33	113,38
MAR	15,85	451,29
PUG	5,19	4,98
SAR	6,66	101,67
SIC	1,18	1,68
TOS	1,56	13,95
DEV.ST mg/kg DW	GAMMA-TOCOPHEROL	ALFA-TOCOPHEROL
CAL	1,30	2,28
CON	0,93	3,33
MAR	0,98	2,55
PUG	1,57	1,58
SAR	1,44	11,79
SIC	2,55	4,73
TOS	0,01	7,40

Alpha-Tocopherol concentrations were found to be substantially greater in leaves than in flowers, with the greatest levels of CAL (528.12 mg/kg) and TOS (521.26 mg/kg) being found in leaves. On the other hand, tocopherol concentrations were significantly lower in flowers, especially in PUG, where alpha-tocopherol was only 4.98 mg/kg. However, MAR flowers had the highest tocopherol accumulation overall, as evidenced by their highest Alpha-Tocopherol concentration (451.29 mg/kg). Flowers had higher fluctuating amounts of gamma-tocopherol; MAR had the highest concentration (15.85 mg/kg), while SIC had the lowest levels (1.18 mg/kg).





Reference

- Nartea, A.; Fanesi, B.; Falcone, P.M.; Pacetti, D.; Frega, N.G.; Lucci, P. Impact of Mild Oven Cooking Treatments on Carotenoids and Tocopherols of Cheddar and Depurple Cauliflower (Brassica Oleracea L. Var. Botrytis). Antioxidants 2021, Vol. 10, Page 196 2021, 10, 196, doi:10.3390/ANTIOX10020196.

1.2.3 Phenolic compounds

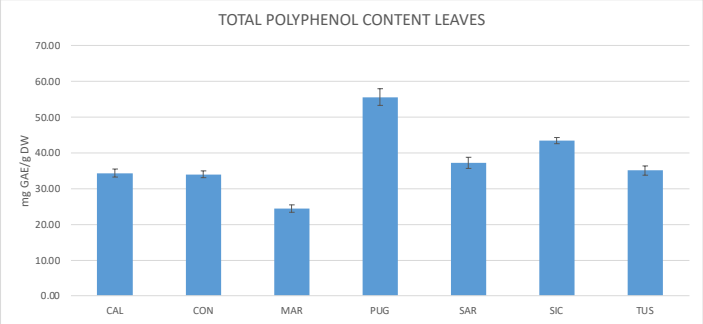
Total Phenolic Content (TPC)

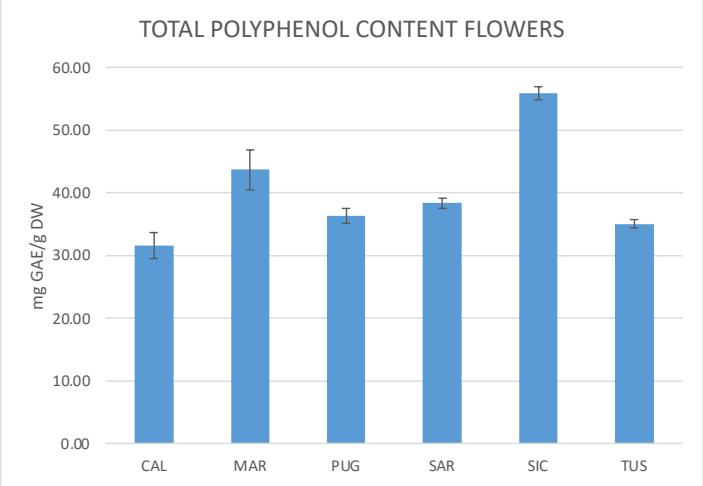
Materials and methods

0.5 g of dried powder of each sample was extracted with 10 mL of deionized water at room temperature in dark conditions, under ultrasound for 15 min, centrifuged, stabilized at -18°C for 2 hours and filtered on regenerated cellulose filter (0,45 um). TPC was carried out with Folin-Ciocalteu method. Liquid extracts were 2-fold diluted with deionized water. 20 µL of each sample were transferred in glass tubes, added with water (1.58 mL), Folin-Ciocalteu reagent (100 µL) and mixed. After 1 minute, 300 µL of sodium carbonate solution was added and the tubes were placed at 40°C for 30 min in darkness before reading absorbance at 765 nm in a spectrophotometer. Blank samples were also analysed. TPC was determined with gallic acid external calibration curve (0-500 mg/mL, R2>0,9994), built in the same condition as the samples. Data were expressed as gallic acid equivalents in mg/mL and mg/g dried weight (DW) plant material.

Results

The results of TPC determination are shown in the following two separate graphs, the first depicting data from leaves and the second showing data from flowers.





The total polyphenol content in leaves varied among the analyzed samples, with PUG exhibiting the highest concentration at approximately 55 mg GAE/g DW, while MAR displayed the lowest content at 25 mg GAE/g DW. Similarly, in flowers, SIC exhibited the highest polyphenol content, reaching approximately 55-60 mg GAE/g DW, whereas CAL and TOS contained the lowest concentrations, both around 30 mg GAE/g DW.

Major hydroxycinnamic acids in leaves, flowers, and extracts

Materials and methods

Aliquots (50 mg) of freeze-dried leaves and flowers were extracted in 0.5 mL of methanol 70% with 0.1% formic acid, with ultrasounds (15 min), vortexed (5 min), and centrifuged (2264×g, 2 min, 21 °C). The supernatant was filtered (0.45 µm, PTFE membrane), and injected (1 µL) in an Acquity UPLC H-class system (Waters Corporation, Milford, US), equipped with PDA and an Ascentis column UPLC C18 (2.1 mm × 100 mm, 1.7 µm). Freeze-dried extracts were dissolved in 0.1 mL of methanol 70% with 0.1% formic acid, centrifuged, and injected. The gradient solvent consisted of water with 0.1% formic acid, and acetonitrile as it is reported by Martins-Noguerol et al. (2022). The flow rate was 0.4 mL/min, column temperature was controlled at 35 °C, and PDA was set at 330 nm. The major hydroxycinnamic acids were identified by comparison of the retention time and absorbance spectrum with pure standards and quantified with external calibration based on chlorogenic acid ($R^2 > 0.999$ in the range of 1–200 µg/mL).

Results

The major hydroxycinnamic acids found in the leaves and flowers of the Italian sea fennel populations herein assayed are reported in table below. In general, flowers of wild populations contained a higher amount of hydroxycinnamic acids than leaves. In leaves, chlorogenic acid was the most abundant compound, with a content ranging from 8.41 to 65.07 mg/g DW.

mg/g DW	Neochlorogenic acid	Chlorogenic acid	Cryptochlorogenic acid	Total chlorogenic acids	3,5-di-O-caffeoylquinic acid
CAL-L-WT	3.78 ± 0.04 cd	20.76 ± 0.21 f	4.72 ± 0.05 c	29.27 ± 0.30 e	6.54 ± 0.07 cf
CON-L-WT	3.31 ± 0.10 d	28.23 ± 0.82 ef	3.84 ± 0.11 c	35.38 ± 1.02 de	2.95 ± 0.09 cf
MAR-L-WT	1.76 ± 0.07 ef	8.41 ± 0.35 g	1.88 ± 0.08 d	12.05 ± 0.50 f	1.86 ± 0.08 f
APU-L-WT	6.96 ± 0.59 a	65.07 ± 5.52 a	8.53 ± 0.72 a	80.57 ± 6.83 a	9.14 ± 0.78 e
SAR-L-WT	5.57 ± 0.44 b	46.06 ± 3.64 bc	6.46 ± 0.51 b	58.09 ± 4.59 b	7.78 ± 0.61 ef
SIC-L-WT	5.24 ± 0.48 b	33.24 ± 3.04 de	6.57 ± 0.60 b	45.04 ± 4.12 cd	5.46 ± 0.50 cf
TUS-L-WT	4.17 ± 0.34 e	26.95 ± 2.22 ef	4.39 ± 0.36 c	35.51 ± 2.93 de	5.39 ± 0.44 cf
MAR-L-C	1.68 ± 0.14 ef	38.93 ± 3.33 cd	1.04 ± 0.09 d	41.65 ± 3.56 cd	47.97 ± 4.10 bc
CAL-F-WT	1.62 ± 0.05 ef	38.45 ± 1.25 cd	1.76 ± 0.06 d	41.83 ± 1.36 cd	41.60 ± 1.36 cd
MAR-F-WT	1.45 ± 0.16 f	39.36 ± 4.39 cd	1.17 ± 0.13 d	41.98 ± 4.69 cd	48.28 ± 5.39 b
APU-F-WT	2.01 ± 0.06 ef	44.45 ± 1.27 c	1.29 ± 0.04 d	47.75 ± 1.37 c	52.08 ± 1.49 ab
SAR-F-WT	2.02 ± 0.15 ef	44.68 ± 3.26 c	1.83 ± 0.13 d	48.53 ± 3.54 bc	46.98 ± 3.43 bcd
SIC–F-WT	2.30 ± 0.07 e	53.63 ± 1.58 b	1.77 ± 0.05 d	57.70 ± 1.69 b	57.73 ± 1.70 a
TUS-F-WT	1.50 ± 0.05 f	34.19 ± 1.07 de	1.24 ± 0.04 d	36.93 ± 1.15 de	41.00 ± 1.28 d

Hydroxycinnamic acids, expressed as mg/g dry weight (DW) of leaves (L) and flowers (F) wild (WT) sea fennel populations sampled in different Italian regions. CAL = Calabria; CON = Marche, Conero Regional Park; MAR = Marche; APU = Apulia; SAR = Sardinia; SIC = Sicily; TUS = Tuscany.

In flowers, 3,5-di-O-caffeoylquinic acid was the predominant compound ranging from 41.00 to 57.73 mg/g DW, followed by chlorogenic acid, ranging from 34.19 to 53.63 mg/g DW.

References

Martins-Noguerol, R.; Matías, L.; Pérez-Ramos, I.M.; Moreira, X.; Muñoz-Vallés, S.; Mancilla-Leytón, J.M.; Francisco, M.; García-González, A.; DeAndrés-Gil, C.; Martínez-Force, E.; et al. Differences in Nutrient Composition of Sea Fennel (Crithmum Maritimum) Grown in Different Habitats and Optimally Controlled Growing Conditions. Journal of Food Composition and Analysis 2022, 106, 104266, doi:10.1016/J.JFCA.2021.104266.

Nartea, A.; Orhotohwo, O.L.; Fanesi, B.; Lucci, P.; Loizzo, M.R.; Tundis, R.; Aquilanti, L.; Casavecchia, S.; Quattrini, G.; Pacetti, D. Sea Fennel (Crithmum Maritimum L.) Leaves and Flowers: Bioactive Compounds, Antioxidant Activity and Hypoglycaemic Potential. Food Biosci 2023, 56, 103417, doi:10.1016/J.FBIO.2023.103417.

1.2.4 Fatty acids

PCA AND PLS-DA leaves, flowers and seeds

Leaves (L; n =9) and flowers (F; n =8) were collected at the plant flowering period in August/September 2023 from wild sea fennel populations (n =9) growing spontaneously in Italian and French coastal areas. Fruits of C. maritimum were collected during the ripening period in October 2023 from wild sea fennel populations (S; n =1) growing spontaneously in Corsica coastal region.

Area of the sample	Abbreviation
Calabria	CAL
Marche, Conero regional park	CON
Marche, Porto Potenza Picena	MAR
Apulia	PUG
Sardinia	SAR
Sicily	SIC
Tuscany	TOS
Corsica	COR
Liguria	LIG

Materials and methods

Oil extraction

The oil extraction of C. maritimum vegetable material was carried out using a Soxhlet extractor. An aliquot of 20 g of C. maritimum powders (leaves and flowers) or 5 g in the case of seed sample was extracted with 100 mL of n-hexane for 5 h. The extract was then dried over anhydrous sodium sulphate and n-hexane was evaporated by means of a vacuum rotary evaporator for further analysis.

Fatty acids composition determination

Fatty acid methyl esters (FAME) were obtained from total lipids through alkaline methylation. Briefly, 20 mg of oil were added of n-hexane (0.5 mL), KOH 2M in methanol solution (0.5 mL) and vortexed. An aliquot (100 µl) of the organic phase was diluted 10 times and then inject in the capillary gas chromatographer. Samples were analyzed in triplicate.

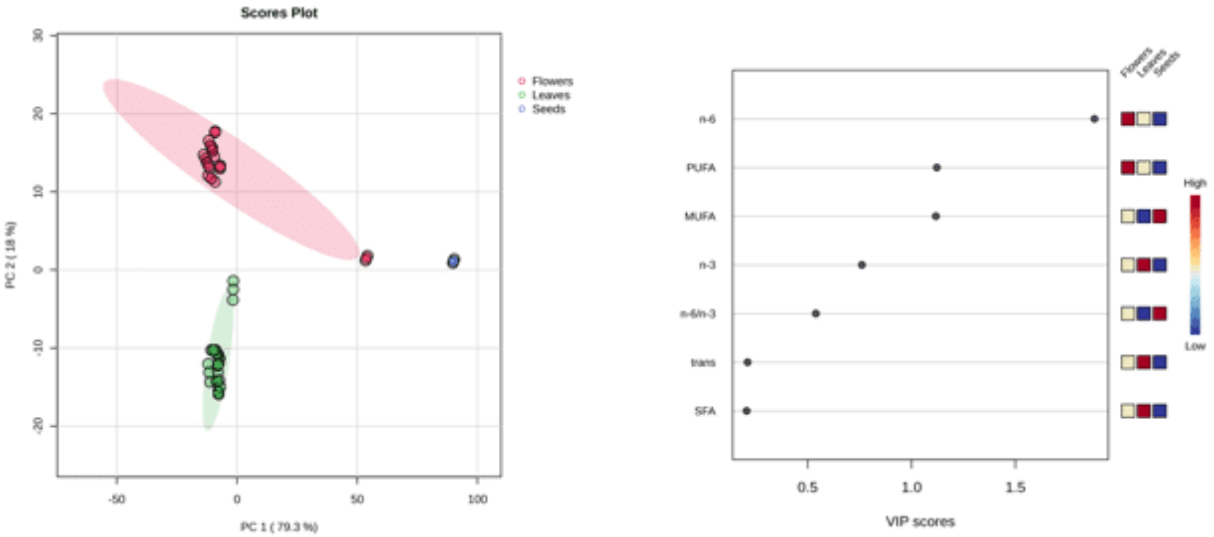
The qualitative analysis of FAMEs (weight% of total fatty acids) was performed by means of gas chromatography using Thermo Scientific TRACE 1300 apparatus (Massachusetts, USA) equipped with a flame ionization detector set at 270°C (FID) and an RT-2560 fused silica capillary column (100 m 0.25 mm i.d., film thickness 0.2 µm; Restek, USA). The carrier gas was helium at a flow rate of 1.6 mL/min. The oven temperature program was: 5 min at 140°C, raised to 240°C at a rate of 4°C/min, then held for 15 min. The injector temperature was 250°C. The identification was performed using F.A.M.E mix C4-C24 (Sigma-Aldrich, St. Louis, USA). The processing software of data was Chromeleon 7.

Experimental results of the performed analysis were processed by the MetaboAnalyst 5.0 online platform. A normalized and scaled dataset was used for total visualization. Principle component analysis (PCA) and

variable importance in projection (VIP) were applied to monitor the variations in the fatty acids profile of leaves, flowers and seeds of SF.

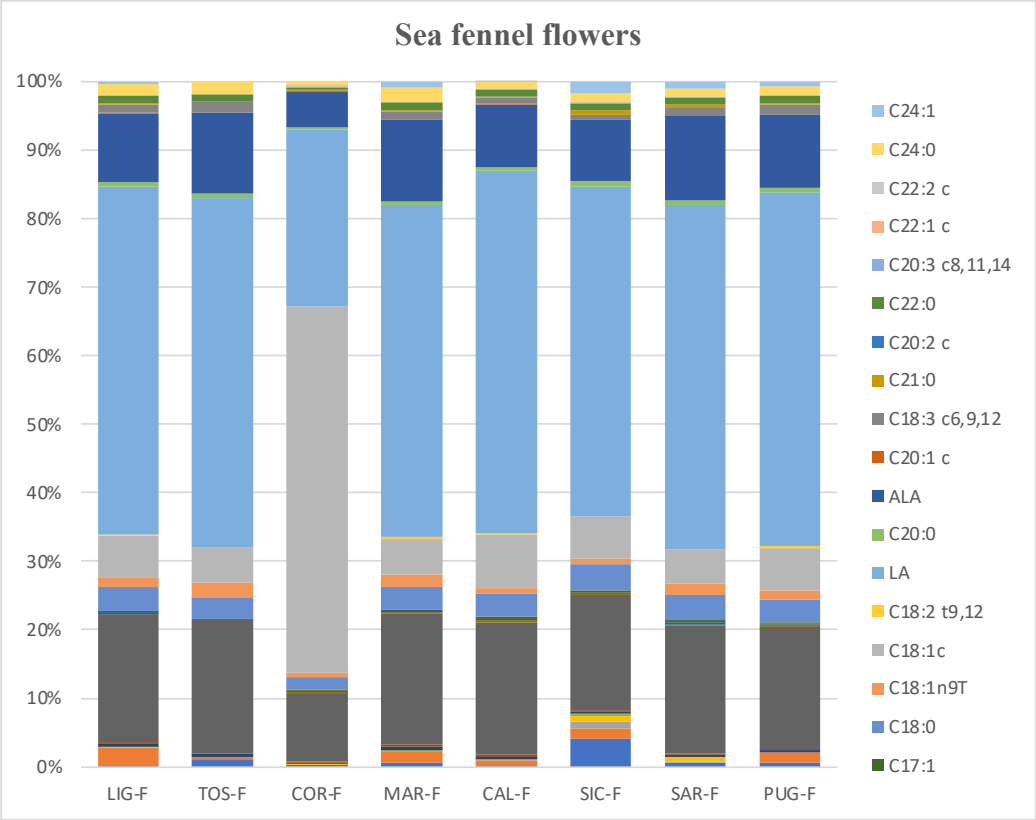
Results

Based on the location of the clusters in the Scores plot, it is clear that the different vegetable parts (leaves, flowers and seeds) of the sea fennel are far from each other in terms of fat composition. It should also be noted that the site of collection, different Italian regions, of the sea fennel samples did not play a significant role in the arrangement of the clusters, only the effect of plant part outstands. Interestingly seeds and flowers from Corsica site are very close in their quadrant of the score plot. The similarity in fatty acid composition between seeds and flowers is because the collection of flower occurred in October during the ripening of fruits, thus their FA composition is more similar to a seed.



The fatty acid composition of each part of sea fennel can be described generally as follows: polyunsaturated fatty acids predominate in the flowers, particularly omega 6, while high levels of omega 3 are present in the leaves. The seeds, in turn, show a high content of monounsaturated fatty acids, while in flowers this indicator is average, and in leaves it is at a low level.

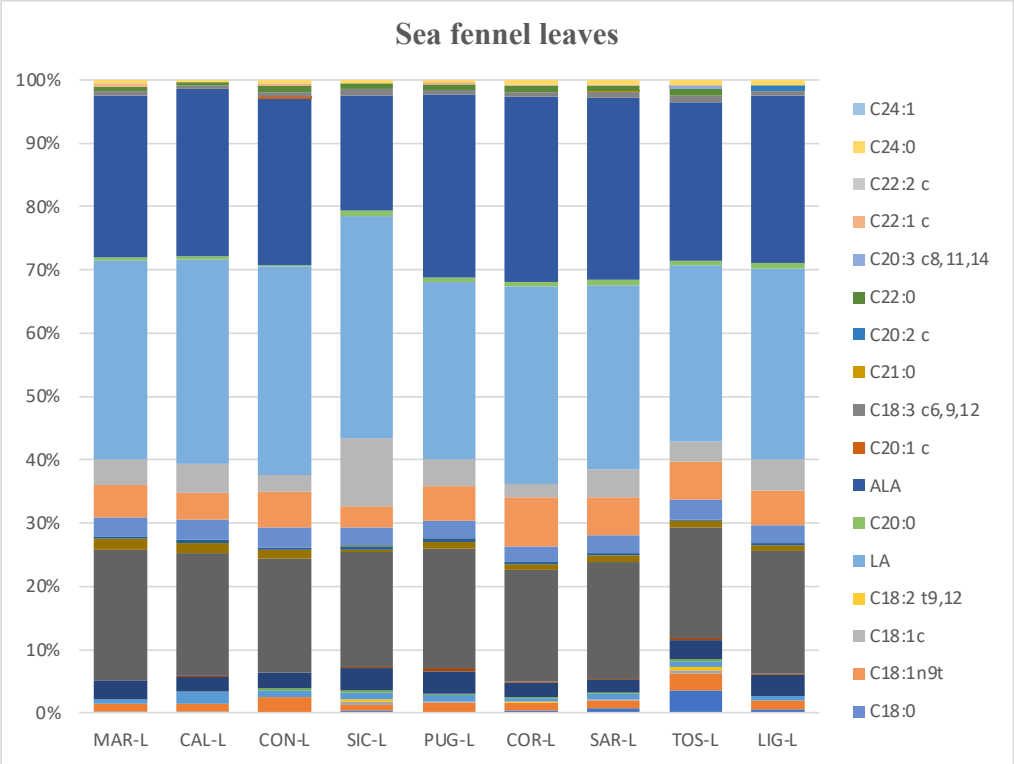
FLOWERS (%FATTY ACIDS)



Fatty acid content in flowers of sea fennel from different regions

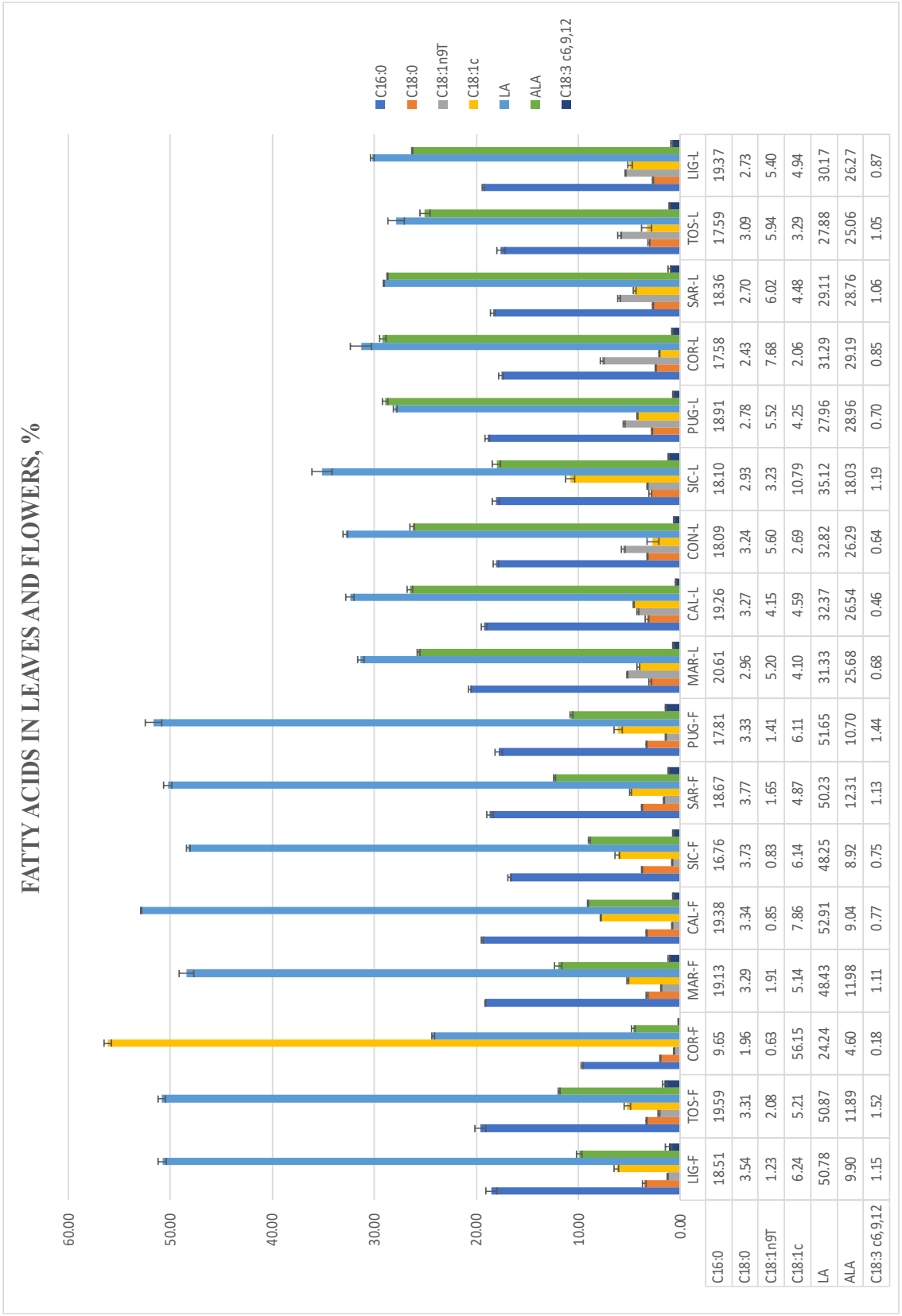
The major proportion of fatty acids in sea fennel flowers from all regions are fatty acids designated as C16:0 (palmitic acid), LA and C18:1c (oleic acid). Their content varies but remains high in most samples. ALA was also present in all samples and the percentage varies from 4.60% (COR-F) to 12.31% (SAR-F), although the amount was significantly lower compared to C16:0 and C18:1c. The samples generally have a similar percentage of fatty acids, also because in all samples the C16:0 fatty acid dominates, while the sample COR-F differs sharply from the other samples because C18:1c was the dominant fatty acid (56.15%). The oleic acid varies from 4.87% to 7.86% in from 4.87% to 7.86% in the other samples. This graph clearly demonstrates how the ripening can influence the chemical composition of plants, in this case the fatty acid content of sea fennel flowers since the flowers of Corsica where collected during the period of the ripening of fruits while the rest of samples were collected at the flowering period.

LEAVES (%FATTY ACIDS)



Fatty acid content in leaves of sea fennel from different regions

In the case of sea fennel leaves, all samples are similar in the percentage of fatty acids and there are no distinctive cases as in the case of flowers. SFA varies from 27.69% to 35.32%, lowest in COR-L and highest in TOS-L region. MUFA ranges from 3.31% to 11.42%, lowest in the COR-L region and highest in SIC-L region. PUFA, also as in case of flowers, are predominant in all regions, highest content in COR-L region (61.32%) and lowest in SIC-L region (54.34%). The dominant fatty acids in the leaves are palmitic acid, linoleic acid and alpha linoleic acid. The minimum values of C16:0 fatty acid are recorded in the samples from COR-L region (17.58%). The minimum value of linoleic acid was found in TOS-L sample (27.88%) and of alpha linoleic acid was found in SIC-L sample (18.03%). The maximum values of these dominant fatty acids are as follows: C16:0 - MAR - L (20.61%), linoleic acid - SIC-L (35.12%), alpha linoleic acid - COR-L (29.19%).



	LIG-F	TOS-F	COR-F	MAR-F	CAL-F	SIC-F	SAR-F	PUG-F	MAR-L	CAL-L	CON-L	SIC-L	PUG-L	COR-L	SAR-L	TOS-L	LIG-L
C16:0	18.51	19.59	9.65	19.13	19.38	16.76	18.67	17.81	20.61	19.26	18.09	18.10	18.91	17.58	18.36	17.59	19.37
C18:0	3.54	3.31	1.96	3.29	3.34	3.73	3.77	3.33	2.96	3.27	3.24	2.93	2.78	2.43	2.70	3.09	2.73
C18:1n9T	1.23	2.08	0.63	1.91	0.85	0.83	1.65	1.41	5.20	4.15	5.60	3.23	5.52	7.68	6.02	5.94	5.40
C18:1c	6.24	5.21	56.15	5.14	7.86	6.14	4.87	6.11	4.10	4.59	2.69	10.79	4.25	2.06	4.48	3.29	4.94
LA	50.78	50.87	24.24	48.43	52.91	48.25	50.23	51.65	31.33	32.37	32.82	35.12	27.96	31.29	29.11	27.88	30.17
ALA	9.90	11.89	4.60	11.98	9.04	8.92	12.31	10.70	25.68	26.54	26.29	18.03	28.96	29.19	28.76	25.06	26.27
C18:3 c6,9,12	1.15	1.52	0.18	1.11	0.77	0.75	1.13	1.44	0.68	0.46	0.64	1.19	0.70	0.85	1.06	1.05	0.87

Main fatty acids in leaves and flowers from different Italian regions. The fatty acids contents are expressed as weight percentages of total FAME. Data are presented as means of three replicas ± standard deviation

For comparison, in particular, the content of LA and ALA in the leaves and flowers of sea fennel, it can be noted that in percentage terms of the total amount of FAME in the flowers of sea fennel, LA predominates, while in the leaves the percentage of ALA from the total amount of FAME is twice as high as in the flowers and the ratio of these two essential fatty acids is almost 1:1. The only sample that differs in the predominant content of monounsaturated fatty acid (C18:1c), instead of polyunsaturated fatty acid LA, is COR-F. The percentage content of LA and ALA in this sample is also 2-fold less in comparison with samples from other regions of sea fennel flowers.

SEEDS (% FATTY ACIDS)

Only one sample from the Corsica region was studied since there were not sufficient amount from other regions to perform a study on the fatty acids composition. As a result, it was decided to deposit the samples in the seed bank.
The table below presents the fatty acid composition of sea fennel seeds from Corsica, showing concentrations in percentages along with standard deviations. C16:0 (Palmitic Acid) was found to account for 4.61%, contributing to the saturated fat content. C18:2 c9,12 (Linoleic Acid) was present at 12.57%. The dominant fatty acid in the seed sample is C18:1c (Oleic Acid), which constitutes 77.08% of the total fat content indicating a high proportion of monounsaturated fats.

	COR_S		
C4:0	3,60	±	0,21
C6:0	0,24	±	0,09
C10:0	0,00	±	0,00
C11:0	0,00	±	0,00
C12:0	0,00	±	0,00
C13:0	0,00	±	0,00
C14:0	0,02	±	0,00
C15:0	0,00	±	0,00
C16:0	4,61	±	0,03
C16:1	0,10	±	0,01
C17:0	0,04	±	0,00
C17:1	0,00	±	0,00
C18:0	1,14	±	0,10
C18:1n9T	0,00	±	0,00
C18:1c	77,08	±	0,11
C18:2 t9,12	0,04	±	0,01

C18:2 c9,12	12,57	±	0,02
C20:0	0,16	±	0,07
C18:3 c9,12,15	0,35	±	0,01
C20:1 c	0,00	±	0,00
C18:3 c6,9,12	0,00	±	0,00
C21:0	0,00	±	0,00
C20:2 c	0,03	±	0,00
C22:0	0,00	±	0,00
C20:3 c8,11,14	0,00	±	0,00
C22:1 c	0,00	±	0,00
C22:2 c	0,00	±	0,00
C24:0	0,03	±	0,00
C24:1	0,00	±	0,00
SFA	9,83	±	0,10
MUFA	77,17	±	0,10
PUFA	12,95	±	0,00
trans	0,04	±	0,01
n-6	12,57	±	0,02
n-3	0,35	±	0,01
n-6/n-3	36,39	±	1,58

Fatty acids composition in sea fennel seeds from Corsica

1.2.5 Volatile Organic Compounds (VOCs)

Materials and methods

Nine samples of leaves and eight of flowers were collected during the plant's flowering period (August/first decade of October 2022 and 2023) from nine wild sea fennel (Crithmum maritimum L.) populations growing spontaneously in the following Mediterranean coastal areas of Italy and France: Calabria (CAL); Marche, Conero Regional Park (CON); Marche, Porto Potenza Picena (MAR); Apulia (APU); Sardinia (SAR); Sicily (SIC); Tuscany (TUS); Liguria (LIG), Corsica (COR). Information on the population were given above, except for LIG and COR; details on these populations were reported in the Table below.

Acronym	Region	Locality	Coordinates	Substrate	Altitude (m a.s.l.)	Exposure
LIG	Liguria	Bergeggi (SV)	44°24'23.42"N - 8°44'53.56"E	dolomite	10	NE
COR	Corsica (France)	Bussaglia	42°16'49,00"N - 8°41'19.00"E	granite	5	W

Samples (about 500 g) of fresh leaves and flowers were stored separately in sterile plastic bags, transported to the laboratory under refrigerated conditions, air-dried in a De Cloet Dryer at < 40°C till reaching a moisture

content lower than 15%, then grounded using by an analytical grinder for less then 10 sec, to prevent heating of the plant material (Raffo et al., 2022). The obtained powder was then stored at -20°C.

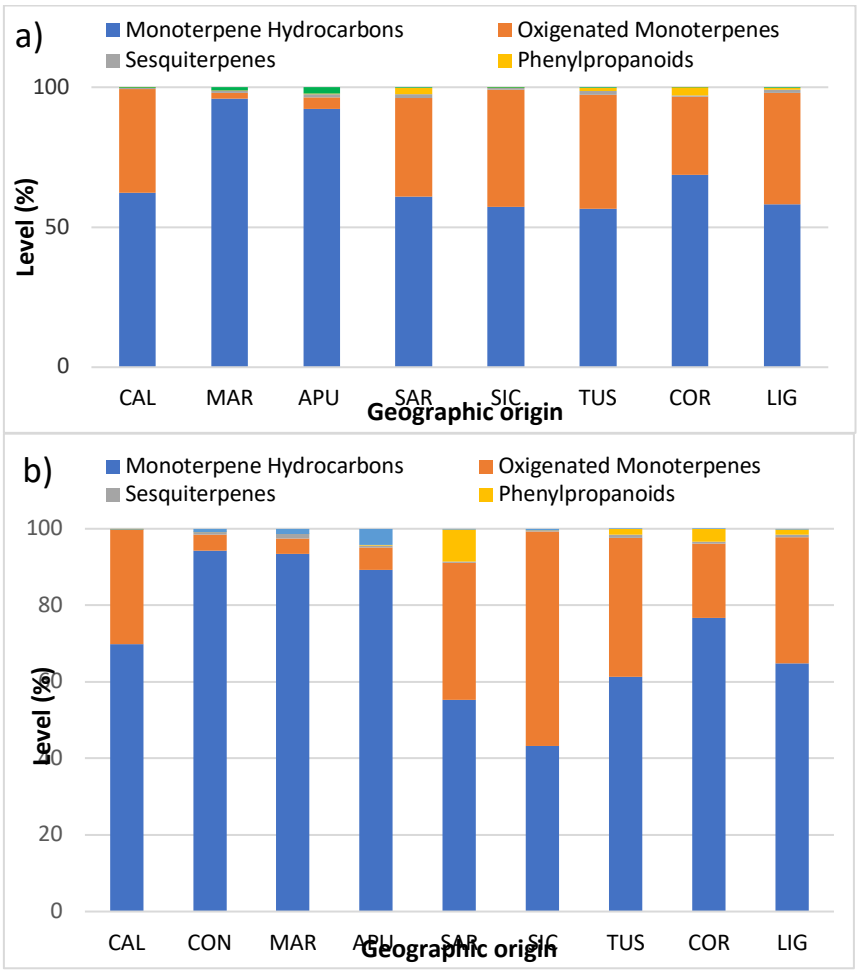
Analysis of VOCs was performed according to the method of Giungato et al. (2019), with some modifications. Isolation of VOCs from powdered leaves and flowers of sea fennel was performed by headspace solid-phase microextraction (HS-SPME). An aliquot of leaves or flowers powder (0.5 g) was transferred into a 15 mL vial capped with a PTFE/silicone septum (Supelco, Sigma-Aldrich Italy) for HS-SPME. The extraction was carried out by exposing an 85 µm CAR/PDMS fibre (Supelco, Sigma-Aldrich, Italy) to the headspace of the powder for 60 min, while keeping the extraction temperature at 40°C by a water bath, after 2 minutes of pre-equilibration. Prior to extraction, each fibre was preconditioned for 12 min in the injector port of the gas-chromatograph at 250°C. At the end of the extraction, the fibre was immediately inserted into the GC split-splitless injection port, for the desorption step, and the GC run was started. The same fibre was used for all the analyses. A triplicate extraction of sea fennel leaves and flowers from each geographic origin was performed.

Gas chromatography–mass spectrometry (GC-MS) analyses were performed with an Agilent 6890 GC 5973 N MS system equipped with a quadrupole mass filter for mass spectrometric detection (Agilent Technologies, Santa Clara, CA). Extracted volatiles were desorbed from the fibre for 5 min within the GC injector provided with a 0.75 mm glass liner suitable for SPME and operating at 250 °C in the split mode (split ratio of 1:50). Carry over and peaks arising from the SPME fibre were checked by frequently running blank samples. A DB-5MS UI column (0.25 mm i.d. × 60 m, 0.25 µm film thickness) (J&W Agilent Technologies - Santa Clara, CA) was used for VOCs separation. The following operating conditions were applied: injector temperature was 250 °C; oven temperature program from 60 °C to 200 °C at 4 °C min^{-1}, then to 280 °C (5 min) at 50 °C min^{-1} (total run time of 41.6 min); helium was used as carrier gas at a constant flow of 1.5 mL min^{-1} corresponding to a linear velocity of 32 cm s^{-1}. The MS detector operated in the electron ionization mode at 70 eV; transfer line, source, and quadrupole temperatures were set at 290, 230, and 150 °C, respectively. Detection was performed in the full scan mode, over the mass range 33-300 amu, for identification purposes.

Identification of compounds was carried out by comparing mass spectra and linear retention indices (LRI) of chromatographic peaks from sea fennel VOCs chromatogram with spectra and retention indices of authentic standards (Merck Life Science S.r.l., Milan, Italy). The list of compounds identified by comparison with an authentic standard is given at Table 2S. When authentic standards were not available, tentative identification was accomplished by comparison with information reported in the NIST/EPA/NIH EI Mass Spectral Library (Version 2.4, 2020) or in the literature. A standard solution of linear alkanes (C7–C30) was run under the same chromatographic conditions as the samples to determine the LRI of the detected compounds. Semi-quantitative determination of the level of VOCs in the headspace of sea fennel powder was based on data of chromatographic peak area.

Results

The volatile profile of sea fennel flowers and leaves was characterised by determination of 42 VOCs isolated from the headspace of dried, powdered plant material using the HS-SPME technique. Regarding distribution of chemical groups within the whole volatile profile, the populations MAR, CON, and APU were dominated by monoterpene hydrocarbons, often exceeding 90%, while other chemical groups made up less than 10% of the volatile fraction.



In contrast, sea fennel from other geographic origins exhibited a more balanced volatile profile, with a notable presence of oxygenated monoterpenes, which ranged from 28% (COR) to 42% (SIC) in the flowers, and from 19% (COR) to 55% (SIC) in the leaves. The proportion of phenylpropanoids was generally low across all populations, with the highest concentrations found in SAR (8.3% in leaves and 2.4% in flowers) and COR (3.3% and 2.9%, respectively). When considering individual VOCs, a two-way ANOVA, excluding the CON population, demonstrated that both geographic origin and plant part (flower or leaf) significantly influenced the level of practically all determined VOCs (Table 2).

Percent content (% area of total area of chromatographic peaks) of individual VOCs as determined by HS-SPME/GC-MS in flowers and leaves of sea fennel from wild population with different geographic origin. Results of 2-way ANOVA analysis[1], with factors represented by plant part and geographic origin.

Volatile Organic Compound	Plant part																
	Flowers								Leaves								
	Geographic origin								Geographic origin								
	CAL	MAR	APU	SAR	SIC	TUS	COR	LIG	CAL	CON	MAR	APU	SAR	SIC	TUS	COR	LIG
3-Methyl-butanal	0.01	0.02	0.02	0.00	0.00	0.01	0.00	0.01	0.01	0.01	0.02	0.04	0.00	0.00	0.00	0.00	0.00
1-Penten-3-ol	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.03	0.06	0.28	0.01	0.01	0.01	0.00	0.00
Pentanal	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.01	0.01	0.03	0.04	0.11	0.01	0.01	0.01	0.00	0.01
Hexanal	0.01	0.08	0.15	0.01	0.01	0.01	0.00	0.02	0.01	0.08	0.13	0.41	0.02	0.02	0.01	0.00	0.01
2-Hexenal	0.01	0.05	0.04	0.01	0.01	0.00	0.00	0.01	0.00	0.02	0.02	0.05	0.01	0.00	0.00	0.00	0.00
Heptanal	0.05	0.23	0.40	0.05	0.04	0.06	0.01	0.03	0.05	0.19	0.34	0.67	0.09	0.08	0.03	0.00	0.02
a-Thujene	0.15	0.32	0.70	0.34	0.74	0.45	0.24	0.69	0.07	0.34	0.07	0.39	0.41	0.61	0.62	0.57	0.61
a -Pinene	2.10	4.28	1.94	1.49	1.34	1.80	1.91	0.53	0.13	0.34	0.20	0.18	0.28	0.09	0.12	0.30	0.15
a -Fenchene	0.02	0.07	0.09	0.01	0.01	0.02	0.01	0.00	0.01	0.02	0.04	0.05	0.01	0.00	0.01	0.01	0.01
Sabinene	0.50	6.46	12.45	2.00	8.89	4.87	1.37	6.05	0.42	5.53	1.36	4.68	5.93	9.42	7.23	7.60	6.22
6-Methyl-5-Hepten-2-one	0.00	0.01	0.03	0.00	0.00	0.00	0.00	0.00	0.02	0.10	0.14	0.31	0.02	0.02	0.01	0.00	0.02
b-Pinene	0.18	0.33	0.19	0.17	0.16	0.17	0.20	0.07	0.03	0.05	0.06	0.07	0.06	0.03	0.04	0.07	0.04
b -Myrcene	0.86	0.94	0.65	1.01	1.02	1.04	1.39	0.90	0.98	0.80	0.94	0.67	0.95	0.60	1.43	1.70	1.26
Octanal	0.11	0.60	1.66	0.09	0.06	0.10	0.02	0.15	0.06	0.44	0.65	2.40	0.12	0.15	0.04	0.01	0.07
a -Phellandrene	0.02	0.03	0.06	0.44	0.05	0.22	0.64	0.04	0.03	0.07	0.12	0.27	0.40	0.05	0.08	0.69	0.18
3-Carene	0.02	0.01	0.02	0.06	0.05	0.05	0.06	0.04	0.02	0.02	0.01	0.00	0.04	0.03	0.04	0.07	0.05
a -Terpinene	0.15	0.22	0.53	0.43	0.54	0.47	0.42	0.53	0.21	0.43	0.66	0.91	0.41	0.39	0.49	0.46	0.44
p-Cymene	10.25	8.80	16.82	11.84	12.25	11.69	7.59	13.24	12.32	25.72	7.53	24.84	16.17	17.19	13.92	6.51	12.64
Z-Ocimene	0.00	0.00	0.00	0.00	1.51	0.00	1.46	1.15	0.00	0.00	0.00	0.00	1.87	0.26	1.31	3.09	1.30
Limonene	26.29	65.78	48.09	1.65	0.00	0.34	0.00	0.00	35.11	45.85	81.33	55.56	0.00	0.00	0.00	0.00	0.00
b -Phellandrene	0.38	0.45	0.45	9.14	0.78	4.88	13.88	0.31	0.44	0.57	0.86	0.87	10.18	0.26	1.27	14.73	3.57
y-Terpinene	21.25	7.91	9.72	32.22	29.87	30.50	39.33	34.35	20.00	14.36	0.18	0.22	18.44	14.18	34.44	40.74	38.12
cis-Sabinene hydrate	0.07	0.24	0.60	0.20	0.16	0.19	0.06	0.19	0.01	0.10	0.07	0.56	0.08	0.64	0.08	0.10	0.11
Terpinolene	0.06	0.24	0.64	0.22	0.13	0.14	0.10	0.19	0.06	0.20	0.16	0.52	0.14	0.19	0.25	0.19	0.21
Linalool	0.09	0.33	0.69	0.27	0.09	0.10	0.14	0.03	0.08	0.11	1.81	1.41	0.29	0.31	0.03	0.04	0.02
trans-Sabinene hydrate	0.04	0.12	0.29	0.11	0.09	0.06	0.03	0.14	0.04	0.11	0.15	0.48	0.06	0.50	0.07	0.06	0.15
4-Terpineol	0.37	0.21	0.47	0.68	0.38	0.36	0.53	0.23	0.08	0.09	0.10	0.34	0.11	0.67	0.07	0.05	0.25
a-Terpineol	0.19	0.48	0.79	0.48	0.21	0.16	0.23	0.09	0.19	0.19	0.50	0.73	0.16	0.53	0.03	0.06	0.07
Thymol Methyl ether isomer	1.65	0.41	0.53	1.42	2.18	1.72	1.06	1.92	1.56	1.53	0.80	0.94	1.21	2.85	1.48	0.81	1.28
Thymol Methyl ether	34.29	0.03	0.03	31.57	38.23	37.75	25.84	37.24	27.12	0.02	0.04	0.02	32.86	49.45	34.06	18.31	30.93
Thymol	0.15	0.32	0.46	0.16	0.23	0.27	0.12	0.05	0.25	0.52	0.12	0.37	0.29	0.38	0.16	0.01	0.06

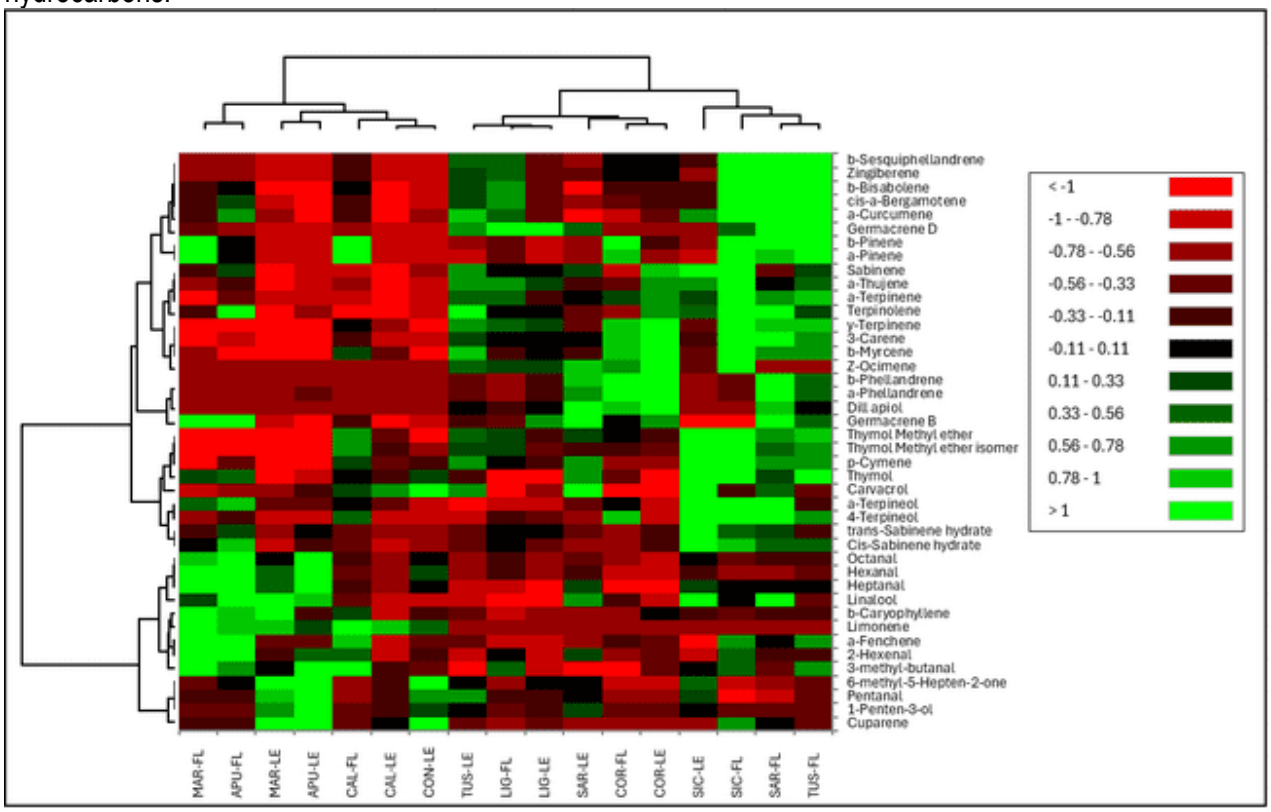
Carvacrol	0.27	0.06	0.27	0.27	0.12	0.15	0.01	0.03	0.61	1.54	0.33	1.05	0.79	0.61	0.40	0.01	0.13
b -Caryophyllene	0.05	0.33	0.20	0.04	0.01	0.03	0.01	0.00	0.01	0.10	0.57	0.17	0.01	0.04	0.03	0.04	0.01
cis-a-Bergamotene	0.09	0.15	0.31	0.24	0.20	0.28	0.09	0.23	0.01	0.07	0.07	0.02	0.06	0.11	0.16	0.10	0.11
a -Curcumene	0.05	0.10	0.18	0.08	0.06	0.11	0.03	0.10	0.01	0.11	0.14	0.08	0.03	0.08	0.10	0.05	0.07
Germacrene D	0.01	0.04	0.01	0.15	0.04	0.10	0.00	0.13	0.00	0.00	0.01	0.00	0.08	0.01	0.08	0.01	0.15
Zingiberene	0.04	0.02	0.02	0.22	0.14	0.23	0.09	0.16	0.00	0.00	0.00	0.00	0.04	0.02	0.09	0.07	0.06
b-Bisabolene	0.09	0.11	0.22	0.20	0.16	0.24	0.07	0.19	0.00	0.05	0.00	0.00	0.00	0.08	0.12	0.08	0.09
Cuparene	0.01	0.02	0.03	0.02	0.02	0.01	0.00	0.00	0.03	0.20	0.33	0.30	0.00	0.00	0.01	0.00	0.01
b -Sesquiphellandrene	0.09	0.08	0.10	0.28	0.21	0.34	0.12	0.21	0.00	0.04	0.03	0.00	0.06	0.09	0.18	0.12	0.11
Germacrene B	0.01	0.05	0.07	0.03	0.00	0.02	0.01	0.01	0.00	0.00	0.00	0.00	0.04	0.00	0.01	0.02	0.03
Dill apiol	0.02	0.06	0.08	2.39	0.00	1.06	2.89	0.73	0.02	0.03	0.03	0.02	8.27	0.03	1.49	3.29	1.42

Volatile Organic Compound	Results of 2-way ANOVA[1] p values		
	Geographic origin	Plant part	Interaction geographic origin × plant part
3-Methyl-butanal	<0.0001	<0.0001	<0.0001
1-Penten-3-ol	<0.0001	<0.0001	<0.0001
Pentanal	<0.0001	<0.0001	<0.0001
Hexanal	<0.0001	0.071	<0.0001
2-Hexenal	<0.0001	<0.0001	<0.0001
Heptanal	<0.0001	<0.0001	<0.0001
a-Thujene	<0.0001	<0.0001	<0.0001
a -Pinene	<0.0001	<0.0001	<0.0001
a -Fenchene	<0.0001	<0.0001	<0.0001
Sabinene	<0.0001	<0.0001	<0.0001
6-Methyl-5-Hepten-2-one	<0.0001	<0.0001	<0.0001
b-Pinene	<0.0001	<0.0001	<0.0001
b -Myrcene	<0.0001	<0.0001	<0.0001
Octanal	<0.0001	<0.0001	<0.0001
a -Phellandrene	<0.0001	<0.0001	<0.0001
3-Carene	<0.0001	<0.0001	<0.0001
a -Terpinene	<0.0001	<0.0001	<0.0001
p-Cymene	<0.0001	<0.0001	<0.0001
Z-Ocimene	<0.0001	<0.0001	<0.0001
Limonene	<0.0001	<0.0001	<0.0001
b -Phellandrene	<0.0001	<0.0001	<0.0001
y-Terpinene	<0.0001	<0.0001	<0.0001
cis-Sabinene hydrate	<0.0001	<0.0001	<0.0001
Terpinolene	<0.0001	<0.0001	<0.0001
Linalool	<0.0001	0.301	<0.0001
trans-Sabinene hydrate	<0.0001	<0.0001	<0.0001
4-Terpineol	<0.0001	<0.0001	<0.0001
a-Terpineol	<0.0001	<0.0001	<0.0001
Thymol Methyl ether isomer	<0.0001	<0.0001	<0.0001
Thymol Methyl ether	<0.0001	<0.0001	<0.0001
Thymol	<0.0001	<0.0001	<0.0001
Carvacrol	<0.0001	<0.0001	<0.0001
b -Caryophyllene	<0.0001	<0.0001	0.000
cis-a-Bergamotene	<0.0001	<0.0001	<0.0001
a -Curcumene	<0.0001	<0.0001	<0.0001
Germacrene D	<0.0001	<0.0001	<0.0001
Zingiberene	<0.0001	<0.0001	<0.0001
b-Bisabolene	<0.0001	<0.0001	<0.0001
Cuparene	<0.0001	0.073	<0.0001
b -Sesquiphellandrene	<0.0001	<0.0001	<0.0001
Germacrene B	<0.0001	<0.0001	<0.0001
Dill apiol	<0.0001	<0.0001	<0.0001

Note: the sample CON leaves was not included in the ANOVA analysis, due to unavailability of data on CON flowers.

A Heat Map visualization (Figure 3) further illustrated the impact of these factors on the overall VOCs profile. The dendrogram displayed vertically (columns) indicated two main clusters based on geographic origin, with one cluster consisting of samples from LIG, SAR, SIC, TUS, and COR, and another cluster including populations from APU, MAR, CAL, and CON. This suggests that geographic origin plays a more substantial role in the volatile profile than the plant part. Further examination revealed that individual VOCs could also be grouped into two primary clusters (horizontal dendrogram, rows), reflecting their association with specific populations. In addition, it is interesting to note that populations placed in the left cluster (MAR, CON, APU),

with the exception of CAL, coincided with those characterised by a dominant presence of monoterpene hydrocarbons.



Reference
Giungato et al. 2019. Characterization of dried and freeze-dried sea fennel (Crithmum maritimum L.) samples with headspace gas-chromatography/mass spectrometry and evaluation of an electronic nose discrimination potential. Food Research International, 115, 65-72. https://doi.org/10.1016/j.foodres.2018.07.067.
Raffo, A., Baiamonte, I., De Benedetti, L., Lupotto, E., Marchioni, I., Nardo, N., & Cervelli, C. (2022). Exploring volatile aroma and non-volatile bioactive compounds diversity in wild populations of rosemary (Salvia rosmarinus Schleid.). Food Chemistry, 404, 134532. https://doi.org/10.1016/j.foodchem.2022.134532

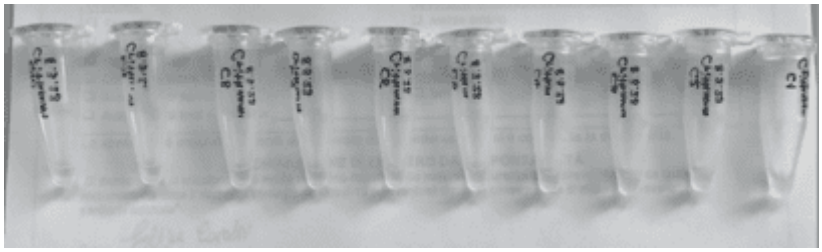
1.3 Molecular analyses

1.3.1 DNA extraction

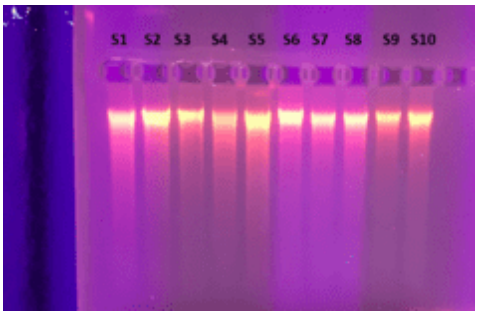
For genomic DNA extraction, approximately 50 gr of leaves from 20 individuals of each sampled population were used for DNA extraction.

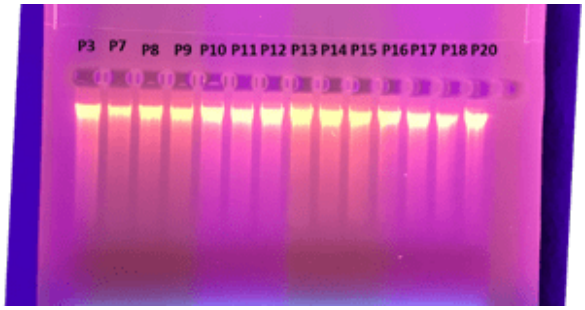
Administrative Region	Location	Code
Calabria	Vasche di Cassiodoro-Copanello (CZ)	V
Marche	Conero (Due Sorelle Bay, AN)	S
Corsica	Bussaglia (Corsica)	Cor
Liguria	Bergeggi (SV)	Lig
Marche	Porto Potenza Picena (MC)	P
Puglia	Calalunga (Peschici, FG)	G
Sicily	Terrasini-Capo Rama (PA)	T
Sardinia	La Licciola (OT)	L
Tuscany	Calafuria (LI)	C

The leaves were dried in silica gel upon their collection (Wilkie et al. 2013). Once the tissue was completely dry, DNA was extracted from 50 mg of dried leaf tissue using DNeasy Plant Pro Kit by Qiagen, following the manufacturer instructions, with some slight changes to obtain higher amount of high molecular weight DNA, as required for next generation sequencing (NGS). Tubes containing extracted DNA in PCR grade water are depicted below.

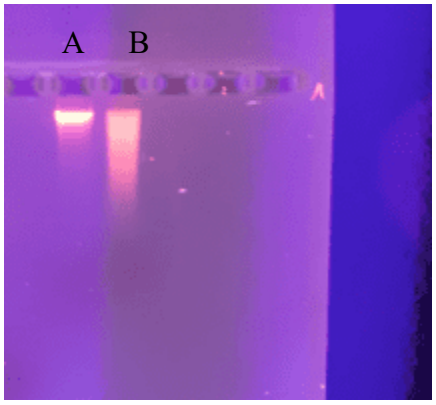


The quantity and purity of the DNA extracts were assessed spectrophotometrically. In order to check the integrity of DNA, the extracts were analysed by electrophoresis on agarose gel, as shown below.





The suitability of DNA extracts in terms of ddRAD analysis was also assessed by digestion with restriction endonuclease HaeIII (see for reference the following image: lane A: DNA not treated with HaeIII; lane B: DNA treated with HaeIII.)



In the following table, the quantity of DNA analysed by Qubit and Nanodrop and the purity values /260/280, 260/230) are recorded.

Sample	Qubit (ng/ul)	Nanodrop (ng/ul)	260/280	260/230	2 ug	H2O_50ul vol fin
P1	184,4	215,1	1,8	2,1	10,9	39,2
P2	154,4	160,1	1,8	2,2	13,0	37,1
P3	234,0	166,6	1,8	2,2	8,6	41,5
P4	157,2	169,2	1,7	1,9	12,7	37,3
P5	138,8	161,4	1,8	2,0	14,4	35,6
P6	140,0	141,0	1,8	2,1	14,3	35,7
P7	14,5	80,8	1,7	1,5	27,3	22,7
P8	147,0	119,2	1,8	2,0	13,6	36,4
P9	104,0	106,6	1,7	1,8	19,2	30,8
P10	197,0	155,8	1,8	2,2	10,2	39,9
P11	123,0	141,1	1,8	2,1	16,3	33,7
P12	241,0	166,8	1,8	2,2	8,3	41,7
P13	241,0	207,1	1,8	2,2	8,3	41,7
P14	231,0	183,0	1,8	2,2	8,7	41,3

P15	173,0	122,0	1,8	2,1	11,6	38,4
P16	159,0	150,9	1,8	2,2	12,6	37,4
P17	142,0	128,9	1,7	2,0	14,1	35,9
P18	96,5	99,1	1,8	2,0	20,7	29,3
P19	143,0	141,8	1,8	2,2	14,0	36,0
P20	233,0	192,4	1,8	2,3	8,6	41,4
S1	166,0	138,4	1,7	1,8	12,1	38,0
S2	174,0	136,5	1,8	1,9	11,5	38,5
S3	106,0	85,1	1,8	2,3	18,9	31,1
S4	72,4	119,0	1,7	1,5	27,6	22,4
S5	181,0	150,0	1,8	2,2	11,1	39,0
S6	132,0	120,3	1,8	2,0	15,2	34,9
S7	132,0	93,4	1,7	1,8	15,2	34,9
S8	85,2	103,5	1,7	1,8	23,5	26,5
S9	87,6	73,1	1,8	1,8	22,8	27,2
S10	93,6	107,4	1,7	1,9	21,4	28,6
S11	161,0	174,8	1,8	2,0	12,4	37,6
S12	216,0	190,4	1,8	2,3	9,3	40,7
S13	117,0	118,6	1,8	1,8	17,1	32,9
S14	88,4	83,6	1,8	1,8	22,6	27,4
S15	158,0	108,0	1,8	1,7	12,7	37,3
S16	131,0	121,0	1,7	1,7	15,3	34,7
S17	93,6	105,9	1,6	1,3	21,4	28,6
S18	170,0	149,4	1,8	2,2	11,8	38,2
S19	177,0	170,5	1,8	2,2	11,3	38,7
S20	123,0	121,7	1,8	2,3	16,3	33,7
C1	158,0	181,7	1,7	1,4	12,7	37,3
C2	204,0	192,9	1,8	1,9	9,8	40,2
C3	95,6	110,5	1,6	1,3	20,9	29,1
C4	55,2	80,4	1,6	1,1	36,2	13,8
C5	57,2	96,1	1,6	1,2	35,0	15,0
C6	118,0	131,8	1,7	1,5	17,0	33,1
C7	102,0	118,8	1,7	1,5	19,6	30,4
C8	158,0	169,3	1,7	1,7	12,7	37,3
C9	169,0	169,6	1,8	1,8	11,8	38,2
C10	112,0	119,7	1,8	1,8	17,9	32,1
C11	95,2	130,1	1,6	1,1	21,0	29,0
C12	74,8	99,5	1,6	1,2	26,7	23,3
C13	136,0	145,3	1,7	1,7	14,7	35,3
C14	124,0	140,4	1,7	1,4	16,1	33,9
C15	98,4	146,4	1,7	1,7	20,3	29,7
C16	178,0	163,8	1,7	1,6	11,2	38,8
C17	153,0	142,3	1,7	1,7	13,1	36,9
C18	160,0	158,9	1,8	1,8	12,5	37,5

C19	124,0	140,9	1,7	1,6	16,1	33,9
C20	198,0	206,7	1,8	1,8	10,1	39,9
G1	112,0	126,3	1,8	1,7	17,9	32,1
G2	101,0	116,7	1,7	1,6	19,8	30,2
G3	276,0	286,9	1,8	1,9	7,2	42,8
G4	224,0	229,4	1,8	2,2	8,9	41,1
G5	171,0	185,7	1,8	1,8	11,7	38,3
G6	155,0	151,6	1,8	2,2	12,9	37,1
G7	80,4	75,9	1,8	1,8	24,9	25,1
G8	116,0	99,0	1,8	2,0	17,2	32,8
G9	157,0	137,1	1,8	1,9	12,7	37,3
G10	85,6	146,0	1,8	1,8	23,4	26,6
G11	143,0	167,0	1,8	2,1	14,0	36,0
G12	232,0	216,0	1,8	1,2	8,6	41,4
G13	124,0	111,0	1,7	1,9	16,1	33,9
G14	129,0	102,7	1,7	1,8	15,5	34,5
G15	94,8	186,7	1,8	1,9	21,1	28,9
G16	132,0	129,5	1,8	2,2	15,2	34,8
G17	154,0	149,2	1,8	2,2	13,0	37,0
G18	86,4	99,6	1,7	1,4	23,1	26,9
G19	90,4	109,9	1,7	1,6	22,1	27,9
G20	216,0	179,2	1,7	1,7	9,3	40,7
V1	182,0	175,0	1,8	2,1	11,0	39,0
V2	137,0	137,0	1,8	1,8	14,6	35,4
V3	180,6	180,6	1,8	1,7	11,1	38,9
V4	122,0	121,0	1,8	1,6	16,4	33,6
V5	144,0	124,4	1,8	1,9	13,9	36,1
V6	181,0	191,6	1,8	2,2	11,0	39,0
V7	192,0	165,7	1,8	2,3	10,4	39,6
V8	115,0	114,6	1,8	2,0	17,4	32,6
V9	110,0	108,6	1,8	2,0	18,2	31,8
V10	216,0	191,6	1,8	2,4	9,3	40,7
V11	178,0	145,8	1,8	2,3	11,2	38,8
V12	133,0	124,1	1,8	1,9	15,0	35,0
V13	146,0	129,6	1,8	2,2	13,7	36,3
V14	126,0	115,5	1,7	1,8	15,9	34,1
V15	75,2	85,3	1,7	1,5	26,6	23,4
V16	100,0	92,0	1,8	1,9	20,0	30,0
V17	125,0	108,4	1,8	2,1	16,0	34,0
V18	86,8	87,3	1,8	1,8	23,0	27,0
V19	52,0	59,7	1,8	1,6	38,5	11,5
V20	101,0	93,0	1,8	1,9	19,8	30,2
Cor1	74,4	78,1	1,7	1,6	26,9	23,1

Cor2	102,0	80,7	1,8	2,0	19,6	30,4
Cor3	120,0	98,2	1,8	2,2	16,7	33,3
Cor4	128,0	90,6	1,8	2,0	15,6	34,4
Cor5	88,0	77,6	1,8	1,7	22,7	27,3
Cor6	80,8	61,6	1,8	2,2	24,8	25,2
Cor7	188,0	140,3	1,8	1,7	10,6	39,4
Cor8	182,0	153,8	1,8	2,2	11,0	39,0
Cor9	127,0	110,8	1,8	2,0	15,7	34,3
Cor10	160,0	136,3	1,7	1,9	12,5	37,5
Cor11	73,6	72,1	1,7	2,2	27,2	22,8
Cor12	130,0	102,2	1,8	2,2	15,4	34,6
Cor13	150,0	122,2	1,8	2,3	13,3	36,7
Cor14	138,0	104,4	1,8	1,9	14,5	35,5
Cor15	120,0	90,5	1,8	2,1	16,7	33,3
Cor16	92,4	78,5	1,8	2,1	21,6	28,4
Cor17	77,6	68,9	1,8	2,3	25,8	24,2
Cor18	102,0	90,9	1,8	2,3	19,6	30,4
Cor19	66,8	67,5	1,7	1,9	29,9	20,1
Cor20	150,0	130,9	1,8	2,2	13,3	36,7
Lig1	96,8	100,8	1,7	1,7	20,7	29,3
Lig2	110,0	127,6	1,6	1,3	18,2	31,8
Lig3	78,0	68,8	1,7	2,0	25,6	24,4
Lig4	78,8	88,4	1,7	1,2	25,4	24,6
Lig5	59,6	57,8	1,8	1,9	33,6	16,4
Lig6	114,0	200,7	1,8	2,0	17,5	32,5
Lig7	119,0	111,7	1,7	1,5	16,8	33,2
Lig8	94,4	88,4	1,7	1,6	21,2	28,8
Lig9	56,0	69,5	1,6	1,0	35,7	14,3
Lig10	104,0	85,3	1,7	1,7	19,2	30,8
Lig11	95,2	116,0	1,7	1,7	17,2	32,8
Lig12	89,6	90,6	1,7	1,5	22,1	27,9
Lig13	85,2	117,3	1,7	2,1	17,1	32,9
Lig14	158,0	166,2	1,8	2,3	12,0	38,0
Lig15	104,0	135,0	1,8	1,7	14,8	35,2
Lig16	166,0	214,1	1,8	2,2	9,3	40,7
Lig17	107,0	146,5	1,7	2,1	13,7	36,3
Lig18	72,4	91,7	1,5	1,1	21,8	28,2
Lig19	144,0	174,2	1,8	2,0	11,5	38,5
Lig20	144,0	139,7	1,7	1,7	14,3	35,7
T1	94,0	98,0	1,7	1,6	21,3	28,7
T2	178,0	159,8	1,8	2,1	11,2	38,8
T3	153,0	128,7	1,7	1,8	13,1	36,9
T4	130,0	123,5	1,7	1,7	15,4	34,6
T5	192,0	171,9	1,8	2,0	10,4	39,6

T6	186,0	164,1	1,8	2,1	10,8	39,2
T7	164,0	151,3	1,8	2,0	12,2	37,8
T8	129,0	119,1	1,8	1,6	15,5	34,5
T9	80,8	121,0	1,7	1,6	24,8	25,2
T10	133,0	112,4	1,7	1,8	15,0	35,0
T11	223,0	217,7	1,7	1,8	9,0	41,0
T12	232,0	184,4	1,8	1,6	8,6	41,4
T13	132,0	102,3	1,8	1,8	15,2	34,8
T14	149,0	127,6	1,7	1,7	13,4	36,6
T15	163,0	130,8	1,8	1,9	12,3	37,7
T16	115,0	119,1	1,8	1,6	17,4	32,6
T17	114,0	137,8	1,7	1,4	17,5	32,5
T18	166,0	121,9	1,9	1,8	12,0	38,0
T19	150,0	158,5	1,8	1,8	13,3	36,7
T20	193,0	151,1	1,8	1,8	10,4	39,6
L1	204,0	276,3	1,7	2,1	9,8	40,2
L2	176,0	194,6	1,8	2,3	11,4	38,6
L3	126,0	166,7	1,7	1,7	15,9	34,1
L4	81,2	114,9	1,7	1,5	24,6	25,4
L5	112,0	119,1	1,8	1,8	17,9	32,1
L6	85,6	92,8	1,8	2,0	23,4	26,6
L7	121,0	109,6	1,8	2,0	16,5	33,5
L8	130,0	160,3	1,8	2,1	15,4	34,6
L9	82,4	104,6	1,7	1,7	24,3	25,7
L10	119,0	119,5	1,8	1,7	16,8	33,2
L11	101,0	134,3	1,7	1,9	19,8	30,2
L12	146,0	136,0	1,9	2,1	13,7	36,3
L13	108,0	139,9	1,7	1,6	18,5	31,5
L14	97,2	118,3	1,7	1,8	20,6	29,4
L15	143,0	133,7	1,9	2,0	14,0	36,0
L16	124,0	144,3	1,7	1,9	16,1	33,9
L17	138,0	153,6	1,7	2,1	14,5	35,5
L18	134,0	139,9	1,8	1,9	14,9	35,1
L19	156,0	160,2	1,8	1,7	12,8	37,2
L20	81,2	96,6	1,8	2,0	24,6	25,4

1.3.2 Sequencing

The sequencing was done by Biomarker Technologies (BMK) Company (Munster, Germany), following SLAF-seq methodology.

Specific locus amplified fragment sequencing (SLAF-seq) is an optimized version of ddRADseq, specifically intended for large-scale genotyping experiments. The enzymes and the sizes of the restriction fragments are optimized with training data to ensure even distribution and avoid repeats. The fragments are also selected over a tight range, to optimize the PCR reaction. The protocol is similar to ddRAD, with a first

digestion with a restriction enzyme (HaeIII), heat inactivation and a second digestion with an other enzyme (Hpy166II).

Then, the ATP and dual-index sequencing adapter were added at the 3' and 5' end of the digested DNA products, respectively. PCR was performed and the products were purified using E.Z.N.A.H Cycle Pure Kit (Omega). The purified products were mixed and incubated with these two restricted enzymes again. After ligation of ATP, and Solexa adapter in the pair-end, the reaction products were purified using a Quick Spin column (Qiagen, Venlo, Netherlands), and segregated on a 2% agarose gel. Fragments with xx-xx bp were isolated using a Gel Extraction Kit (Tiangen). These SLAFs were subjected to PCR to add barcode. The PCR products were re-purified and then prepared for paired-end sequencing on an Illumina HiSeq sequencing platform (Illumina, San Diego, CA,USA).

Data analysis

The raw reads were further processed with a bioinformatic pipelinetool, BMKCloud(www.biocloud.net) online platform.

Quality control

Raw data (raw reads) of fastq format were firstly processed through fastp software. In this step, clean data(clean reads) were obtained by removing reads containing adapter, reads containing ploy-N and low quality reads from raw data. At the same time, Q30, GC-content of the clean data were calculated. All the downstream analyses were based on clean data with high quality.

Reads mapping to the reference genome

The adaptor sequences and low-quality sequence reads were removed from the data sets. Raw sequences were transformed into clean reads after data processing. These clean reads were then mapped to the reference genome sequence. bwa soft were used to map with reference genome.

SNP/INDEL Calling

The SNP/INDEL calling was performed using GATK(v3.8 McKenna et al.,2010) and SAMtools packages (Li et al., 2009) (v1.9.1). A total of 448436 SNPs with a minor allele frequency (MAF) and integrity was retained.

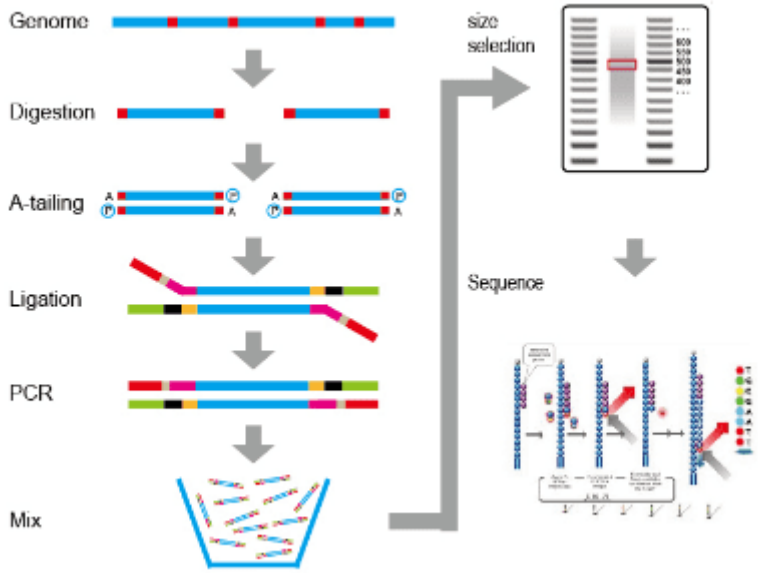
SNP/INDEL Annotation

SNP annotation was performed on the basis of the reference genome using snpEff software(3.6c (build 2014-05-20)) (Cingolani et al., 2012), and SNPs were categorized into intergenic regions, upstream or downstream regions, and exons or introns. SNPs in coding exons were further classified as synonymous SNPs or nonsynonymous SNPs. InDels in exons were grouped according to whether they led to a frameshift.

Gene functional annotation

Gene function was annotated based on the following databases: Nr (NCBI non-redundant protein sequences);Pfam (Protein family); KOG/COG (Clusters of Orthologous Groups of proteins); Swiss-Prot (A manually annotated and reviewed protein sequence database); KO (KEGG Ortholog database); GO (Gene Ontology).

This methodology allows: i) deep sequencing for genotyping accuracy; ii) reduced representation strategy to reduce sequencing costs; iii) pre-designed reduced representation scheme to optimize marker efficiency; iv) double barcode system for large populations



Methodology used for sequencing

References

Sun, X., Liu, D., Zhang, X., Li, W., Liu, H., Hong, W., ... & Zheng, H. (2013). SLAF-seq: an efficient method of large-scale de novo SNP discovery and genotyping using high-throughput sequencing. PloS one, 8(3), e58700.

Wilkie, P., Poulsen, A. D., Harris, D., & Forrest, L. L. (2013). The collection and storage of plant material for DNA extraction: the teabag method. Gardens' Bulletin Singapore, 65(2), 231-234.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., ... & 1000 Genome Project Data Processing Subgroup. (2009). The sequence alignment/map format and SAMtools. bioinformatics, 25(16), 2078-2079.

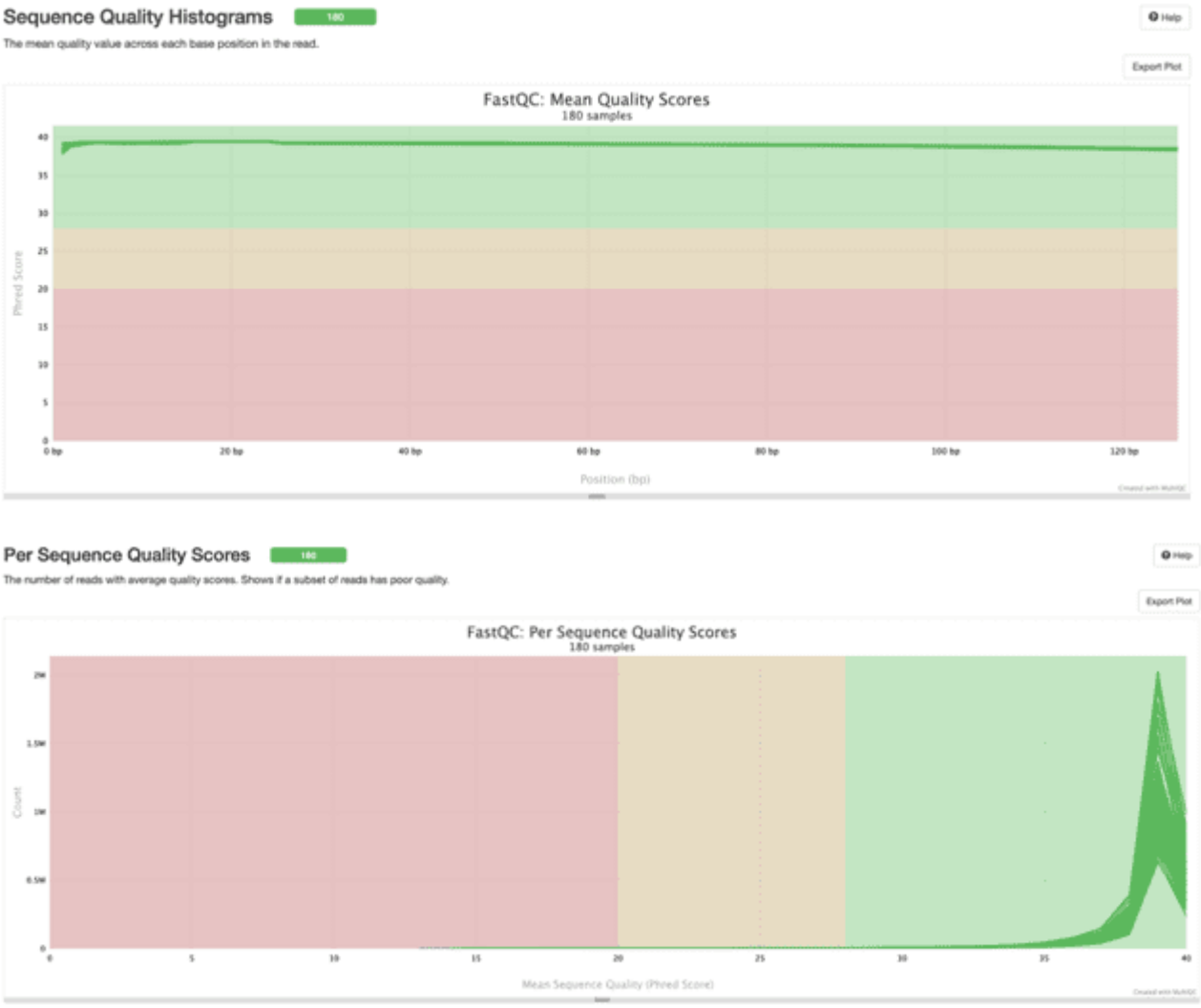
McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., ... & DePristo, M. A. (2010). The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome research, 20(9), 1297-1303.

Cingolani, P., Platts, A., Wang, L. L., Coon, M., Nguyen, T., Wang, L., ... & Ruden, D. M. (2012). A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. fly, 6(2), 80-92.

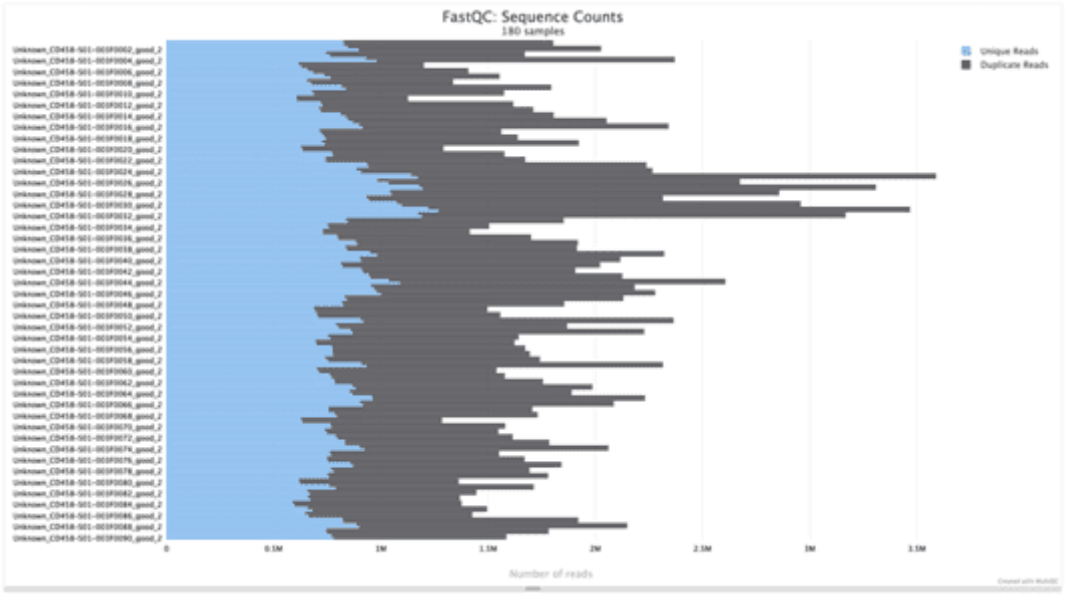
1.3.3 Analysis of sequences

A total of 90 samples were processed by the company Biomarker Technology (BMK) in order to generate raw SLAF-seq data. Actually, clean data were obtained through fastp software by removing reads containing adapters and poly-N and low quality reads from raw data. At the same time, Q30 and GC content were calculated on clean reads. To produce a summarizing report on the quality of data, we applied multiqc on cleaned reads.

Overall, data were of good quality. Indeed, data profiles reported in "Sequence Quality Histograms" were all close to 40 of PHRED score.



Secondly, a high estimate of duplicates emerged from sequence counts. This result is not surprising due to the fact that a genome of a neighboring species, Apium graveolens, was used to perform the step of SNP calling. The use of a non-specific reference genome may lead to an apparent increase in duplicates as reads from repetitive regions may not be resolved correctly and "mapped" incorrectly or assigned to multiple positions.



Results

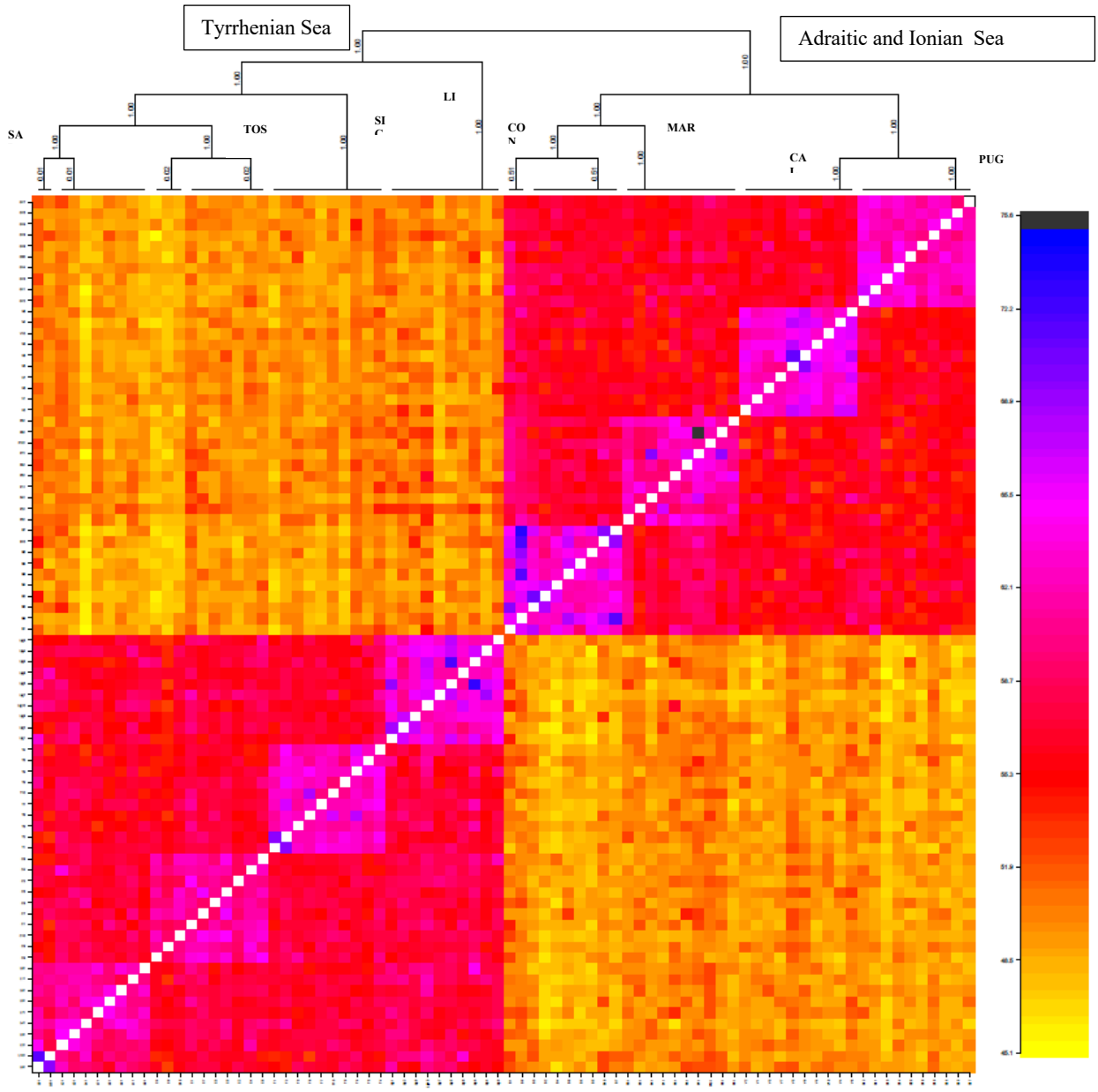
The results of the genetic sequence analyses confirm, although not completely, the results of the morphological data analyses (see 1.4.1).

The Coancestry analysis shows that the populations are clearly differentiated into two groups:

a Tyrrhenian group that includes the populations of Sardinia (SAR), Tuscany (TOS), Sicily (SiC), and Liguria (LIG), and an "eastern" or Adriatic-Ionian group that includes the populations of Marche (MAR), Conero (CON), Puglia (PUG), and Calabria (CAL).

In the first "Tyrrhenian" group, it is confirmed that SAR and TOS are similar populations, while the Sicilian and Ligurian populations are more clearly structured and separated. The Corsican population was not included in these analyses as it is not considered among the Italian populations.

In the Adriatic-Ionian group, the populations of the central Adriatic (CON and MAR) are more closely related to each other, while the more southern populations (PUG and CAL) are grouped together while maintaining a certain autonomy.



Finally, it can be concluded that there are two "geographical types" for Italian populations: a Tyrrhenian type, which groups together the populations living along the Tyrrhenian coasts (Sardinia, Tuscany, Sicily, Liguria, and Corsica), and an Adriatic-Ionian type, which groups together the Adriatic populations (Puglia, Marche, and Conero) and the Ionian population (Calabria).
See paragraph 6 for comparisons with the populations of the partner countries.

1.4 Statistical elaboration of data

1.4.1 *Statistical elaboration of morphological and genetic data*

The morphological data have been statistically elaborated in order to explore if there are differences among the Italian population of sea fennel.

Material and methods

To assess the variability of each morphometric trait between populations, conditional box plots were generated.

To compare all populations simultaneously in terms of morphometric variability, the mean value of each trait was used as a column in a data matrix, with each population represented as a row.

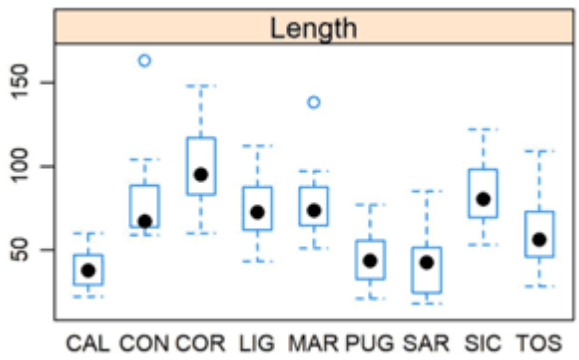
Standardised principal component analysis (PCA) was then performed on the data matrix to explore patterns of variation.

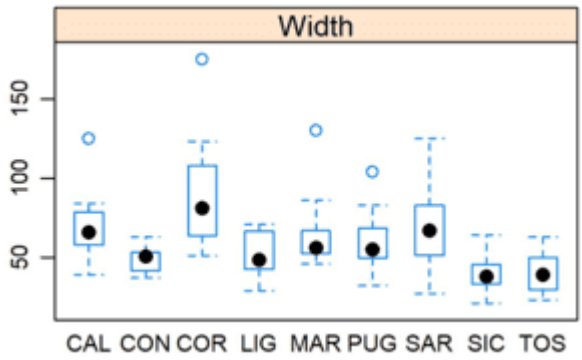
Results

Below are the boxplots performed on the individual measured characters.

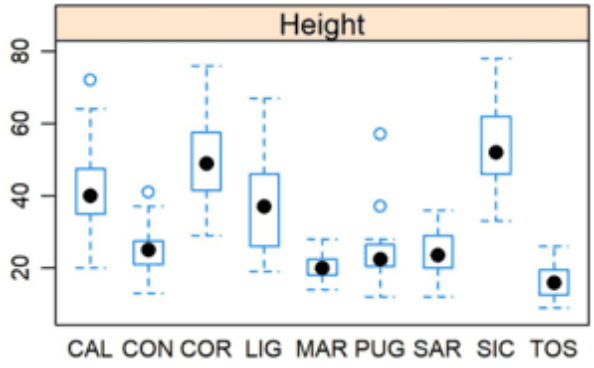
Whole plant

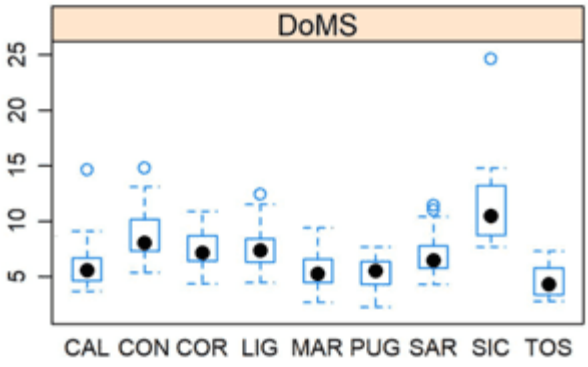
As dimensional traits, the length, width and height of the entire plant were measured as well as the diameter of the main stem.



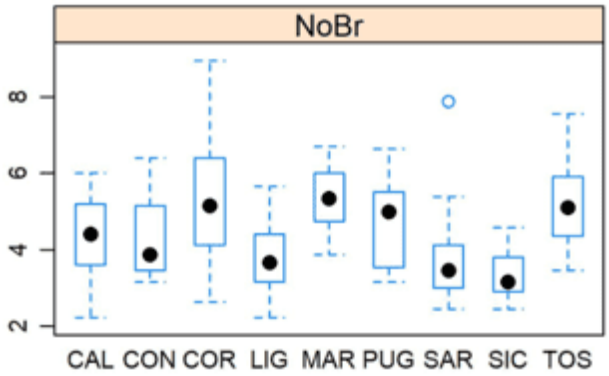


The population with the largest plants (Length and Width) is that of Corsica (COR) while the population with the smallest plants is that of Sicily (SIC).

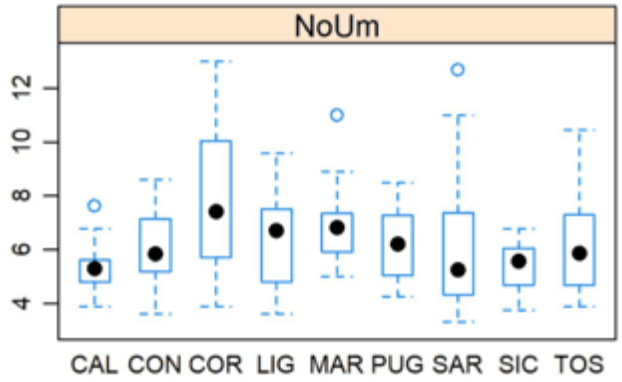




The tallest plants are found in the populations of Sicily (SIC) and Corsica (COR) while the shortest ones are found in the population of Tuscany (TOS). The cliffs on which the SIC and COR populations develop are formed by very large blocks of rock between which the plants take refuge and develop and therefore remain protected from the winds; this allows the plants to develop more in height. The sandstone cliff on which the population of Tuscany (TOS) develops, on the contrary, is not formed by large blocks and therefore the plants are more exposed to the winds. As regards the diameter of the main stem (DoMS), Sicily (SIC) has larger stems; this means that the plants of the SIC population are older. This is probably due to the fact that the sampling site is located in a protected nature reserve (WWF reserve of Capo Rama) and therefore the human disturbance is very limited. The opposite is found for the populations of Tuscany (TOS), Calabria (CAL), Marche (MAR) and Sardinia (SAR) where the sites are more disturbed as they are frequented by tourists and bathers.



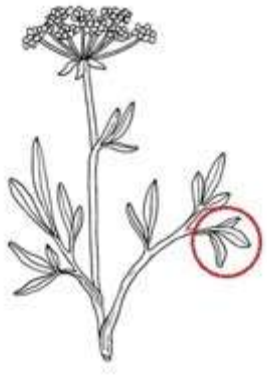
The graph above refers to the number of branches (NoBr). As can be observed, there is a more or less large variability within each population. In general, the COR, MAR and TOS populations have plants with a greater number of branches while SIC, LIG, CAL and SAR have a low number of branches.



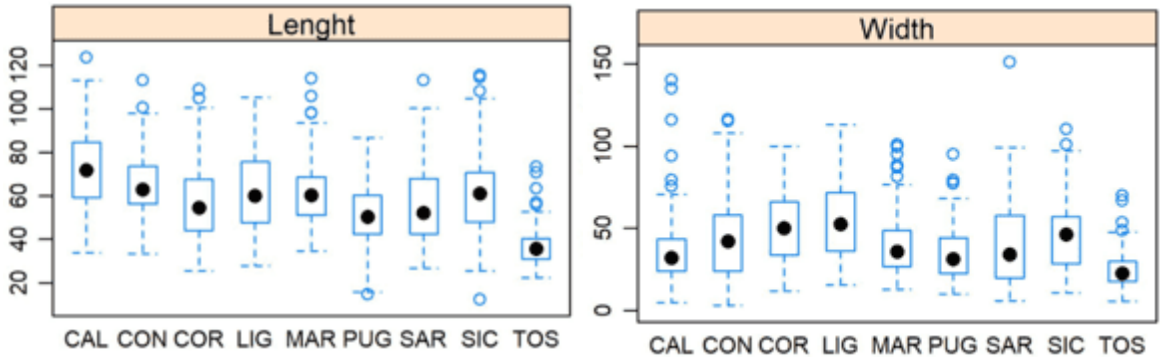
The graph above refers to the number of umbels (NoUm). Again, the population with the highest number of umbels is COR while CAL and SIC are those with the lowest number of umbels.

Leaves

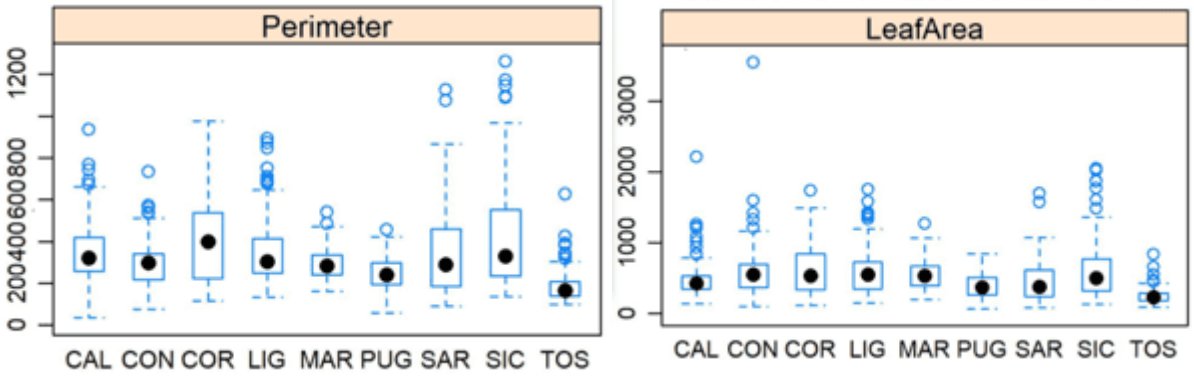
As previously mentioned, the basal leaves of the plants were collected. Sea fennel has bipinnate or tripinnate compound leaves. The measurements were not taken on the entire leaf but on the first order leaflets, in particular on the first lower leaflet (fig. 2).



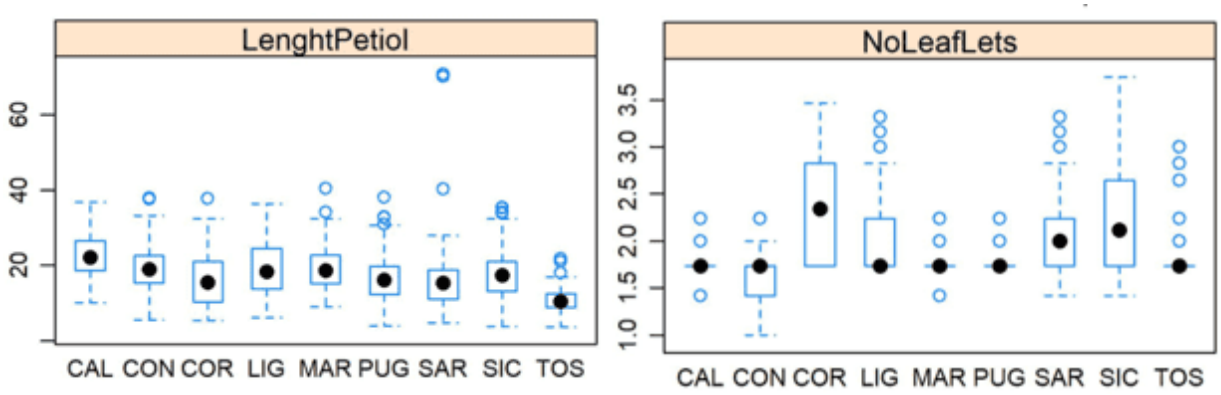
The red circle indicates the portion of the leaf (leaflet) on which the measurements were taken.



The longest leaflets are those of the CAL population while the widest ones are those of the LIG population. The shortest and narrowest leaflets are those of the TOS population.

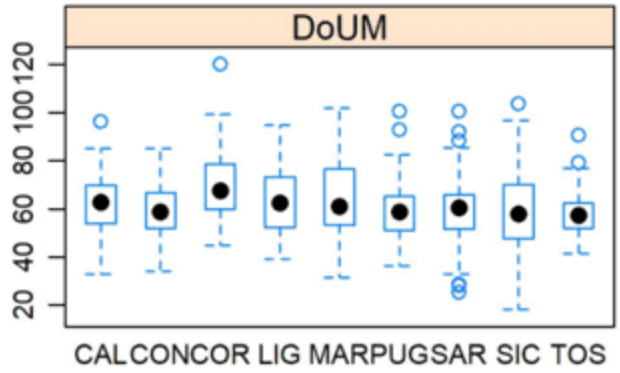


The leaves with the largest perimeter were found in the CAL, COR and SIC populations while those with the smallest perimeter were found in the TOS population. As regards the area, the leaves with a larger surface were found in the LIG population.

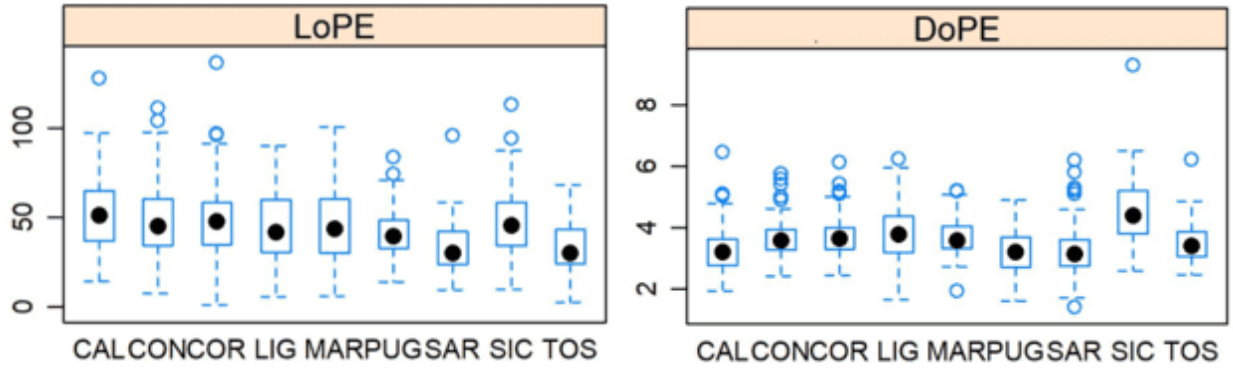


The leaves with the longest petiole are those of the CAL population while those with the shortest petiole are those of the TOS population.
On average, the leaflets are composed of three lobes; in the COR and SIC populations, most of the leaflets have more than three lobes while in the CON population a high number of leaflets have less than three lobes.

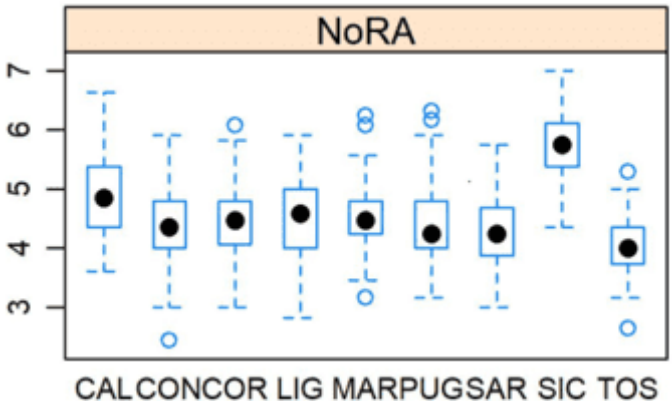
Umbels and umbellets



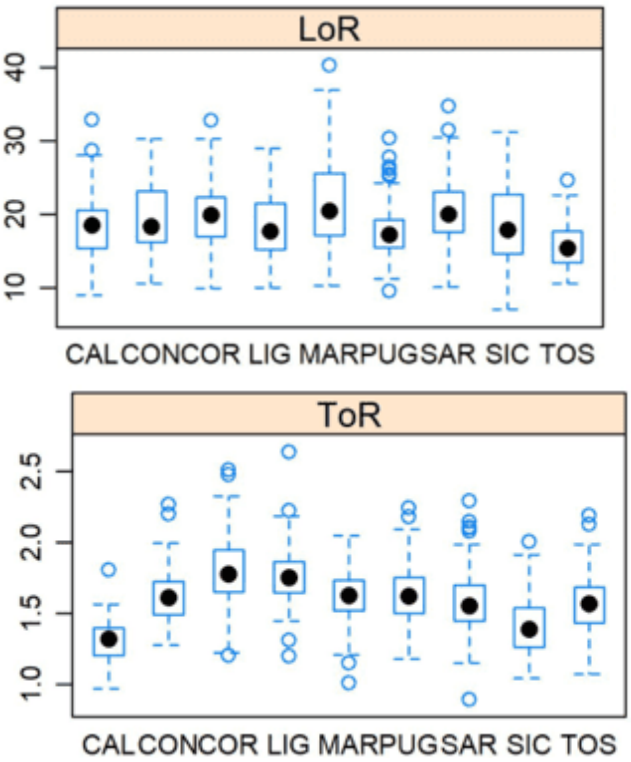
The population characterized by having umbels with a larger diameter (DoUM) is COR which therefore confirms that it is composed of large plants. The umbels with a smaller diameter are found in the SAR and TOS populations.



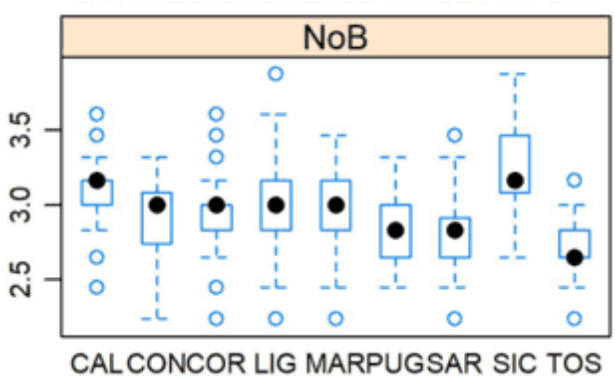
The two graphs above refer to the length (LoPE) and diameter (DoPE) of the peduncle that subtends the terminal umbel. The longest peduncle is found in the CAL population while the shortest is found in the SAR and TOS populations. The peduncles with the largest diameter are found in the SIC population while the thinnest are found in the PUG population.



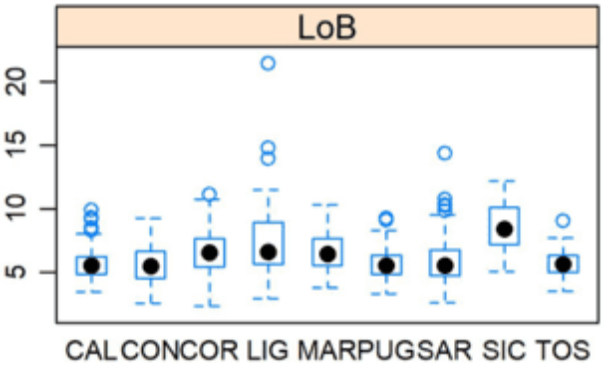
The box plot above refers to the number of rays of which the umbel is composed (NoRA). The umbels of the SIC population are those that have the highest number of rays in absolute terms, on average 33 rays in each umbel. The TOS population instead has umbels with a very low number of rays, on average 16-17 rays.

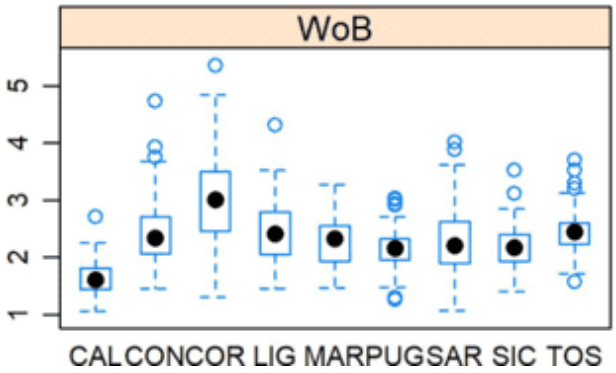


The umbels with the longest ray length (LoR) are those of the MAR population while the shortest ones are found in the TOS population. The umbels with the largest mean ray diameter (ToR) are found in the COR population while the CAL population has umbels with thinner rays.

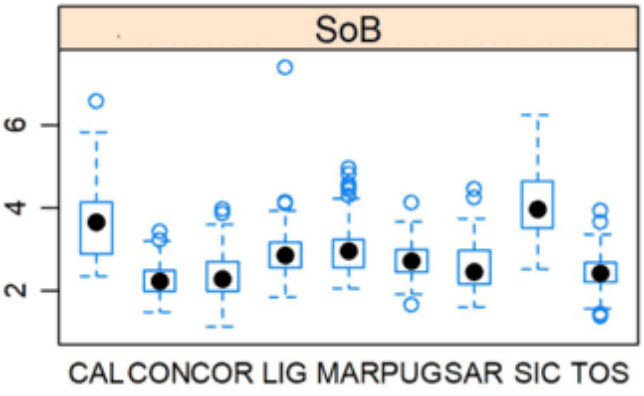


Some interesting characters that differentiate the populations are found in the bracts located at the base of the umbels. First, the bracts were counted.
The population with the highest number of bracts (NoB) (on average 10 bracts) is that of Sicily (SIC) while the TOS population has an average number of bracts of 7.
The dimensions of the bracts (length and width, respectively LoB and WoB) were measured and the ratio between length and width (SoB) was calculated.



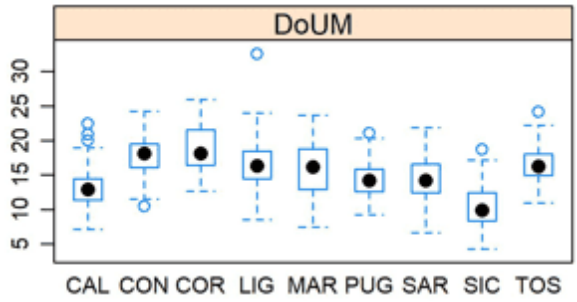


The SIC population has bracts that are on average longer than the other populations, while the widest bracts are found in the COR population. The shortest bracts are recorded in the CON population, while the narrowest are found in the CAL population.

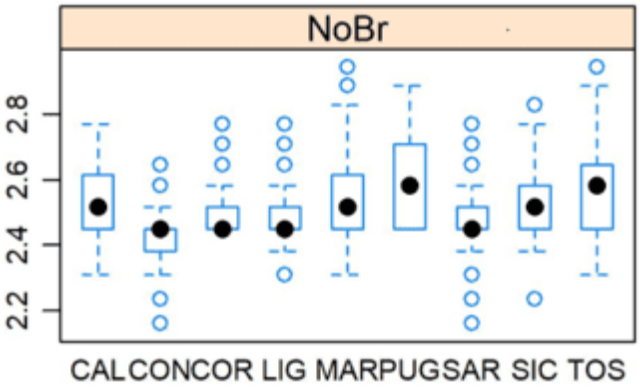


Considering the ratio between length and width (SoB) it appears that the SIC population, having the highest ratio, has long and narrow bracts while the population with the lowest ratio and therefore bracts that tend to be more rounded-oval are found in the CON population.

In addition to the size of the umbels characters, the characters of the umbellets were also considered. The average diameter (DoUM) of these was measured first.

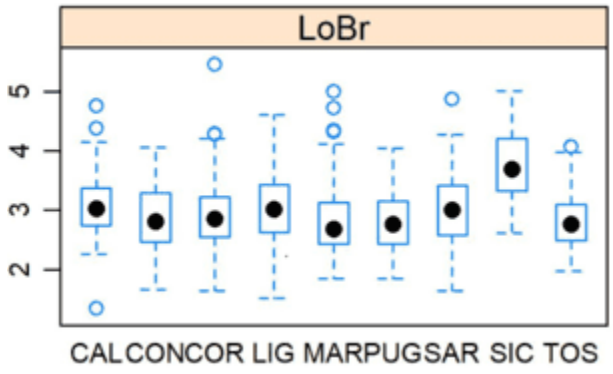


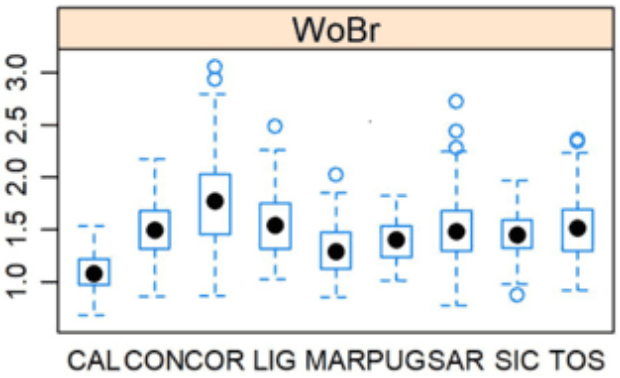
As can be observed, the largest umbellets, i.e. with a larger diameter, are found in the COR population while the SIC population has smaller umbellets (but, as seen, more numerous).



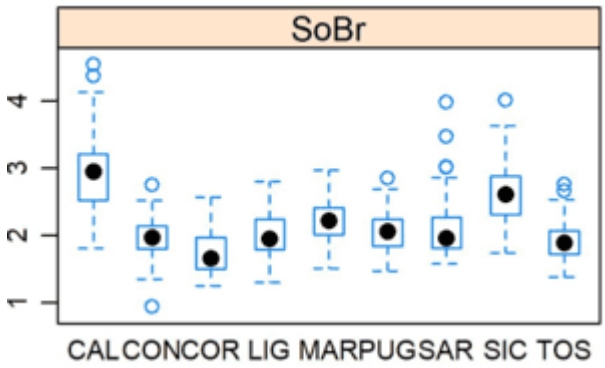
The bracteoles are generally 6; in the CON population a certain number of umbellets with a number of bracts less than 6 are observed while in the PUG population most of the umbellets have more than 6 bracteoles.

The dimensional values of the bracteoles will be discussed below.



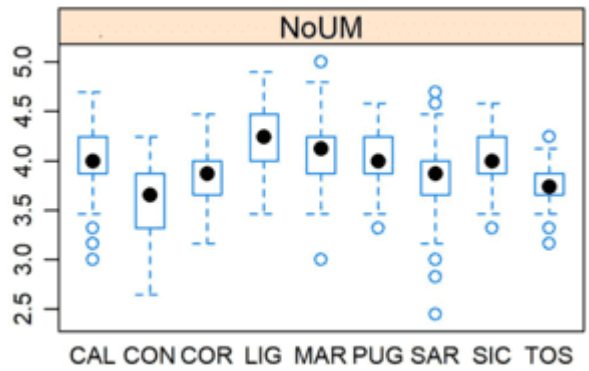


The longest bracteoles (see LoBr) are observed in the SIC population while the shortest ones in the PUG population. The widest bracteoles are found in the COR population while the narrowest ones in the CAL population.

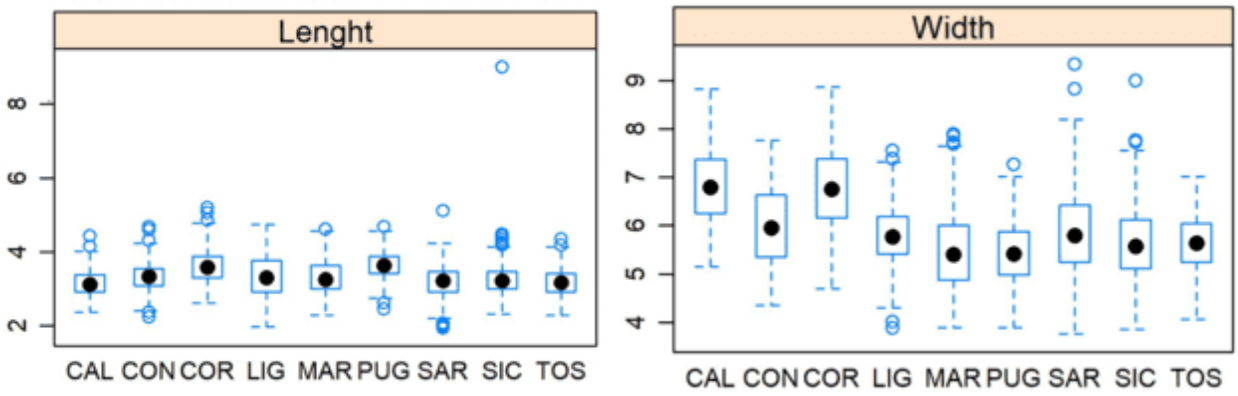


Considering the ratio between length and width of the bracteoles (SoBr), it emerges that the CAL population has bracteoles of a more lanceolate shape on average while the COR population has more rounded-oval bracteoles as the length/width ratio is lower.

Fruits



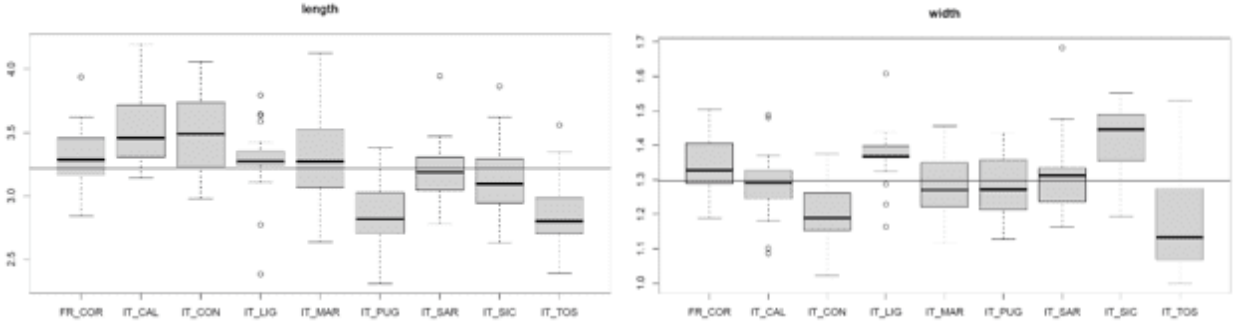
First, the average fruits carried by the umbellets were counted and it emerged that the population with umbellets that carry more fruits is the LIG population while the population with umbellets that on average have "poorer" fruits is the CON population.



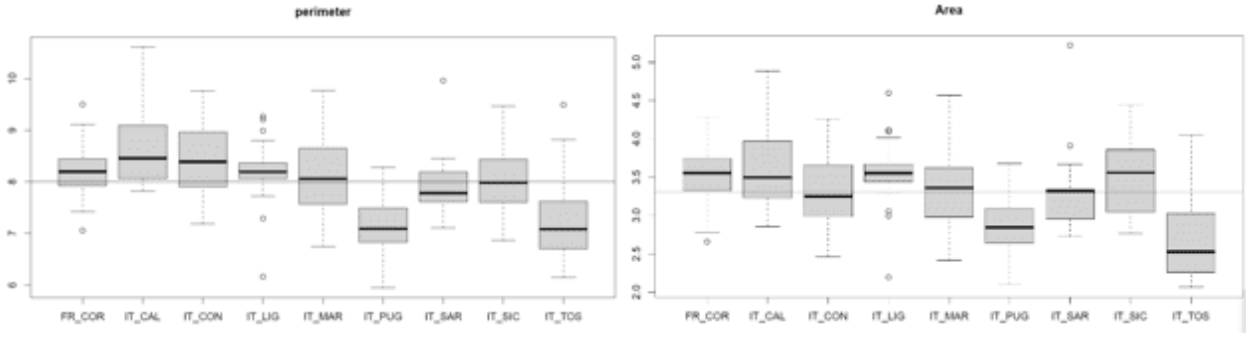
As regards the dimensional characteristics, the population in which the longest fruits are found is CAL while the shortest ones are recorded in the PUG population. The widest fruits are observed in the PUG population while the narrowest ones are recorded in the CAL population. It follows that the CAL population has the slenderest fruits while the PUG population has the most rounded fruits.

Seeds

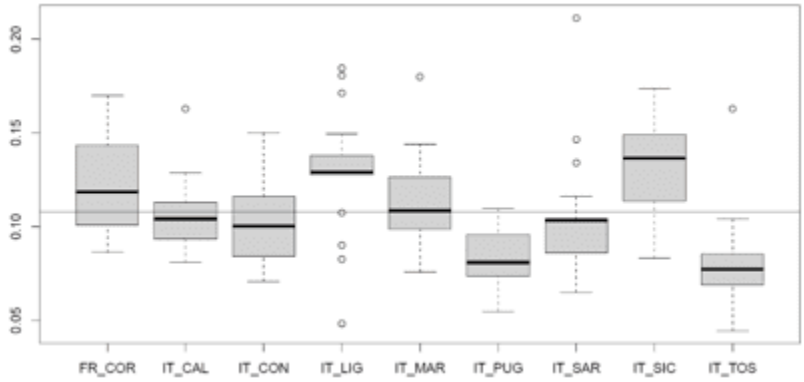
The seeds were extracted from the fruits. All the fruits contain two seeds.
The seeds were measured and weighed. Below are the results of the processing.



As regards length and width, it emerges that the longest seeds are observed in the CAL population while the shortest ones are observed in the PUG and TOS populations; the widest seeds are recorded in the SIC population while the narrowest ones in the TOS population.

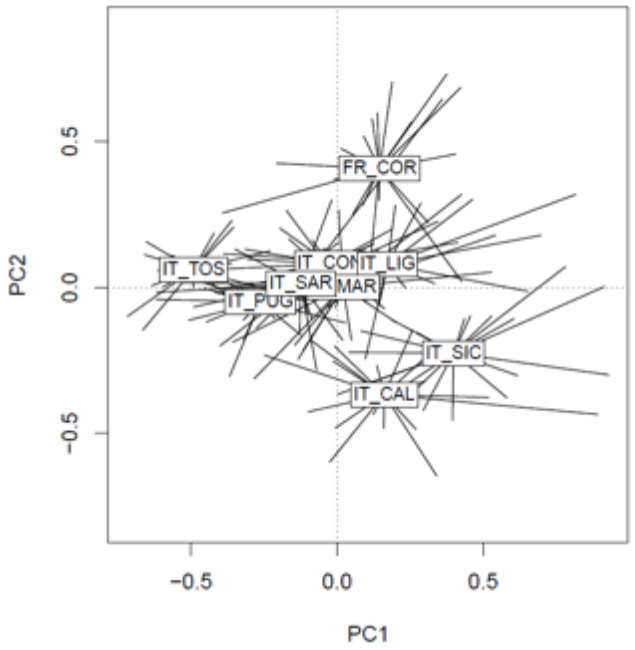


The population with seeds with a larger perimeter is CAL while the one with a smaller perimeter is PUG; as regards the area, the LIG population has seeds with a larger surface area while the TOS population has a smaller surface area.

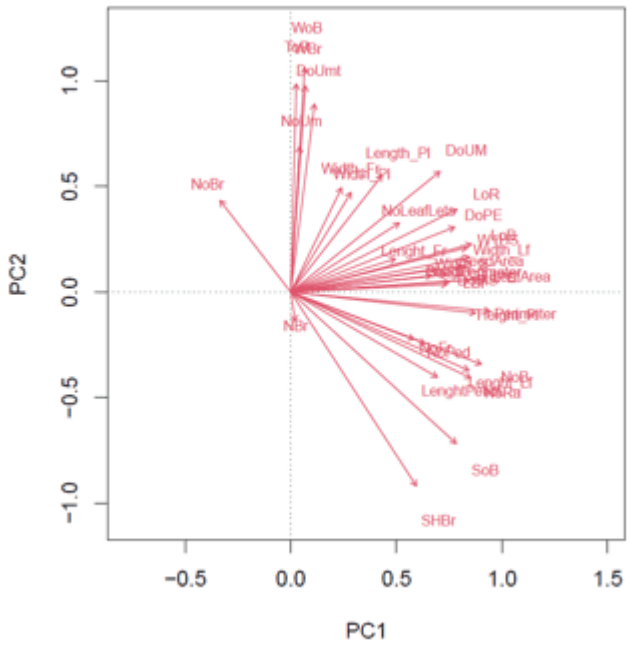


As regards the weight, calculated on 100 seeds, the population with the heaviest average seeds is LIG while the one with the lightest seeds is TOS.

To get a more complete idea of the similarities/differences between different populations and to see if there are patterns of variation, we subjected our data to principal components analysis.



PC1 axis explains 27% of the variability while PC2 explains approximately 14.25%.
As regards populations, based on PC1, the most dissimilar populations are TOS and SIC to which LIG, CAL and PUG are also added. TOS and PUG are below the mean while SIC, LIG and CAL are above.
Based on PC2, the populations that deviate from the mean are COR, CAL and SIC with Cor above the mean and SIC and CAL below the mean.



The second PCA graph highlights the most discriminating morphological characters among the populations.

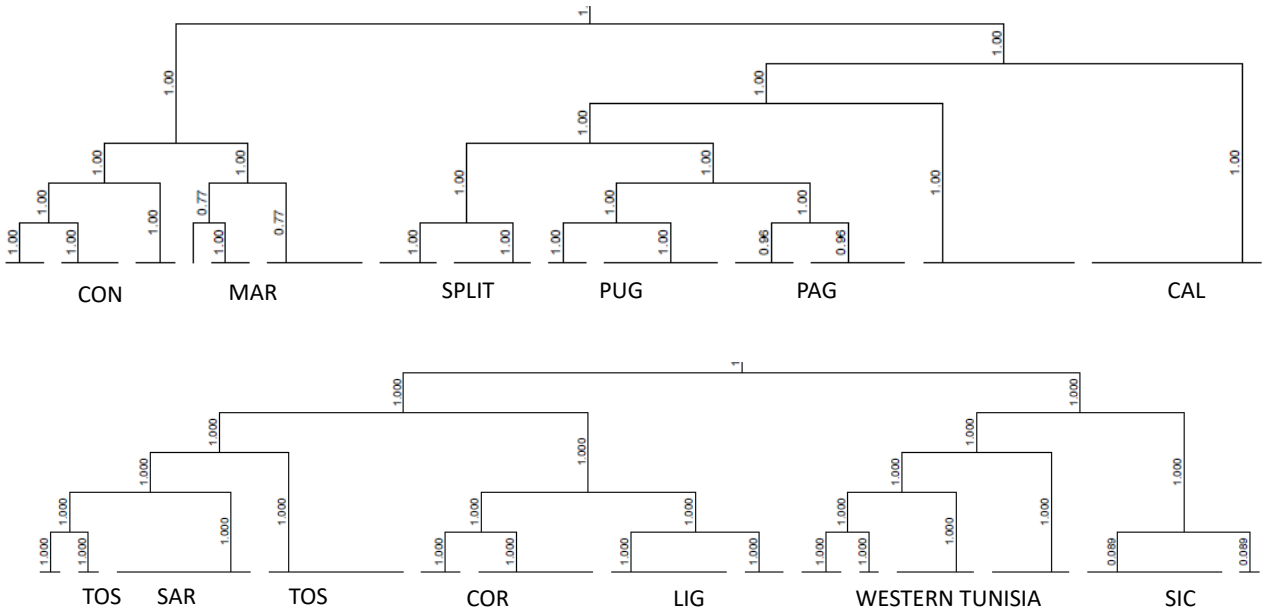
The PC1 axis highlights that the most discriminating characters are those listed below:
Perimeter= perimeter of the leaf
Leaf Area
Width_Lf = width of the leaf
Lenght_Lf= length of the leaf
Lenght Petiol
NoB= Number of bracts
LoB = length of the bracts
SoB = shape of bracts
LBr = length of the bracteoles
NoRa = Number of rays
LoR = length of rays
LoPE= length of the peduncle
DoUM= diameter of the umbels
DoPE= diameter of the peduncle
W1HS= weight of 100 seeds
Seed Area
Seed Perimeter
Width_S= width of the seed
DoMS = diameter of the main stem

PC2 axis identifies these other characters as more discriminating:
NoUm = number of umbels
DoUmt = Diameter of the umbellets
ToR = Thickness of the rays
WoB = width of the bracts
SoB = Shape of the bracts
WBr = width of the bracteoles
ShBr = Shape of the bracteoles.

In conclusion, the populations that seem to be most different for many morphological traits are SIC and CAL and TOS and COR.

Results of genetic data elaboration

- Two distinct groups: a group of Tyrrhenian populations that are linked to those of Corsica and Tunisia and a group of Adriatic populations that are linked to the Croatian ones;
- The population of Calabria (CAL) joins the Adriatic group;
- The populations of Conero (CON) and Porta Potenza (MAR) constitute a distinct group in which a certain structuring of the two distinct populations is recognized;
- The population of Gargano (PUG) is included in the group of Croatian populations;
- The populations of Sardinia (SAR) and Tuscany (TOS) constitute a distinct group;
- The populations of Corsica (COR) and Liguria (LIG) constitute a distinct group;
- The population of Sicily (SIC) joins the group of populations of western Tunisia.



Conclusion:

- Excellent correspondence between morphological analyses and genetic analyses;
- Two distinct morphological and genetic groups: an Adriatic group and a Tyrrhenian group;
- The population of Sicily is connected to those of western Tunisia;
- The population of Corsica is connected to that of Liguria;
- The population of Sardinia and that of Tuscany are connected;
- The Adriatic populations of central Italy are similar;
- The population of Puglia is connected to some Croatian populations and not to those of the Marche region

2 Croatian sea fennel populations

Wild sea fennel populations sampled across Croatian coasts are listed in the following string. They are named according to the sampling location.

Location	Krk	Senj	Pag	Šibenik	Split	Drašnice	Korčula	Pelješac	Neretva	Cavtat

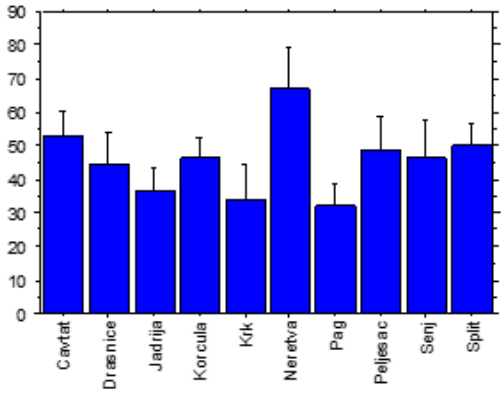
Analogously to what done by the other partners involved in this WP, these populations were subjected to chemical and molecular analyses.

2.1 Morphological characterization

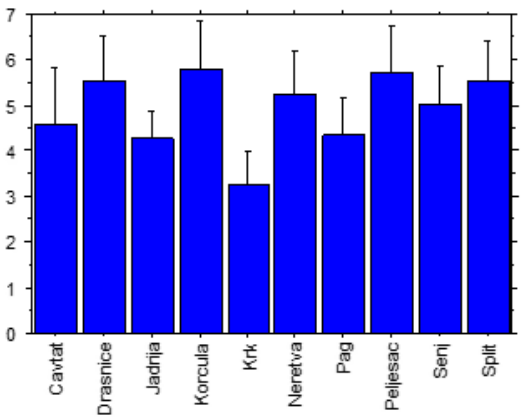
Materials and methods

The morphometric characterization of the 9 Italian wild sea fennel populations was done according to the procedure detailed in Annex I (see the end of this deliverable). Such a procedure has been agreed and shared among all partners involved into WP3 activities using one drive.

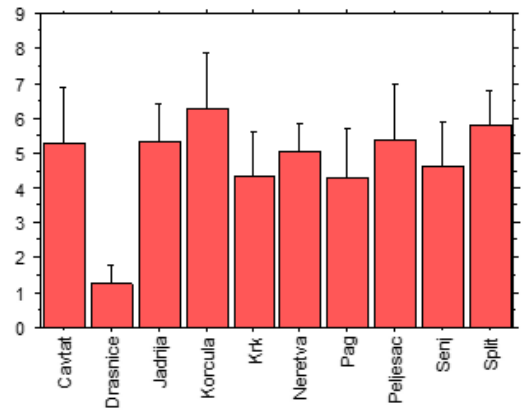
Results



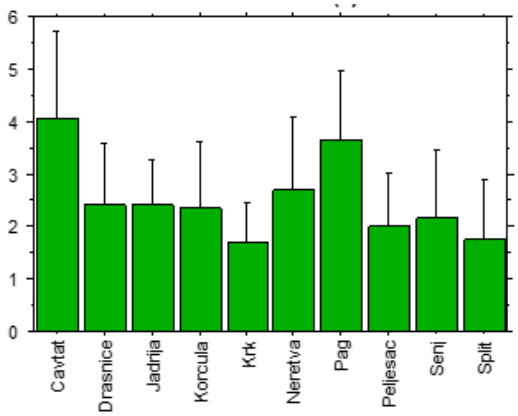
Plant height (cm)



Stem diameter (mm)



Number of branches



Number of umbells

Seed traits of Croatian populations are reported in the following table.

Location	Weight of 100 seeds (g)	Seed lenght (SL; mm)	seed width (SW; mm)	SL/SW	coefficient form
Cavtat	0,19	2,73 (2,05-3,70)*	1,17 (0,90-1,48)	2,33	0,73
Drašnice	0,124	2,89 (2,16-3,70)	1,21 (0,93-1,49)	2,39	0,72
Korčula	0,08	2,69 (2,10-3,57)	1,09 (2,10-3,45)	2,47	0,7
Krk	0,091	2,61 (2,10-3,43)	1,09 (0,77-1,45)	2,39	0,72
Neretva	0,16	3,43 (2,57-4,58)	1,19 (0,90-1,53)	2,88	0,63
Pag	0,133	2,90 (2,25-3,84)	1,20 (0,92-1,57)	2,42	0,71
Prapatno	0,18	3,04 (2,25-4,02)	1,21 (0,90-1,61)	2,51	0,69
Senj	0,125	2,94 (2,33-3,59)	1,12 (1,48-0,74)	2,63	0,67
Šibenik	0,135	3,08 (2,22-4,08)	1,24 (0,99-1,55)	2,48	0,7
Split (Žnjan)	0,174	2,74 (2,08-3,89)	1,30 (1,04-1,69)	2,11	0,77

*Values in brackets are min-max variations

2.2 Chemical analyses

The plant material for the determination of carotenoids, tocopherols, phenolic compounds, and fatty acids was freeze-dried before extraction of the target compounds, whereas the plant material for the isolation of essential oils was air-dried to avoid loss of volatiles.

A) Sea fennel freeze drying

B) Sea fennel air drying

2.2.1 Carotenoids

Materials and methods

The extraction of carotenoids from sea fennel was done according to Nartea et al. (2021) with slight modifications. Briefly, 100 mg of the sample was extracted in 5 mL of acetone, stored at 4 °C for 15 min and centrifuged at 1370 rpm for 10 min; the whole procedure was repeated again. Afterwards, the sample was filtered through a 0.45 µm PVDF filter, and the solvent evaporated under nitrogen. Prior to analysis, the dried residue was re-suspended in 0.5 mL acetone for the determination of β-carotene and in 80% methanol for the determination of neoxanthine, violaxantine and lutein.

The analysis was performed by Thermo-scientific ultrahigh performance liquid chromatography coupled with UV-VIS DAD detector (Ultimate 3000RS, Thermo Fisher Scientific, Waltham, Massachusetts, SAD), on a reversed phase column (Halo C30; 150 x 3 mm, 2.7 µm; Advanced Materials Technology, DE, USA). Carotenoids were separated by gradient elution with solvent A (1 % acetic acid in water), solvent B (MeOH) and solvent C (ACN) as follows: 70 % C isocratically for 0.2 min; 0.2-8 min to 100 % C; 8-9 min to 30 % B and 70 % C; 9-17 min 30 % B and 70 % C. The total analysis time was 17 min, including 0.1 min for start conditions and 4.9 min for re-equilibration. The carotenoids in the sample extracts were monitored at 415, 425 and 450 nm, the flow rate was set to 1 mL/min and 10 µL of the sample was injected into the system. Peaks were identified by comparing their retention times with those of authentic standards and spiked samples using standard solutions. Calibration curves for neoxanthine, violaxantine and lutein were constructed using 6 calibration points in the concentration range 0.05-20 µg/mL from standard solutions using methanol/water (80:20, v/v), and for β-carotene from 0.05-5 µg/mL by diluting the standard solution in acetone. Cromeleon software was used to collect, record, process and integrate the data. Sample concentrations were expressed as mg of compound tested per gram of dry extract (mg/g).

Results

Carotenoid profiles (mg/g) of Croatian sea fennel leaves from different locations along the Adriatic coast are shown in the following table.

The most abundant compounds in all tested samples, collected from all locations, was xanthophyll lutein (107.1-435.4 mg/kg), followed by β-carotene (16.8-80.2 mg/kg) and neoxanthin (0.5-8.4 mg/kg). Sea fennel samples from south locations showed higher abundance of all quantified carotenoids: the sample from peninsula Pelješac had the highest amount of lutein and neoxanthin, while β-carotene was the most abundant in this sample and sample from the southernmost location of Cavtat. Sea fennel samples from Split had generally showed the lowest carotenoid content among all tested locations, with the absence of neoxanthin.

Compound (mg/kg)	Krk	Senj	Pag	Šibenik	Split	Dražnice	Korčula	Pelješac	Neretva	Cavtat
Neoxantin	-	-	-	0.77±0.01	-	0.68±0.02	3.96±0.48	8.40±0.73	-	0.54±0.04
Lutein	249.97±0.08	127.67±0.21	147.30±2.81	244.84±0.97	107.14±2.01	178.37±0.46	273.94±1.74	435.38±0.00	151.38±0.21	236.27±0.66
β-carotene	51.02±0.01	23.37±0.05	23.47±0.22	54.22±0.65	16.81±0.04	35.40±0.75	74.64±0.34	79.59±0.58	20.96±0.17	80.21±0.26

Reference

Nartea, A.; Fanesi, B.; Falcone, P.M.; Pacetti, D.; Frega, N.G.; Lucci, P. Impact of Mild Oven Cooking Treatments on Carotenoids and Tocopherols of Cheddar and Depurple Cauliflower (Brassica oleracea L. var. botrytis). Antioxidants 2021, 10, 196. https://doi.org/10.3390/antiox10020196

2.2.2 Tocopherols

Materials and methods

Tocopherols were isolated by direct acetone extraction and saponification as reported by Knecht et al. (2015). Ascorbic acid (1 g), sodium sulfate (0.1 g), ethanol (20 mL), and potassium hydroxide solution 60% (4 mL) were added to sea fennel leaf powder (0.4 g). The suspension was saponified in a water bath (at 85°C for 30 min), shaken from time to time, and cooled to room temperature. Then water was added (12 mL) and triplicate extraction with n-hexane (10 mL) was performed. The organic phases were pooled, washed four times with water (10 mL), dried by rotavapor at 35°C, and finally dissolved in n-hexane.

Samples were injected (20 µL) into the HPLC system (Series 200, Perkin Elmer, Walthamn, Massachusetts, USA) equipped with a fluorimetric detector (FLD Series 200, Perkin Elmer) and an Ultra-silica column (250×4.6 mm, 5 µm, Restek Corporation, USA). Linear elution was performed with solvent A (hexane) and solvent B (isopropanol) in ratio 96:4 at a flow rate of 0.8 mL/min. The temperature of the column was maintained at 25 ºC. Detection was performed using a fluorescence detector (excitation 290 nm and emission 330 nm). The α-, β-, and γ- tocopherols were identified based on the retention times of the standards. The calibration curve was used for α-tocopherol, while the concentrations of β-, and γ-tocopherol were reported relative to its concentration. The obtained results are expressed as mg of detected compound per kg of dry plant material (mg/kg). All analyses were performed in triplicate and the final results are expressed as the mean ± standard deviation.

Results

Tocopherols (mg/g) determined in Croatian sea fennel leaves from different locations along the Adriatic coast are shown in the following table.

Compound (mg/kg)	Krk	Senj	Pag	Šibenik	Split	Drašnice	Korčula	Pelješac	Neretva	Cavtat
α-tocopherol	38.25±0.45	14.98±0.33	13.31±0.14	9.68±0.18	18.66±0.81	23.29±1.01	37.43±0.43	53.91±0.99	22.76±0.08	27.26±0.28
β-tocopherol	5.18±0.00	4.70±0.13	3.95±0.08	10.11±0.23	5.15±0.46	5.56±0.49	6.40±0.16	2.22±0.04	9.17±0.21	4.44±0.04
γ-tocopherol	1.92±0.27	1.33±0.00	1.34±0.00	1.19±0.02	1.71±0.03	1.54±0.00	1.34±0.23	1.65±0.09	2.43±0.22	1.27±0.10

Analysis of sea fennel samples from different locations along the Croatian Adriatic coast identified α-, β- and γ-tocopherol isomers. α-Tocopherol was predominant in all samples except the sample from Šibenik, which is consistent with previous studies on halophyte species. The content of α-tocopherol was lowest in the sample from Šibenik (9.68 mg/kg) and highest in the sample from Pelješac (53.91 mg/kg). The content of β-isomer ranged from 2.22 mg/kg (Pelješac) to 10.11 mg/kg (Šibenik) and that of γ-isomer from 1.19 mg/kg (Šibenik) to 2.43 mg/kg (Neretva). It should be noted that the sea fennel from Šibenik has the lowest content of α-tocopherol, while the other two isomers were present in the highest amounts in this sample.

References

Knecht, K.; Sandfuchs, K.; Kulling, S.E.; Bunzel, D. Tocopherol and tocotrienol analysis in raw and cooked vegetables: A validated method with emphasis on sample preparation. Food Chem. 2015, 169, 20–27.

2.2.3 Phenolic compounds

Materials and methods

The homogenised, dry sea fennel samples (0.5 g) were extracted with methanol (80:20 v/v, 5 mL) by sonication (15 minutes at room temperature) followed by mixing in orbital shaker (3 h at room temperature). The samples were left overnight at +4 °C in the dark, filtered (Chromafil™ Xtra PTFE Syringe filters, 0.45 µm, Macherey-Nagel, Düren, Germany) and further used for the detection of the individual phenolic compounds.
High-performance liquid chromatographic (HPLC) analyses of the individual phenolic compounds were performed on Shimadzu Nexera HPLC system LC-40 (Shimadzu, Kyoto, Japan) equipped with a UV/VIS detector using Phenomenex C18 (250 mm×4.6 mm, 5 µm; Torrance, CA, USA) reverse-phase column. The mobile phase flow rate was 1.0 mL/min and the temperature was maintained at 35 C. The mobile phase (A) was 0.2% phosphoric acid in water while mobile phase (B) was methanol-acetonitrile (1:1, v/v). The elution started isocratically with 4% B, and then the gradient program was set as follows: 0-16 min (linear gradient up to 15% B), 16-50 min (linear gradient up to 35% B), 50-62 min (linear gradient up to 4% B), and 62-65 min (4% B). The initial conditions were established in 2 min and maintained for 10 min to equilibrate the column. Six-point standard curves were prepared by diluting the working standard solution with methanol/water (80:20, v/v). Calibration curves for neochlorogenic and 4-O-caffeoylqunic acid were prepared in a concentration range of 0.1-50 µg/mL, for chlorogenic acid from 5-500 µg/mL and for all other phenolics tested in a range from 0.1 to 10 µg/mL. Sample concentrations were expressed as mg of the tested compound per gram of dry extract (mg/g). The phenolic compounds were identified by comparing the retention times of the sample peaks and their absorbance spectra (at 220 and 320 nm) with those obtained for the standard compounds tested under the same conditions, while quantifications were performed using an external standard calibration curve. The presented results are obtained from two injections and are expressed as milligrams of compound per gram of dry plant material (mg/g) (as mean value ± standard deviation).

Results

Phenolic profiles (mg/g) of Croatian sea fennel leaves from different locations along the Adriatic coast are shown in the following table.

According to the results, chlorogenic acid (3-O-caffeoylquinic acid) and its derivatives, cryptochlorogenic acid (4-O-caffeoylquinic acid) and neochlorogenic acid (5-O-caffeoylquinic acid), were the predominant phenolic compounds in the sea fennel samples studied, which is in agreement with our previous reports on Croatian sea fennel samples, as well as with studies by other authors on samples from other Mediterranean countries. The highest content of chlorogenic acid was determined in the Korčula sample (248.44 mg/g), followed by the Neretva sample (154.65 mg/g). High concentrations (over 80 mg/g) were also found in the samples collected from Pelješac and Cavtat, so it can be concluded that all samples from the southern areas were extremely rich in this compound. All other samples contained significantly lower amounts of chlorogenic acid (between 24.65 and 48.38 mg/g), with the exception of samples collected from island of Krk were the presence of a higher amount of chlorogenic acid was confirmed (81.15 mg/g).

	Krk	Senj	Pag	Jadrija	Split	Drašnice	Korčula	Pelješac	Neretva	Cavtat
Gallic acid	0.02±0.000	0.05±0.00	0.04±0.00	0.09±0.00	0.01±0.00	0.03±0.00	0.03±0.00	0.02±0.00	0.03±0.00	0.01±0.00
Protocatechuic acid	0.16±0.001	0.23±0.01	0.04±0.00	0.37±0.18	0.17±0.00	0.12±0.04	0.23±0.00	0.10±0.00	0.14±0.01	0.09±0.00
Neochlorogenic acid	7.47±0.297	4.26±0.01	2.96±0.01	3.85±0.01	4.27±0.00	2.63±0.08	30.05±0.02	9.71±0.08	9.54±0.00	5.57±0.02
p-Hydroxybenzoic acid	1.30±0.148	0.90±0.01	0.80±0.01	1.56±0.00	1.81±0.00	1.40±0.14	3.36±0.00	1.55±0.02	0.91±0.00	0.59±0.02
Chlorogenic acid	81.15±0.274	48.38±0.02	32.40±0.01	43.93±0.00	27.04±0.00	24.65±0.00	248.44±0.05	97.08±0.69	154.65±0.00	82.04±0.02
4-O-Caffeoylquinic acid	10.43±0.03	6.73±0.01	4.70±0.00	6.05±0.00	5.94±0.14	4.35±0.00	42.06±0.02	13.67±0.11	15.93±0.01	8.92±0.07
Caffeic acid	0.09±0.00	0.37±0.30	0.20±0.00	0.06±0.00	0.02±0.01	0.06±0.00	0.09±0.02	0.73±0.01	0.27±0.00	0.50±0.03
Ferulic acid	0.57±0.00	0.47±0.04	0.25±0.0	0.46±0.00	0.27±0.00	0.38±0.10	1.42±0.13	0.72±0.10	0.46±0.07	0.53±0.00
Sinapic acid	0.09±0.00	0.22±0.04	0.10±0.00	0.37±0.01	0.12±0.00	0.20±0.10	0.59±0.13	0.35±0.05	0.22±0.07	0.05±0.00
Rutin	2.11±0.07	9.04±0.01	2.04±0.03	2.69±0.04	1.49±0.04	2.36±0.03	3.65±0.05	2.34±0.10	3.12±0.04	6.78±0.07

2.2.4 Fatty acids

Materials and methods

The analysis of fatty acids in sea fennel samples was performed through the analysis of FAMEs prepared by methylation. The lipids were extracted from dry sea fennel samples (1 g) using 2-propanol (4 mL) and by heating (at 80°C for 15 min) the suspensions. Then, hexane (6 mL) and sodium sulphate (6.7 %, w/v, 5 mL)

were added, and the suspension was shaken vigorously. After the centrifugation (3000 rpm, 3 min) and the upper phase was transferred to clean tube. The aqueous phase was extracted again with hexane: 2-propanol mixture (7:2 v/v, 7.5 mL), and the upper phase was combined with the previously one. After removing solvent, the fatty acids methylation was performed by the addition methanol: toluene: sulphuric acid (88:10:2 v/v/v, 3 mL) and sample heating (at 80 °C during 1 h). The FAMEs were extracted from the samples cold to the room temperature using heptane (2×1 mL) and analysed by GC-FID.

The prepared FAMEs were analysed using a gas chromatograph (model 3900; Varian Inc., Lake Forest, CA, USA) equipped with flame ionization detector and an RTX 2330 capillary column (30 m, 0.25 mm, 0.2 µm; Restek Corp., Bellefonte, PA, USA) by the injection of 1 µL of the sample. The split ratio was 1:50 and helium (flow rate 2 mL/min) was used as carrier gas. The oven temperature was 140 °C, held 4 min, raised to 210 °C at the rate 4 °C/min and held at 210 °C for 11 min. The total run time was 32,5 min. The injector and detector temperature was 250 °C. FAME set of standards was used for the identification of the compounds. Analyses were performed in duplicate, and the results are expressed as the mean of percentage of fatty acid (calculated as the ratio between FAME peak area and the total peaks area) ± standard deviation.

Results

Fatty acids profiles (%) of Croatian sea fennel populations from different locations along the Croatian coast are shown below.

Total of 21 fatty acids have been identified in different proportions; among them two polyunsaturated fatty acids (PUFAs), namely linoleic (C18:2n6) and linolenic acid (C18:3n3) and six monounsaturated fatty acids (MUFAs), namely pentadecenoic (C15:1n10), palmitoleic acid (C16:1n7), cis-7-hexadecenoic acid (C16:1n9), heptadecenoic acid (C17:1), oleic acid (C18:1), and eicosenoic acid (C20:1). All other detected compounds were saturated fatty acids (SFAs). From all detected compounds, only four of them were detected in high amount. The dominant SFAs in samples were palmitic acid (C16:0) with the amounts ranging from 19.0% in Senj sample to 22.4% in Pag sample, and stearic acid (C18:0) with concentrations from 15.8% in Neretva sample to 26.6% in Split sample. The content of linoleic acid ranged from 19.6 to 24.6% with the highest found in Korčula sample, while the content of linolenic acid ranged from 17.4 to 23.0%, with the highest found again in Korčula sample. Total SFAs of the samples ranged from 47.6 to 56.4%, MUFAs from 5.2 to 6.6% and PUFAs from 37.7 to 47.6%.

	Krk	Senj	Pag	Jadrija	Split	Drašnice	Korčula	Pelješac	Neretva	Cavtat
C8:0	0.1±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
C10:0	0.3±0.1	0.4±0.0	0.1±0.0	0.2±0.0	0.2±0.1	0.7±0.0	0.5±0.0	0.1±0.0	0.2±0.2	0.2±0.1
C11:0	0.1±0.0	0.2±0.0	0.2±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.2±0.0
C12:0	2.6±0.1	2.5±0.0	1.7±0.1	1.5±0.1	1.3±0.1	1.7±0.0	1.4±0.1	1.1±0.1	1.0±0.0	0.8±0.0
C13:0	0.7±0.0	0.8±0.0	0.7±0.0	0.6±0.0	0.6±0.0	0.6±0.0	0.5±0.0	0.6±0.0	0.7±0.0	0.8±0.0
C14:0	4.0±0.0	5.4±0.0	5.4±0.0	5.1±0.2	5.1±0.2	3.7±0.1	3.6±0.0	3.1±0.0	4.1±0.4	1.3±0.0
C15:0	0.3±0.0	0.3±0.0	0.4±0.0	0.3±0.0	0.4±0.1	0.3±0.0	0.2±0.0	0.2±0.0	0.4±0.0	0.3±0.1
C15:1	1.5±0.0	1.1±0.0	1.5±0.0	0.8±0.0	0.8±0.0	0.8±0.0	1.4±0.0	1.0±0.0	1.3±0.0	0.8±0.0
C16:0	21.0±0.0	19.0±0.3	22.3±0.2	21.0±0.1	22.1±0.2	20.3±0.1	22.5±0.1	20.7±0.1	21.3±0.2	22.1±0.1
C16:1 ω-7	1.5±0.0	1.1±0.0	1.4±0.0	1.3±0.0	1.4±0.0	1.2±0.0	1.5±0.0	1.5±0.0	1.3±0.0	1.9±0.1
C16:1 ω-9	0.0±0.0	0.2±0.3	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.1±0.0
C17:0	0.6±0.0	0.6±0.0	0.8±0.0	1.9±0.1	0.5±0.0	0.5±0.0	0.6±0.0	0.9±0.0	0.4±0.0	0.9±0.0
C17:1	0.1±0.0	0.1±0.1	0.2±0.0	0.1±0.0	0.2±0.0	0.3±0.2	0.1±0.0	0.1±0.0	0.3±0.0	0.3±0.0
C18:0	16.4±0.0	25.5±0.2	23.8±0.0	22.1±0.2	15.8±0.1	20.0±0.1	19.1±0.0	19.4±0.0	26.6±0.2	20.8±0.1
C18:1	2.8±0.0	2.9±0.0	2.4±0.0	2.5±0.0	3.0±0.0	4.1±0.0	2.9±0.0	2.4±0.0	2.0±0.0	2.1±0.0
C18:2	23.5±0.0	20.0±0.1	20.3±0.5	22.2±0.2	23.6±0.1	23.7±0.1	23.9±0.1	24.6±0.1	19.7±0.2	24.3±0.0
C20:0	0.1±0.0	0.1±0.0	0.1±0.0	0.0±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.1	0.1±0.0	0.0±0.0
C18:3	22.7±0.0	18.3±0.2	17.5±0.1	18.5±0.2	23.1±0.1	20.7±0.1	20.3±0.0	22.9±0.1	18.8±0.1	22.1±0.1
C20:1	0.3±0.0	0.3±0.1	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.2±0.0	0.3±0.0	0.2±0.0
C22:0	0.2±0.0	0.2±0.0	0.1±0.0	0.2±0.0	0.4±0.0	0.3±0.0	0.3±0.0	0.2±0.0	0.1±0.0	0.1±0.0
C24:0	1.2±0.0	0.9±0.0	0.8±0.0	1.0±0.1	1.0±0.1	0.7±0.1	0.6±0.1	0.6±0.0	1.2±0.1	0.5±0.0

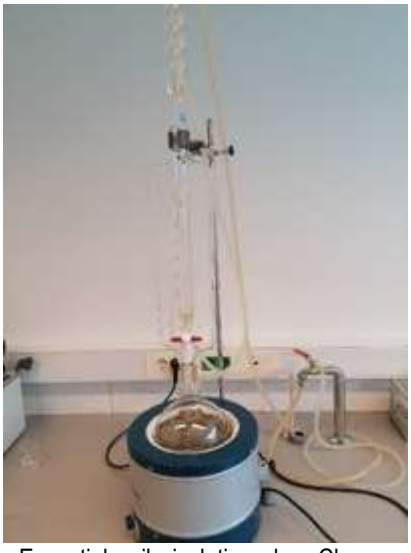
2.2.5 Volatile organic compounds

Materials and methods

The dry sea fennel leaves were hydrodistilled in a Clevenger apparatus for 3 hours. The isolated essential oils were taken up in pentane/diethyl ether (1:1), dried over anhydrous sodium sulphate and stored at -4 °C until analysis.

Separation and analysis of the components of the sea fennel essential oils were performed at GC-MS using a gas chromatograph (model 8890 equipped with an automatic liquid injector model 7693A, and a tandem mass spectrometer (MS) model 7000D GC/TQ (Agilent Inc., Santa Clara, CA, USA) and equipped with a non-polar column HP-5MS UI (5% phenylmethylpolysiloxane, 30 m×0.25 mm, 0.25 µm, Agilent Inc.). Helium was used as the carrier gas at a flow rate of 1 mL/min. The temperature programme of the column was used as follows: 3 min at 60 °C and then heating to 246 °C at 3 °C/min, where it was kept isothermal for 25 min. The inlet temperature was 250 °C, the sample injection volume was 1 µL, and the split ratio was 1:50. MS the following conditions were used: Ion source temperature 200 °C, ionisation energy 70 eV, full-scan range (33-350 m/z). Individual peaks were identified by comparing their retention indices with the series of n-hydrocarbons and by computer matching of mass spectra with commercial databases (Wiley 7 MS library, Wiley, NY, USA) and NIST02 (Gaithersburg, MD, USA) and comparison of their mass spectra and retention indices with literature data (Adams, 2017). All analyses were performed in triplicate. The percentages of the identified compounds were calculated as the mean ± standard deviation.

A) Plant material drying



B) Essential oil isolation by Clavenger apparatus

Results

EO components (%) of Croatian sea fennel leaves from different locations along the Adriatic coast are shown below.

A total of 35 constituents were found during the chemical analysis of the chemical composition of the sea fennel leaves EOs collected from various locations along the Adriatic coast, of which the most abundant compounds belong to the group of monoterpenes and monoterpenoids (Table 2). Sesquiterpenes and phenolic compounds are present in lower amounts. Limonene was identified as the most abundant constituent of the

leaf oil (24.36 to 93.20%). The highest concentration of limonene was found in the essential oils of the samples collected on the southern and central Adriatic coast, in Neretva (93.20%), Split (79.13%), Korčula (79.44%) and Senj (71.07%), and the lowest in the southernmost region, in sample from Cavtat (24.36%). A high percentage of sabinene (0.80-31.18%) and a slightly lower percentage of γ-terpinene (0.38-8.74%) and terpinen-4-ol (1.55-18.96%) were also found. All investigated essential oils belong to the chemotype II since no dillapiole was detected in their chemical composition (Pateira et al., 1999), while according to Renna et al. (2017), they belong to the monoterpene hydrocarbon essential oil chemotype.

EO component	Krk	Senj	Pag	Šibenik	Split	Drašnice	Korčula	Pelješac	Neretva	Cavtat
α-thujene	0.19±0.01		0.29±0.10	0.43±0.05				0.54±0.01		
α-pinene	0.88±0.02	0.38±0.12	0.51±0.14	0.47±0.06				2.84±0.12	0.49±0.00	0.47±0.04
sabinene	18.66±1.89	16.48±3.79	23.74±1.95	31.81±2.81	0.80±0.10	18.21±1.51	3.07±0.83	15.89±2.10	3.39±1.63	1.24±0.14
ß-pinene		0.16±0.01		0.12±0.01						
octanal			0.29±0.01	0.11±0.01	0.69±0.01	0.32±0.02		0.99±0.11		0.49±0.06
α-terpinene	1.2±0.32	0.87±0.31	1.45±0.01	2.01±0.47		0.56±0.02		1.60±0.13		
p-cymene	1.34±0.57	0.1±0.01	1.40±0.271	0.86±0.30		3.76±0.45	5.00±2.69	4.30±0.25		0.15±0.04
limonene	63.10±8.92	71.07±10.93	39.71±6.35	35.29±4.81	79.13±2.10	44.81±1.94	79.44±9.07	45.32±5.13	93.20±2.90	24.36±2.35
(E)-ß-ocimene	1.13±0.37	3.32±1.80	0.30±0.02	0.12±0.01	0.42±0.09		0.79±0.31	0.34±0.05	0.46±0.01	
Benzeneacetaldehyde			0.10±0.01	0.13±0.01		0.63±0.03		0.39±0.01		1.58±0.22
γ-terpinene	5.41±1.42	2.69±1.26	6.64±1.13	6.00±0.99	0.55±0.11	5.43±0.58	8.74±3.22	7.81±1.40	0.89±0.50	0.38±0.05
cis-sabinene hydrate	0.19±0.01		0.87±0.02	0.78±0.245		±		0.23±0.01		0.56±0.06
terpinolene	0.48±0.08	0.13±0.01	0.65±0.02	0.73±0.16		0.29±0.02		0.60±0.02		
linalool	0.19±0.01		0.99±0.29	0.83±0.31				0.37±0.00		0.68±0.05
cis-p-mentha-2,8-dien-1-ol			0.22±0.02	0.10±0.01	0.62±0.07			0.25±0.03		0.33±0.02
trans-p-menth-2-en-1-ol	0.29±0.01		0.93±0.24	0.84±0.29	1.40±0.40	1.30±0.19	0.21±0.01	0.75±0.25		10.32±2.08
cis-verbenol			0.63±0.14	0.09±0.01	0.90±0.20	0.51±0.02		0.23±0.01		6.29±0.31
trans-verbenol	0.22±0.01			0.50±0.23	1.04±0.04	0.82±0.07	0.22±0.01	0.88±0.09		2.12±0.55
pinocarvone			0.09±0.09	0.09±0.01						
terpinen-4-ol	5.75±2.60	4.12±3.04	18.96±4.64	17.07±2.38	1.55±0.05	15.69±1.69	2.30±1.36	13.19±2.01	1.56±1.10	2.35±0.15
isocarveol				0.08±0.01	1.14±0.07	0.45±0.04				6.44±1.77
α-terpineol	0.22±0.01	0.52±0.38	0.69±0.02	0.47±0.18	1.63±0.04	1.33±0.68	0.21±0.01	0.96±0.06		3.64±0.19
myrtenol			0.13±0.13	0.20±0.01	0.89±0.04	0.55±0.02		0.23±0.01		2.69±0.26
trans-carveol			0.10±0.10	0.10±0.01	1.82±0.10	0.94±0.05		0.33±0.02		7.82±0.98
cis-carveol				0.11±0.01		0.36±0.03				
verbenone			0.12±0.12	0.11±0.02	1.57±0.21	0.64±0.04		0.25±0.03		12.01±1.91
trans-chrysanthenyl acetate			0.20±0.20	0.16±0.02		0.44±0.07				
carvone					0.47±0.01					2.08±0.21
carvacrol						0.41±0.02				
myrtenyl acetate			0.11±0.11	0.10±0.01	0.64±0.01	0.37±0.05		0.34±0.04		
10-(acetylmethyl)-3-carene			0.11±0.11	0.09±0.01	0.91±0.03	0.61±0.03		0.38±0.01		1.82±0.36
ß-longipinene				0.09±0.01	0.82±0.01	0.52±0.03		0.30±0.01		1.05±0.17
cuparene					0.66±0.01					1.72±0.16
ß-vatirenene					0.63±0.02					
spathulenol			0.08±0.08			0.55±0.07		0.49±0.05		1.63±0.40
TOTAL	99.25	99.84	99.31	99.89	98.28	99.50	99.98	99.80	99.99	94.84

EOs of Croatian sea fennel flowers (%) from different locations along the Adriatic coast

The EOs from sea fennel flowers were extracted by hydrodistillation and subjected to GC-MS analysis which resulted in 16 identified volatile organic compounds (VOCs). The detected compounds belong to monoterpenes and monoterpenoids. Limonene was the predominant compound in EOs from flowers from all locations and constituted from 50.82% (Pag) to 97.3% (Neretva). The lowest content of limonene was found in EOs from the sea fennel flowers collected in the locations Pag and Šibenik, but those samples contained the highest amounts of sabinene. Its content in EOs of the flowers from the southern locations was quite low (0.3 to 3.5%). Significant amounts of γ-terpinene were found in the EOs from the flowers from the island of Pag (7.08%) and the island of Korčula (8.93%), These results showed variability in the EO profiles depending on the sites where the plants were collected from

EO component	Krk	Senj	Pag	Šibenik	Split	Drašnice	Korčula	Pelješac	Neretva	Cavtat
α-thujene			0.12±	0.36±0.15						
α-pinene	1.81±0.59	2.61±0.96	3.17±0.93	0.88±0.08	0.91±0.04	1.13±0.07	1.90±0.33	3.01±1.46	1.32±0.34	1.80±0.72
sabinene	18.87±2.66	16.62±2.42	31.43±1.24	31.73±4.47	1.65±0.33	5.05±2.83	0.68±0.15	3.46±2.13	0.75±0.22	0.32±0.01
ß-pinene			0.11±0.01							
ß-myrcene		0.25±0.01	0.15±0.01	0.33±0.01						
octanal					0.37±0.01					
α-terpinene	0.60±0.13	0.25±0.01	1.12±0.42	0.84±0.14						
p-cymene			0.67±0.27	0.54±0.10		0.94±0.25	1.34±0.21	0.66±0.01		
limonene	72.37±6.43	72.94±6.73	50.82±4.82	55.22±4.96	93.39±1.29	87.86±4.54	85.92±4.32	85.42±7.88	97.31±0.84	96.78±0.37
(Z)-ß-ocimene				0.44±0.01			0.09±0.01			
(*E*)-ß-ocimene	1.30±0.58	2.89±1.06	0.17±0.01	0.77±0.05	3.03±1.18	0.42±0.01	1.12±0.57	1.57±0.84	0.61±0.28	
γ-terpinene	2.27±0.90	2.43±0.86	7.08±1.43	2.79±0.43		2.83±1.09	8.93±3.14	5.54±2.45		0.51±0.02
cis-sabinene hydrate			0.19±0.01	0.31±0.05						
terpinolene			0.19±0.01	0.36±0.08						
linalool			0.16±0.01							
terpinen-4-ol	2.77±1.56	2.00±0.93	4.58±1.90	4.77±1.50		0.6±0.01		0.33±0.01		
TOTAL	99.99	99.99	99.96	99.64	99.99	99.28	99.98	99.99	99.99	99.41
Yield %, w/w	0.71	1.31	1.87	0.59	0.40	0.23	0.99	0.92	0.85	0.19

VOCs of Croatian sea fennel seeds (%) from different locations along the Adriatic coast

The aromatic or volatile organic compounds (VOCs) of the pickled sea fennel were extracted via headspace–solid-phase microextraction (HS-SPME) and detected via GC-MS. The analysis resulted in 30 identified compounds (15 compounds in Neretva sample). Limonene was again the predominant compound in samples from all locations with the amounts ranged from 35 to 80%. Among other compounds significant amounts of α-pinene and p-cymene were detected, as well as sabinene which was present in higher amounts in samples from north. Again, variability in the VOCs profiles depending on the sites where the plants were collected from can be established.

Compound	RI	Krk	Senj	Pag	Šibenik	Split	Dražnice	Korčula	Neretva	Pelješac	Cavtat
Heptanal	901	0.12±0.02	1.44±0.02	0.39±0.20	0.93±0.15	-	1.24±0.18	0.56±0.03	0.45±0.05	0.41±0.08	0.72±0.05
α-Thujene	931	2.14±0.35	2.60±0.77	3.10±0.77	3.06±0.05	-	0.86±0.18	1.75±0.73	-	0.55±0.12	0.68±0.03
α-Pinene	936	10.49±2.75	18.74±3.23	11.97±0.15	11.57±1.67	9.09±0.72	9.24±1.36	10.90±0.37	3.03±0.15	11.47±1.83	10.54±0.66
Camphene	942	0.05±0.01	0.64±0.10	0.13±0.07	-	0.40±0.03	0.13±0.02	0.31±0.01	0.22±0.01	tr	0.12±0.01
Sabinene	975	10.06±0.75	4.34±0.68	13.85±2.33	10.03±0.00	2.19±0.25	1.53±0.24	6.50±2.20	-	1.22±0.31	1.75±0.04
α-Terpinene	1001	1.45±0.30	0.96±0.05	1.73±0.43	0.35±0.06	tr	tr	tr	-	-	tr
Octanal	1003	1.54±0.22	5.14±0.04	2±0.38	4.43±0.79	0.95±0.40	5.01±0.91	2.03±0.39	2.87±0.51	2.11±0.26	3.42±0.17
p-Cymene	1025	10.76±0.36	5.90±0.20	3.65±0.91	3.64±0.35	0.97±0.23	4.68±0.05	20.55±4.92	-	13.07±2.33	13.02±0.1
Limonene	1030	46.86±3.40	35.01±3.64	53.05±6.37	52.32±3.08	76.02±1.82	66.27±3.13	45.82±7.02	80.51±3.89	54.74±1.44	53.80±3.34
β-Ocimene	1040	0.59±0.14	tr	0.89±0.40	tr	1.74±0.31	1.70±0.28	-	-	-	-
γ-Terpinene	1064	11.15±0.94	1.88±0.01	3.60±0.89	2.43±0.09	-	1.02±0.32	5.87±1.28	-	9.22±0.29	2.99±0.59
(Z)-Sabinene hydrate	1069	0.54±0.02		1.56±0.28	2.43±0.58	-	0.68±0.16	0.78±0.10	-	0.38±0.07	0.66±0.13
α-Terpinolene	1086.	0.81±0.14	2.83±0.05	-	0.94±0.30	-	-	0.13±0.01	-	-	-
(E)-Sabinene hydrate	1097	0.54±0.06	tr	1.02±0.19	2.36±0.75	-	0.76±0.02	0.34±0.02	-	0.31±0.07	0.60±0.18
(E)-p-Mentha-2,8-dien-1-ol	1122	0.70±0.14	2.48±0.38	0.77±0.12	1.04±0.28	0.83±0.01	1.02±0.28	0.69±0.13	1.83±0.31	0.6±0.13	2.10±0.54
α-Campholenal	1125	tr	2.09±0.25	0.29±0.04	0.34±0.09	0.85±0.02	0.29±0.08	0.10±0.02	tr	tr	-
(Z)-p-Mentha-2,8-dien-1-ol	1137	-	1.27±0.14	tr	0.54±0.20	0.69±0.33	0.70±0.01	0.44±0.04	1.48±0.45	0.34±0.04	1.23±0.29
(E)-Limonene oxide	1138	-	-	tr	tr	1.37±0.67	-		0.58±0.17	-	-
Sabina ketone	1159	0.42±0.05	1.06±0.01	0.82±0.15	1.45±0.46	-	0.76±0.01	0.43±0.01	-	-	0.12±0.04
(E)-2-Nonenal	1163	0.38±0.09	0.56±0.00	-	1.06±0.45	-	0.96±0.09	0.41±0.00	0.66±0.22	-	0.37±0.12
Borneol	1164	tr	0.93±0.02	tr	tr	-	tr	0.31±0.02	tr	0.22±0.03	tr
Terpinen-4-ol	1176	0.29±0.06	0.81±0.02	0.27±0.04	0.40±0.11	-	0.35±0.09	0.31±0.02	-	0.27±0.03	tr
(Z)-p-Mentha-1(7),8-dien-2-ol	1189	-	1.20±0.10	-	-	1.09±0.66	-	-	0.60±0.16	0.3±0.01	1.12±0.36
α-Terpineol	1192	-	2.21±0.02	tr	-	-	-	-	0.94±0.18	0.73±0.15	1.17±0.28
(E)-Carveol	1220	tr	1.56±0.32	tr	tr	0.10±0.06	0.64±0.15	tr	1.40±0.28	0.63±0.15	1.22±0.34
(Z)-Carveol	1224	tr	1.26±0.05	tr	tr	0.10±0.06	0.21±0.05	tr	0.48±0.11	0.32±0.07	0.55±0.17
(E)-p-Mentha-1(7),8-dien-2-ol	1230	-	0.90±0.14	-	-	-	-	tr	-	0.17±0.05	-
Methyl thymyl ether	1236	-	-	-	-	-	-	tr	-	1.09±0.39	tr
Carvone	1248	0.22±0.03	0.62±0.02	-	-	0.80±0.48	0.80±0.21	-	2.13±0.33	0.24±0.07	1.25±0.29
(E)-2-Decenal	1265	0.43±0.20	-	-	-	-	-	-	-	-	-
Isobornyl acetate	1289	0.39±0.09	0.34±0.02	0.21±0.04	tr	0.81±0.49	-	-	0.11±0.02	0,5±0.13	0.56±0.1
Total chromatogram identified %		99.88	96.68	99.29	99.30	97.98	98.87	98.21	97.26	98.84	97.94

2.3 Molecular analyses

2.3.1 DNA extraction

For genomic DNA extraction, approximately 10 gr of leaves from 10 individuals of each sampled population listed below was taken

Region	Location	Code
South Dalmatia	Cavtat	Cav
South Dalmatia	Korčula	Kor
Central Dalmatia	Split	St
Central Dalmatia	Šibenik	Šib
North Dalmatia	Senj	Senj

North Dalmatia	Krk	Krk

The leaves were dried in silica gel upon their collection. Once the tissue was completely dry, we extracted DNA from 60 mg of powdered dried leaf tissue using DNeasy Plant Pro Kit by Qiagen, following the manufacturer instructions (with some slight changes) to obtain higher amount of high molecular weight DNA, as required for next generation sequencing (NGS). The process of DNA grinding and extraction is shown below.

Tubes containing extracted DNA in elution buffer

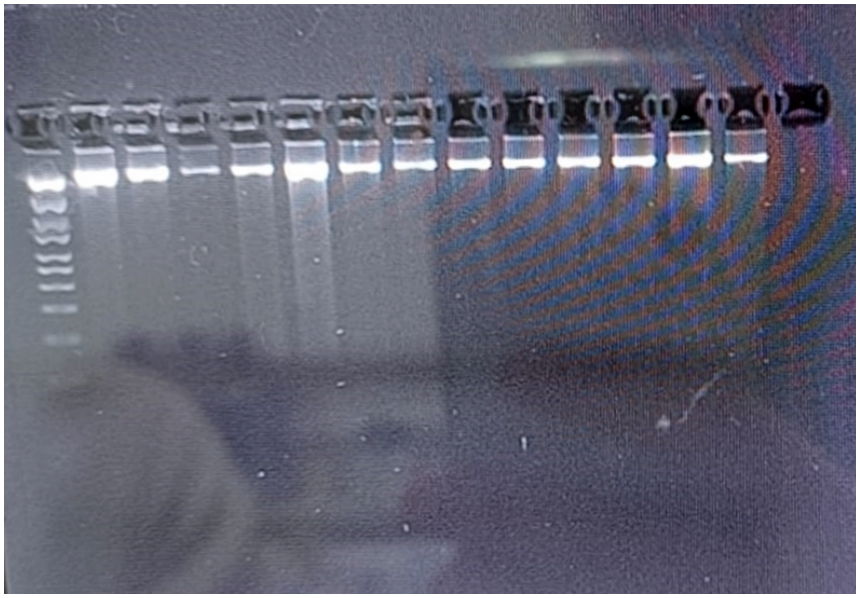
The quantity and purity of the DNA extracts were assessed spectrophotometrically. The quantity of DNA was analysed by Nanodrop and purity values of 260/280 were recorded.

Sample	Nanodrop (ng/µL)	260/280
CAV1	135,3	1,81
CAV2	86,8	1,79
CAV3	35,5	1,96
CAV4	41,2	1,79
CAV5	90,1	1,87
CAV6	39,3	1,76
CAV7	70,7	1,77

CAV8	53,4	1,66
CAV9	40,4	1,96
CAV10	38	1,72
KOR1	35,2	1,79
KOR2	42,7	1,73
KOR3	48,7	1,83
KOR4	50	1,79
KOR5	37,1	1,78
KOR6	123,9	1,66
KOR7	46,3	1,85
KOR8	41,8	1,74
KOR9	62,3	1,8
KOR10	50	2,17
SPL1	139,3	1,82
SPL2	71,6	1,72
SPL3	81,7	1,76
SPL4	37,3	1,83
SPL5	47,2	1,85
SPL6	57,6	1,84
SPL7	77,4	1,63
SPL8	205,5	1,81
SPL9	34,9	1,76
SPL10	106,8	1,82
ŠIB1	47,4	1,71
ŠIB2	42,1	1,81
ŠIB3	54,7	1,82
ŠIB4	60,9	1,7
ŠIB5	54,6	1,83
ŠIB6	104,9	1,78
ŠIB7	35,7	1,71
ŠIB8	35,2	1,73
ŠIB9	34,9	1,64
ŠIB10	36,2	1,75
PAG1	48,6	1,82
PAG2	61	1,75
PAG3	35,6	1,83
PAG4	61	2,22
PAG5	42,4	1,78
PAG6	40	2,35
PAG7	68,3	1,81
PAG8	37	1,65
PAG9	35,1	1,77

PAG10	96,1	1,65
KRK1	103,2	1,79
KRK2	130,9	1,79
KRK3	83,1	1,76
KRK4	66,4	1,8
KRK5	69,7	1,77
KRK6	58,3	1,96
KRK7	107,2	1,8
KRK8	44,6	1,83
KRK9	41,2	1,78
KRK10	59,6	1,78

In order to check the integrity of DNA, the extracts were analysed by electrophoresis on agarose gel.



2.3.2 Sequencing

The sequencing was done by Biomarker Technologies (BMK) Company (Munster, Germany), following SLAF-seq methodology.

Specific locus amplified fragment sequencing (SLAF-seq) is an optimized version of ddRADseq, specifically intended for large-scale genotyping experiments. The enzymes and the sizes of the restriction fragments are optimized with training data to ensure even distribution and avoid repeats. The fragments are also selected over a tight range, to optimize the PCR reaction. The protocol is similar to ddRAD, with a first digestion with a restriction enzyme (HaeIII), heat inactivation and a second digestion with an other enzyme (Hpy166II).

Then, the ATP and dual-index sequencing adapter were added at the 3' and 5' end of the digested DNA products, respectively. PCR was performed and the products were purified using E.Z.N.A.H Cycle Pure Kit (Omega). The purified products were mixed and incubated with these two restricted enzymes again. After

ligation of ATP, and Solexa adapter in the pair-end, the reaction products were purified using a Quick Spin column (Qiagen, Venlo, Netherlands), and segregated on a 2% agarose gel. Fragments with xx-xx bp were isolated using a Gel Extraction Kit (Tiangen). These SLAFs were subjected to PCR to add barcode. The PCR products were re-purified and then prepared for paired-end sequencing on an Illumina HiSeq sequencing platform (Illumina, San Diego, CA,USA).

Data analysis

The raw reads were further processed with a bioinformatic pipelinetool, BMKCloud(www.biocloud.net) online platform.

Quality control

Raw data (raw reads) of fastq format were firstly processed through fastp software. In this step, clean data(clean reads) were obtained by removing reads containing adapter, reads containing ploy-N and low quality reads from raw data. At the same time, Q30, GC-content of the clean data were calculated. All the downstream analyses were based on clean data with high quality.

Reads mapping to the reference genome

The adaptor sequences and low-quality sequence reads were removed from the data sets. Raw sequences were transformed into clean reads after data processing. These clean reads were then mapped to the reference genome sequence. bwa soft were used to map with reference genome.

SNP/INDEL Calling

The SNP/INDEL calling was performed using GATK(v3.8 McKenna et al.,2010) and SAMtools packages (Li et al., 2009) (v1.9.1). A total of 448436 SNPs with a minor allele frequency (MAF) and integrity was retained.

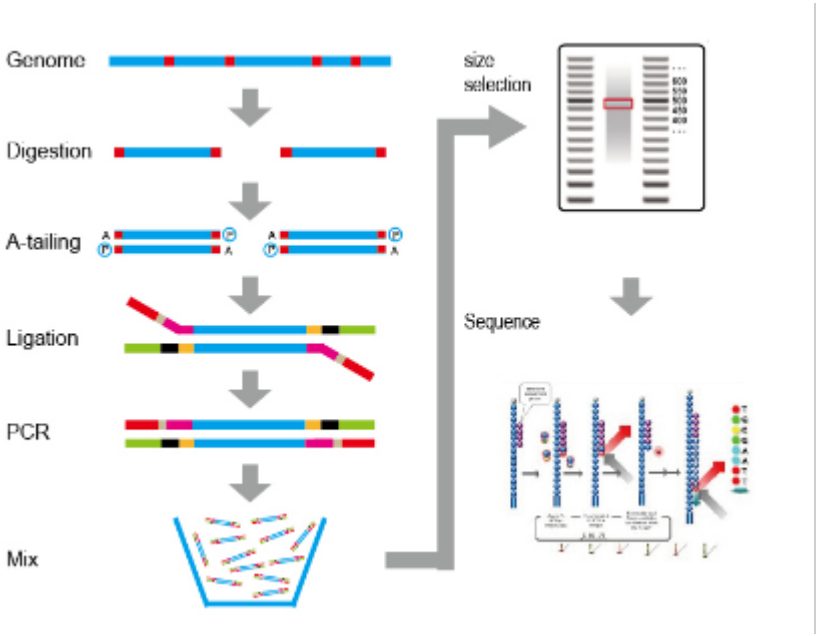
SNP/INDEL Annotation

SNP annotation was performed on the basis of the reference genome using snpEff software(3.6c (build 2014-05-20)) (Cingolani et al., 2012), and SNPs were categorized into intergenic regions, upstream or downstream regions, and exons or introns. SNPs in coding exons were further classified as synonymous SNPs or nonsynonymous SNPs. InDels in exons were grouped according to whether they led to a frameshift.

Gene functional annotation

Gene function was annotated based on the following databases: Nr (NCBI non-redundant protein sequences);Pfam (Protein family); KOG/COG (Clusters of Orthologous Groups of proteins); Swiss-Prot (A manually annotated and reviewed protein sequence database); KO (KEGG Ortholog database); GO (Gene Ontology).

This methodology allows: i) deep sequencing for genotyping accuracy; ii) reduced representation strategy to reduce sequencing costs; iii) pre-designed reduced representation scheme to optimize marker efficiency; iv) double barcode system for large populations



Methodology used for sequencing

References

Sun, X., Liu, D., Zhang, X., Li, W., Liu, H., Hong, W., ... & Zheng, H. (2013). SLAF-seq: an efficient method of large-scale de novo SNP discovery and genotyping using high-throughput sequencing. PloS one, 8(3), e58700.

Wilkie, P., Poulsen, A. D., Harris, D., & Forrest, L. L. (2013). The collection and storage of plant material for DNA extraction: the teabag method. Gardens' Bulletin Singapore, 65(2), 231-234.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., ... & 1000 Genome Project Data Processing Subgroup. (2009). The sequence alignment/map format and SAMtools. bioinformatics, 25(16), 2078-2079.

McKenna, A., Hanna, M., Banks, E., Sivachenko, A., Cibulskis, K., Kernytsky, A., ... & DePristo, M. A. (2010). The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome research, 20(9), 1297-1303.

Cingolani, P., Platts, A., Wang, L. L., Coon, M., Nguyen, T., Wang, L., ... & Ruden, D. M. (2012). A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. fly, 6(2), 80-92.

2.3.3 Analysis of sequences

See paragraph 6 for comparisons with the populations of the partner countries.

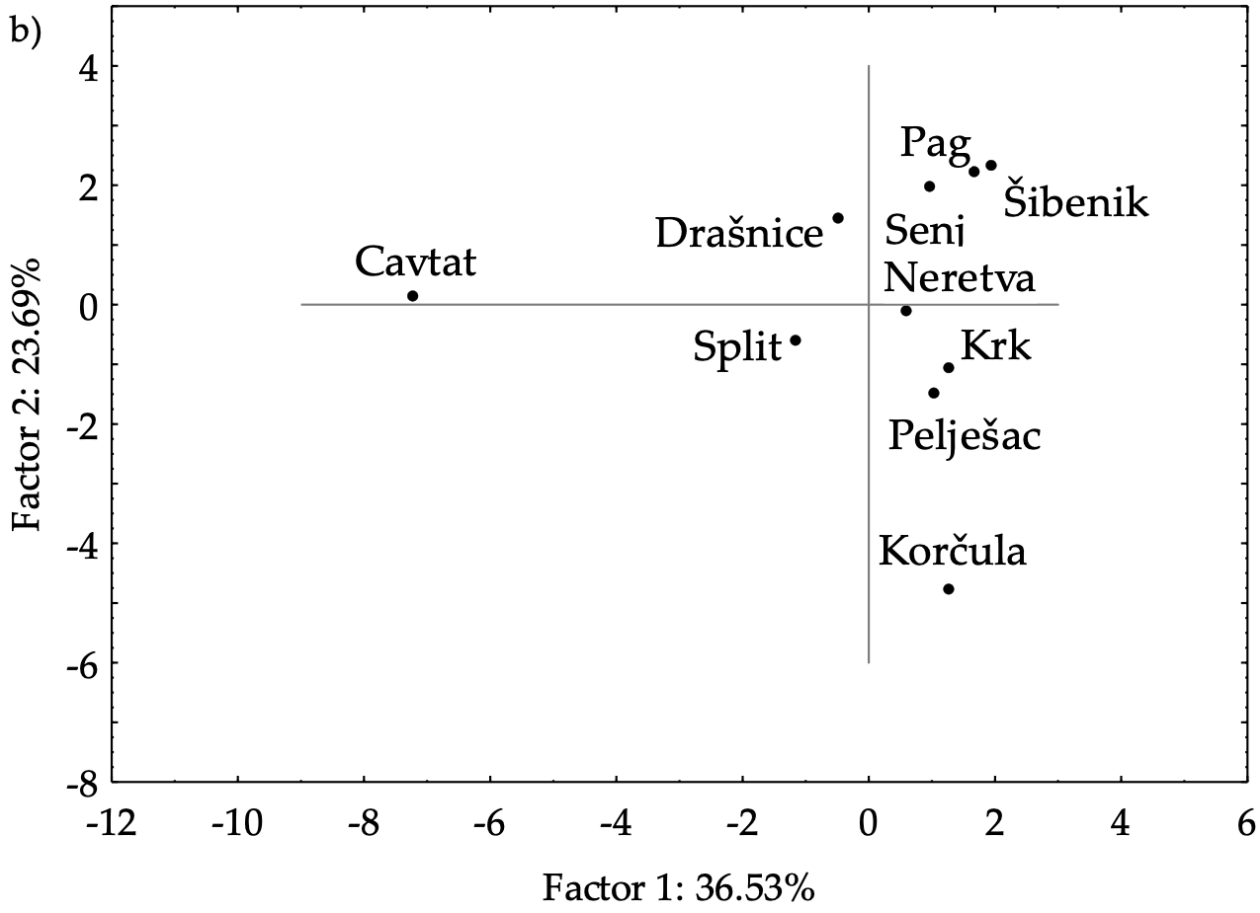
2.4 Statistical analysis for the Croatian populations

Materials and methods

Statistic tools were used to compare and differentiate phytochemicals from the investigated sea fennel populations as well as to obtain information about the phytochemical factors responsible for variations between Croatian populations.
The statistical difference between samples in terms of significant difference in fatty acid shares, phenolic concentrations, essential oil composition and pigment concentration between the sampling area was determined using the analyses of variance (one-way ANOVA, followed by Fisher's least significant difference test). Analyses were performed using software Statgraphics Centurion-Ver.16.1.11 (StatPoint Technologies, Inc., Warrenton, VA, USA). On the other hand, the relationship between the dominant fatty acids (>15%, n=4), phenolics (chlorogenic acid, n=3), essential oil components (>5%, n=8), tocopherols (α- and β- form, form, n=2) and pigments (n=3) in relation to sampling areas was determined by principal component analysis (PCA) using the software STATISTICA® (version 13, StatSoft Inc, Tulsa, OK, USA). Before analyses the data were log-transformed.

Results

Principal component analysis (PCA) was used to describe the variations between the dominant fatty acids (palmitic, stearic, linoleic and linolenic acids), phenolics (chlorogenic acid, neochlorogenic acid and criptochlorogenic acid), essential oil components (limonene, sabinene, γ-terpinene, terpinen-4-ol, verbenone, cis-verbenol, trans-p-menth-2-en-1-ol, trans-carveol), tocopherols (α-tocopherol and β-tocopherol) and pigments (lutein, β-carotene, neoxanthin) in relation to sampling areas. The results of the PCA, the correlation plot (a) and the scoreplot (b) of the dominant are shown in Figure 2. The first two PCs described 60.22% of the initial data variability. The highest variable contribution to Factor 1 was observed for trans-p-menth-2-en-1-ol, cis-verbenol, trans-carveol, verbenone, neoxantin, lutein and β-carotene, while phenolic acids (neochlorogenic, chlorogenic and cryptochlorogenic) and α-tocopherol contributed to Factor 2.
A clear separation along PC1 was observed between samples from Cavtat and other locations. These were positioned on the left part of the multivariate space, followed by Split and Drašnice samples, and with the rest of the samples positioned in the right part of the score plot. The main difference between the sample from Cavtat and the other samples is the content of essential oil components. This sample contained the lowest amount of limonene (only 24%), while components that were present in other samples only in small amounts or not at all were present in the sea fennel from Cavtat in amounts of over 5% (such as trans-p-menth-2-en-ol, cis-verbenol, trans-carveol, isocarveol, verbenone). On the other hand, the samples from Split and Drašnice had the lowest content of chlorogenic acid. Interestingly, the distribution along PC2 showed a clear separation of Korčula sample (highest case contribution) in the lower right area and Pag and Šibenik in the upper right area. The Korčula sample is characterised by an extremely high content of chlorogenic acid and its two derivatives (for example, a concentration more than 10 times higher than in the Drašnice sample). The highest content of lutein was also found in sea fennel from Korčula, while the samples from Pag and Šibenik also had a low content of chlorogenic acid, but also a low content of α-tocopherol and limonene, but a high content of sabinene.



Score plots of the dominant fatty acids, phenolics, essential oils and pigments in relation to sampling area.

References
Generalić Mekinić, I., Politeo, O., Ljubenkov, I., Mastelić, L., Popović, M., Veršić Bratinčević, M., Šimat, V., Radman, S., Skroza, D., Ninčević Runjić, T., Runjić, M., Dumičić, G., Urlić, B. The alphabet of sea fennel: Comprehensive phytochemical characterisation of Crithmum maritimum L. Food Chemistry: X 22 (2024), 101386.

3 French sea fennel populations

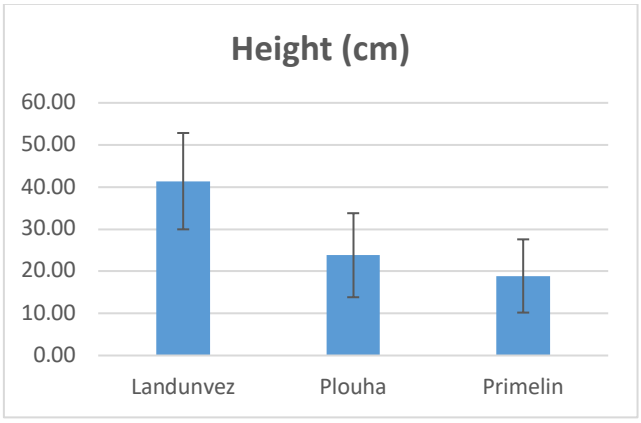
3.1 Morphological characterization

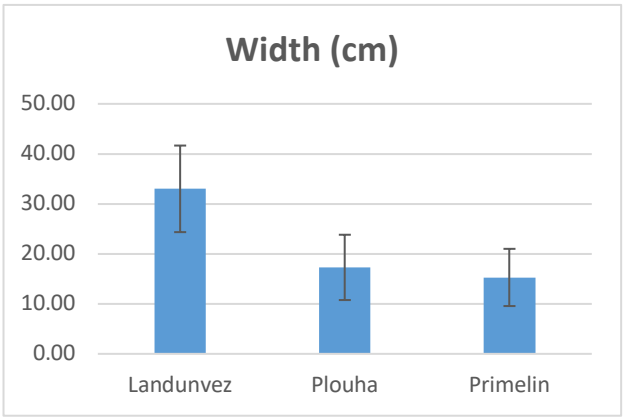
Materials and methods

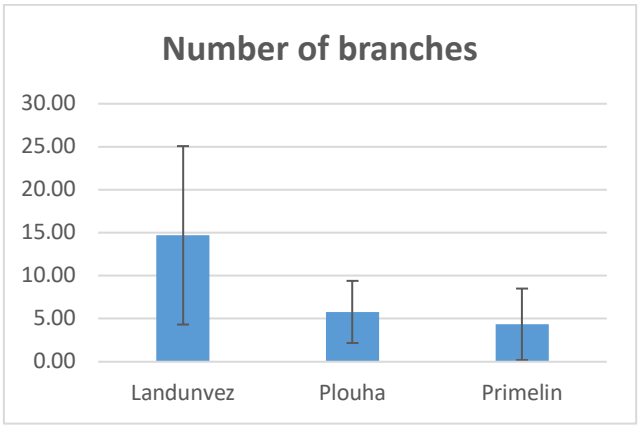
The morphometric characterization of the 3 French wild sea fennel populations sampled across the Atlantic coast was done according to the procedure detailed in Annex I (see the end of this deliverable). Such a procedure has been agreed and shared among all partners involved into WP3 activities using one drive. Thus, as in the other partner sites, measurements of the whole plant (length, width, height and diameter of the main stem), as well as counting the number of branches and number of umbels were performed in the field. For

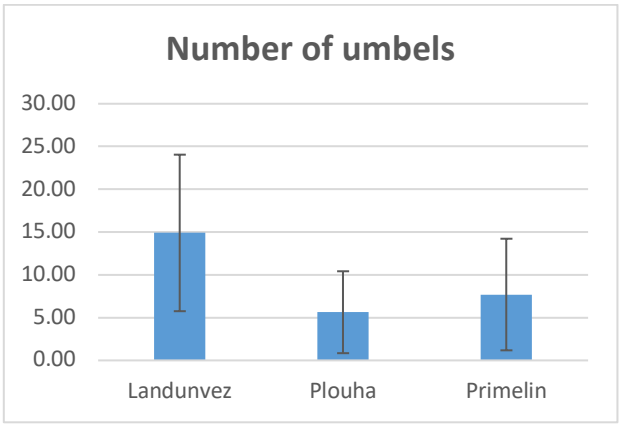
each of the 20 plants sampled in each location, 5 main umbels and 5 basal leaves were collected for measurements of some traits performed in the laboratory. In total, 100 umbels and 100 leaves were collected for each sampling location.

Results

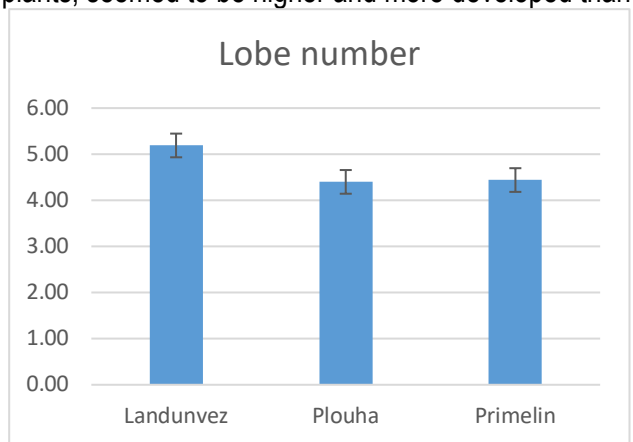


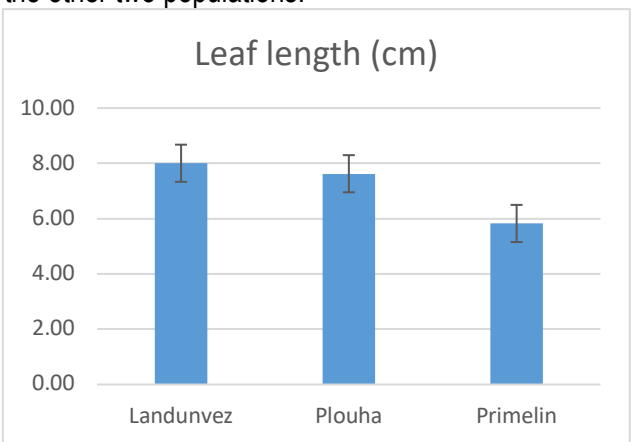


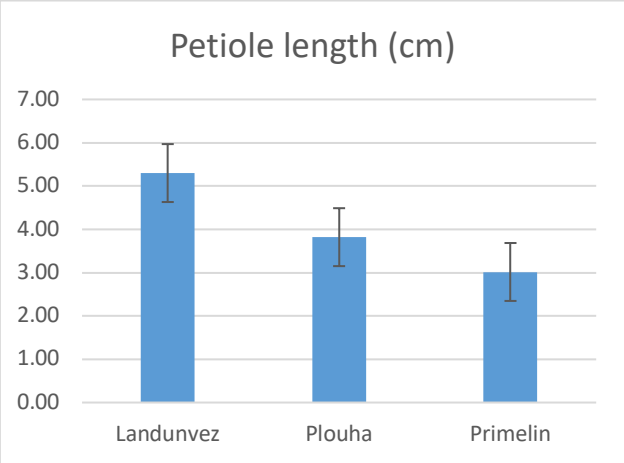


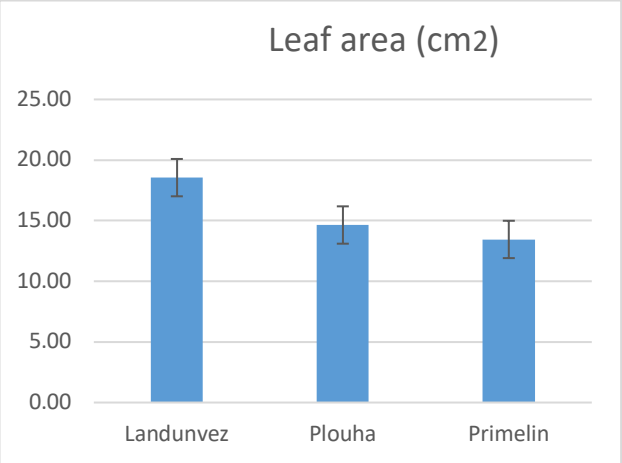


At the whole plant level, sea fennel plants from Landunvez population, although being the most exposed plants, seemed to be higher and more developed than the other two populations.









Results obtained with the leaves confirm that seafennel plants from Landunvez were more developed than those of the other two populations.
Finally, the study of reproductive organs parameters did not show any significant differences between the three populations, plants exhibiting similar pedoncule length (3.4±1 cm), pedoncule length (2.7±0.5 mm), flower diameter (4.4±1 cm), ray number (12±3), and flower number/umbellet (11±1).

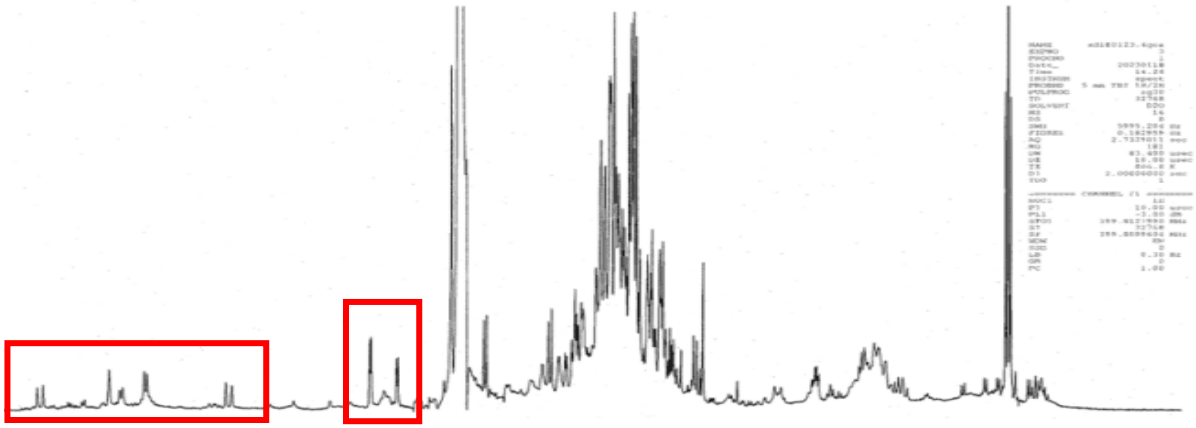
3.2 Chemical analysis

3.2.1 Metabolic profile

Materials and Methods

Raw extracts of sea fennel plants from the three populations was first analysed through NMR profile. Dry extracts were dissolved in 700 µL of 99.5% D_2O and placed in 5 mm NMR tubes. ^{1}H and ^{13}C NMR spectra were obtained at 298°K on a Bruker DRX-400 spectrometer (400 MHz), equipped with a 5 mm dual $^{1}H/^{13}C$ probe head, using standard pulse sequences available in the Bruker Software (Brüker, Wissembourg, France). A typical 1D ^{1}H NMR spectrum consisted of 32 scans, and 2,2,3,3-tetradeuterio-3-(trimethylsilyl)-propanoic acid sodium salt was used as an internal standard. For 2D ^{1}H NMR analyses (COSY, HMBC, HMQC, TOCSY, and J-MOD), experiments were carried out at 298°K on a Bruker Avance III HD500 spectrometer equipped with an inverse 5 mm TCI cryoprobe with z-gradients. Data were processed using TopSpin® software, version 4.0 (Bruker).

Results



Typical 1H-NMR spectra of sea fennel leaf extract from French populations. Red squares show aromatic (left) and carbohydrate signals.

French populations of seafennel plants were characterized with a large signals of aromatic compounds and carbohydrates. As a consequence, those plants should be rich in phenolic compounds and soluble carbohydrates (mainly sucrose and glucose).

3.2.2 Chlorogenic acid

Materials and methods

Chlorogenic acid level in sea fennel leaves from the three French populations was determined from the 1H-NMR spectrum of leaf extract (sea the spectrum above), by comparing the signal size to that of pure chlorogenic acid solution. Calibration curve for chlorogenic acid was prepared in a concentration range of 1-100 µg/mL. Chlorogenic acid concentrations were expressed as mg per gram of dry extract (mg/gDW).

Results

French sea fennel plants appeared to be rich in chlorogenic acid, with concentrations of about 20 mg/gDW found in the leaves and up to 20 mg/gDW in the flowers. On that point, no significant difference was found between the three populations studied. However, no chlorogenic acid was found in fruit coats.

3.2.3 Antioxidant activities

Material and methods

DPPH scavenging activity

The scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was determined by the method of Marwah et al. (2007). Briefly, the reaction medium contained 100 µL of 100 µM DPPH$^{\cdot}$ solution in ethanol and 100 µL of plant extract at different concentrations (or water for the control). The reaction mixture was incubated in the dark for 15 min and the absorbance was recorded at 517 nm with a Multiskan FC microplate reader (Thermo Scientific Technologies, China). The decrease in absorbance upon addition of test samples was used to calculate the inhibition percentage (%IP) of DPPH radical. Finally, the antiradical activity was expressed by the IC_{50} (extract concentration resulting in a 50% inhibition) value for each sample.

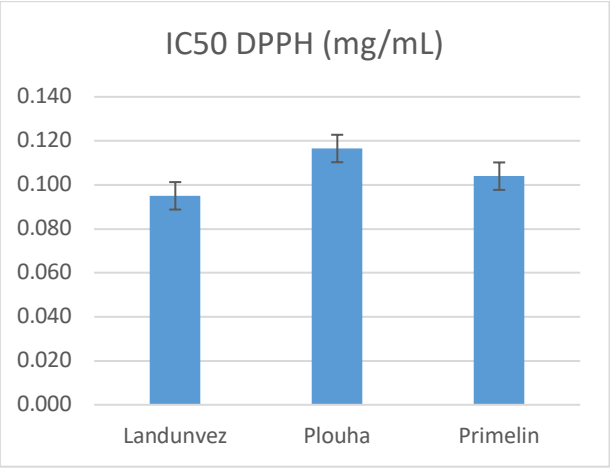
ABTS scavenging activity

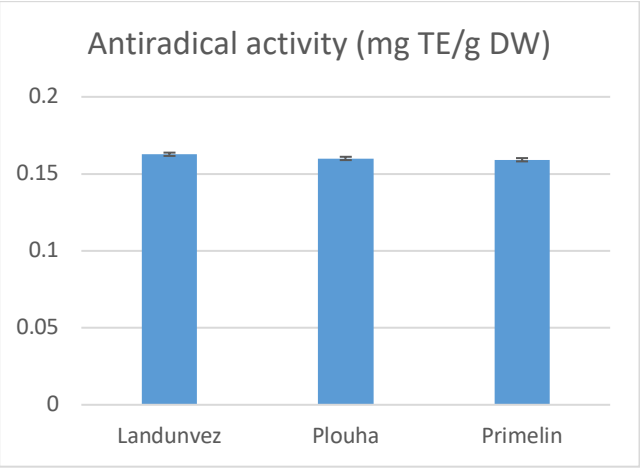
The ABTS radical scavenging assay was based on the method described by Re et al. (1999) with a slight modification. Briefly, a 7 mM ABTS solution in 80% ethanol reacted with 2.45 mM potassium persulfate in ethanol:water (1:3 v/v) and allowed to stand in dark at room temperature for 16-20 h to prepare ABTS radical cation ($ABTS^{\cdot+}$). This ABTS radical solution was diluted to an absorbance at 734 nm of 0.70 ± 0.02. Finally, the absorbance of a mixture consisting of sample (or water for the blank) and ABTS reagent was followed at 734 nm. The antiradical capacity of the samples was expressed as Trolox equivalents (mg TE/g DW).

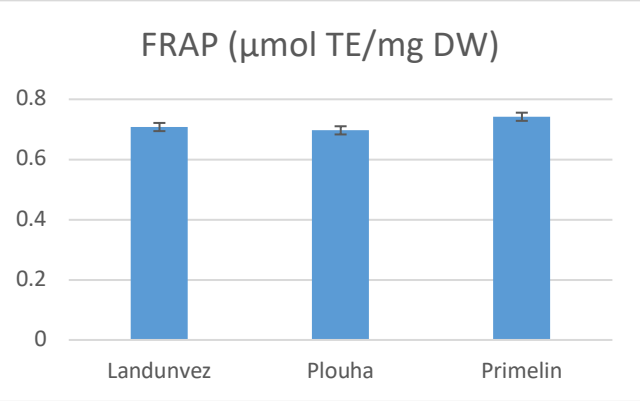
Ferric reducing activity (FRAP)

The assay is based on the reaction of Fe^{2+} with 2,4,6-tri(pyridyl)-s-triazine (TPTZ) to form a violet-blue colour with maximal absorbance at 593 nm. The FRAP solution was prepared by mixing 10 volumes of acetate buffer (300 mM, pH 3.6) with 1 volume of TPTZ (40 mM in HCl) and 1 volume of ferric chloride (20 mM in water). A 280 µL aliquot of this solution was mixed with 20 µL of samples in a 96-well microplate, then incubated at 37°C in the dark for 30 min and read at 593 nm. The increase in absorbance upon addition of test samples was used to calculate the % of reducing capacity (%EP), as follows: $\%EP = [(A_s - A_c) / A_c] \times 100$,
where A_s and A_c are the absorbances of the control and the test sample, respectively. Finally, the reducing capacity of each sample was expressed as EC_{50} (extract concentration resulting in a 50% efficacy).

Results







French sea fennel plants display strong antioxidant activities, either as antiradical (IC50 <= 0,1 mg/mL) or reducing (0,75 µmol Trolox eq./mg DW) capacities. On that point, no significant difference was found between the three populations studied.

3.3 Molecular analyses

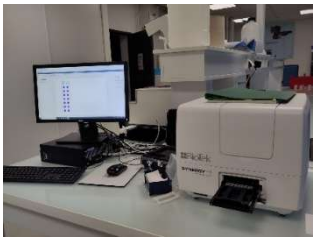
Sea fennel leaves sampled from Brittany shoreline at Landunvez (Finistère, France) were dried in silica gel upon their collection.

3.3.1 DNA extraction

Once dry, DNA was extracted using DNeasy Plant Pro Kit by Qiagen for molecular analyses. Approximately 50 gDW of leaves from 10 individuals were used for DNA extraction.

DNeasy Plant Pro Kit (Qiagen)

Samples of DNA extracts



Testing DNA quality in each extract

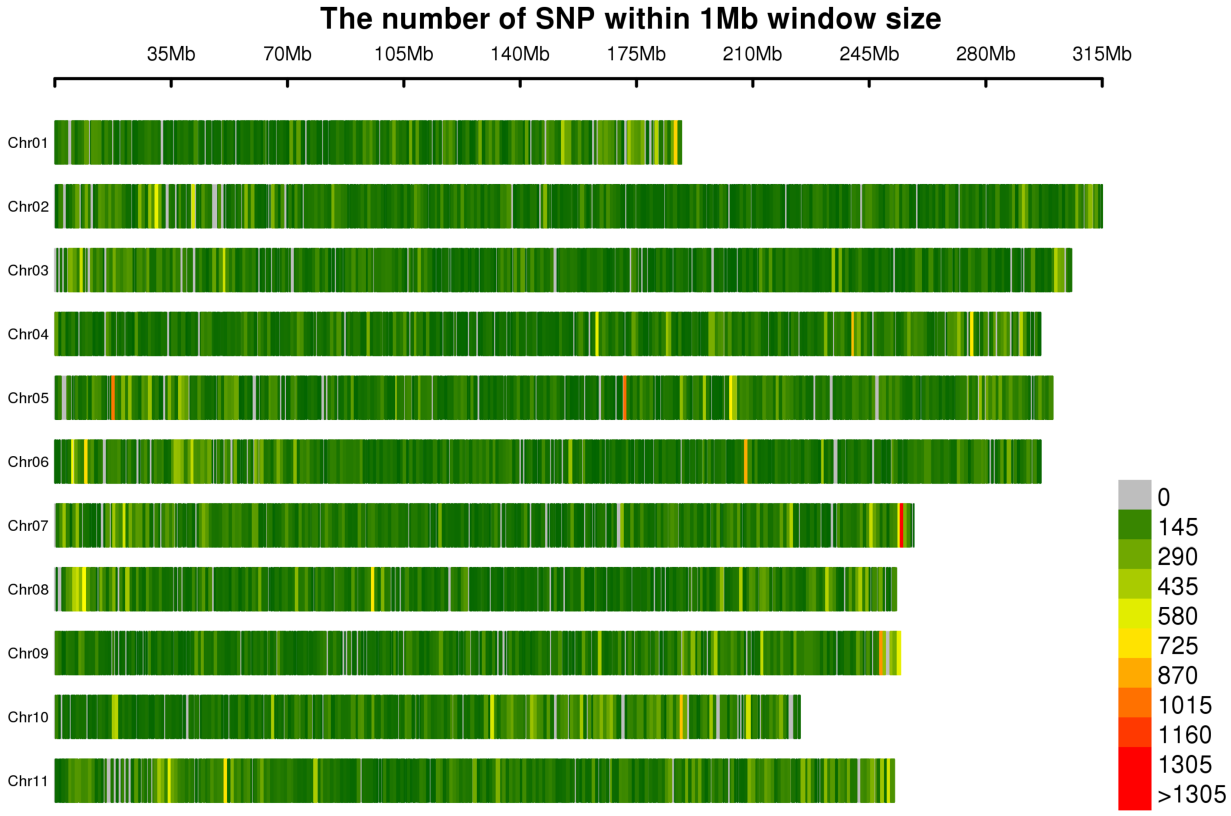
Conc (ng/gl)	Total (ug)	OD260/280
>35	>1.6 (Volum >15µl)	1.6-2.5

3.3.2 Sequencing

The following results were obtained from the samples sent to BMK.

Sample ID	BMK ID	SNP num	Integrity	Heter ratio
FR-1	FR-1	we obtain a	53.63%	23.13%
FR-10	FR-10	168918	51.18%	22.10%
FR-11	FR-11	161001	48.79%	21.11%
FR-12	FR-12	171697	52.03%	21.98%
FR-13	FR-13	234494	71.05%	29.50%
FR-14	FR-14	172214	52.18%	22.06%
FR-15	FR-15	177576	53.81%	22.45%
FR-16	FR-16	183373	55.56%	22.85%
FR-17	FR-17	169480	51.35%	22.35%
FR-18	FR-18	193875	58.75%	23.82%
FR-19	FR-19	177563	53.80%	22.36%
FR-2	FR-2	167992	50.90%	22.20%
FR-20	FR-20	172783	52.36%	21.58%
FR-21	FR-21	158274	47.96%	20.46%
FR-22	FR-22	187287	56.75%	23.18%
FR-23	FR-23	113135	34.28%	16.42%
FR-24	FR-24	159357	48.29%	20.68%
FR-25	FR-25	82635	25.04%	13.53%
FR-26	FR-26	142742	43.25%	20.02%
FR-27	FR-27	141497	42.88%	19.67%
FR-28	FR-28	159815	48.43%	22.06%
FR-29	FR-29	160525	48.64%	21.09%
FR-3	FR-3	162942	49.37%	21.87%
FR-30	FR-30	161298	48.88%	21.46%
FR-4	FR-4	168333	51.01%	22.32%
FR-5	FR-5	175797	53.27%	22.77%
FR-6	FR-6	169929	51.49%	21.66%
FR-7	FR-7	172022	52.12%	21.92%
FR-8	FR-8	155764	47.20%	20.36%
FR-9	FR-9	170320	51.61%	23.10%
Total_SNP	330021			

We can see that for the SNP, an heterozygosis ratio between 13.53 and 29.50 was obtained for an integrity comprised between 34 and 71%.



It seems that a sequence on chromosome 7 (SNP number >1300), and to a lesser extent on chromosome 5 (SNP number >1000), were characteristic of French population.

3.3.3 Analysis of sequences

For the mapping, we obtained a coverage between 34 and 48 % with 19 to,35% properly mapped (see table below).

Sample ID	Total reads	Mapped(%)	Properly map
FR-1	3858876	39.48	26.62
FR-10	3230076	42.19	28.85
FR-11	3088174	41.56	28.10
FR-12	3053946	43.02	29.84
FR-13	10454768	39.53	25.74
FR-14	3724070	44.73	32.25
FR-15	3871682	43.34	30.42
FR-16	3845828	46.40	33.30
FR-17	3298328	42.80	29.79
FR-18	4385324	44.50	30.59
FR-19	3765504	48.39	35.66
FR-2	4255652	40.48	26.09
FR-20	4216702	37.01	25.56
FR-21	2885964	37.28	24.91
FR-22	4222688	43.80	30.18
FR-23	1362476	39.18	26.96
FR-24	2933830	40.84	27.06
FR-25	842678	42.13	29.30
FR-26	3075860	41.82	26.43
FR-27	3502734	45.11	29.81
FR-28	3909060	34.23	19.48
FR-29	3514682	37.08	22.44
FR-3	3868084	39.44	25.23
FR-30	4167268	34.59	20.71
FR-4	3806800	40.46	26.51
FR-5	4243420	44.22	31.36
FR-6	3944658	42.10	27.66
FR-7	3178702	44.99	31.96
FR-8	2705902	47.58	35.02
FR-9	3782614	40.42	26.93

3.4 Statistical analysis for French populations

The morphological data have been statistically analysed in order to explore if there are differences among the French populations of sea fennel. The variability of each morphometric trait between populations was assessed through standard deviation analysis (see results above). Data are represented as mean ± SD of 20 individuals for each trait.

4 Tunisian sea fennel populations

Five Tunisian Sea fennel populations were considered for morphological, biochemical, and molecular analysis.

Region	GPS Coordinates
Bizerte	37,25265°N, 9,94500°E
Tabarka	36,95951°N, 8,75302°E
Cap-Negro	37,10278°N, 8,98423°E
Haouaria	37,05143°N, 10,94242°E
Monastir	35,78414°N, 10,83414°E

Geographic distribution of the five Tunisian populations

4.1 Morphological characterization

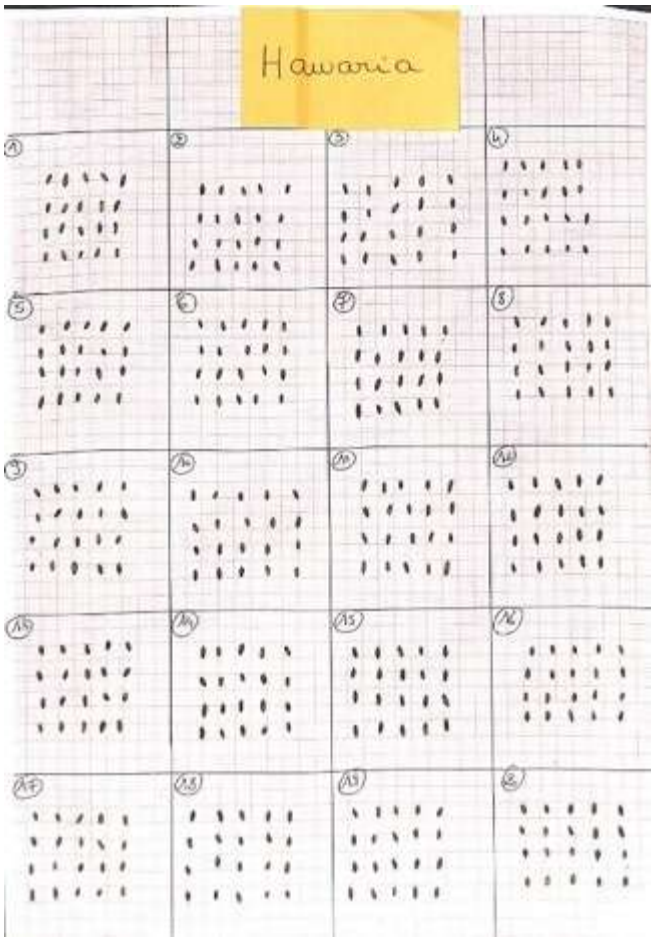
Materials and methods

The morphometric characterization of the 5 Tunisian wild sea fennel populations was done according to the procedure detailed in Annex I (see the end of this deliverable). Such a procedure has been agreed and shared among all partners involved into WP3 activities. In order to do so, different traits were considered based on the International Plant Genetic Resources Institute (IPGRI) descriptors (1998). The hole plant characterization was performed in the field considering its height, width, length, and main stem diameter. 20 plants were sampled in every location.

Following field measurements, umbels, umbellets, leaves, flowers, fruits, and seeds characterization was performed in the lab. Scans of the different parts of the plants were performed using a Fujitsu ScanSnap SV600 overhead scanner at a 600Dpi resolution.

A total number of 100 leaves were sampled in each population and measurements were performed on the first order leaflets of the plant's basal leaves.

For seeds characterization, 20 seeds per plant were used to measure their length, width, area, and perimeter using Mesurim software.

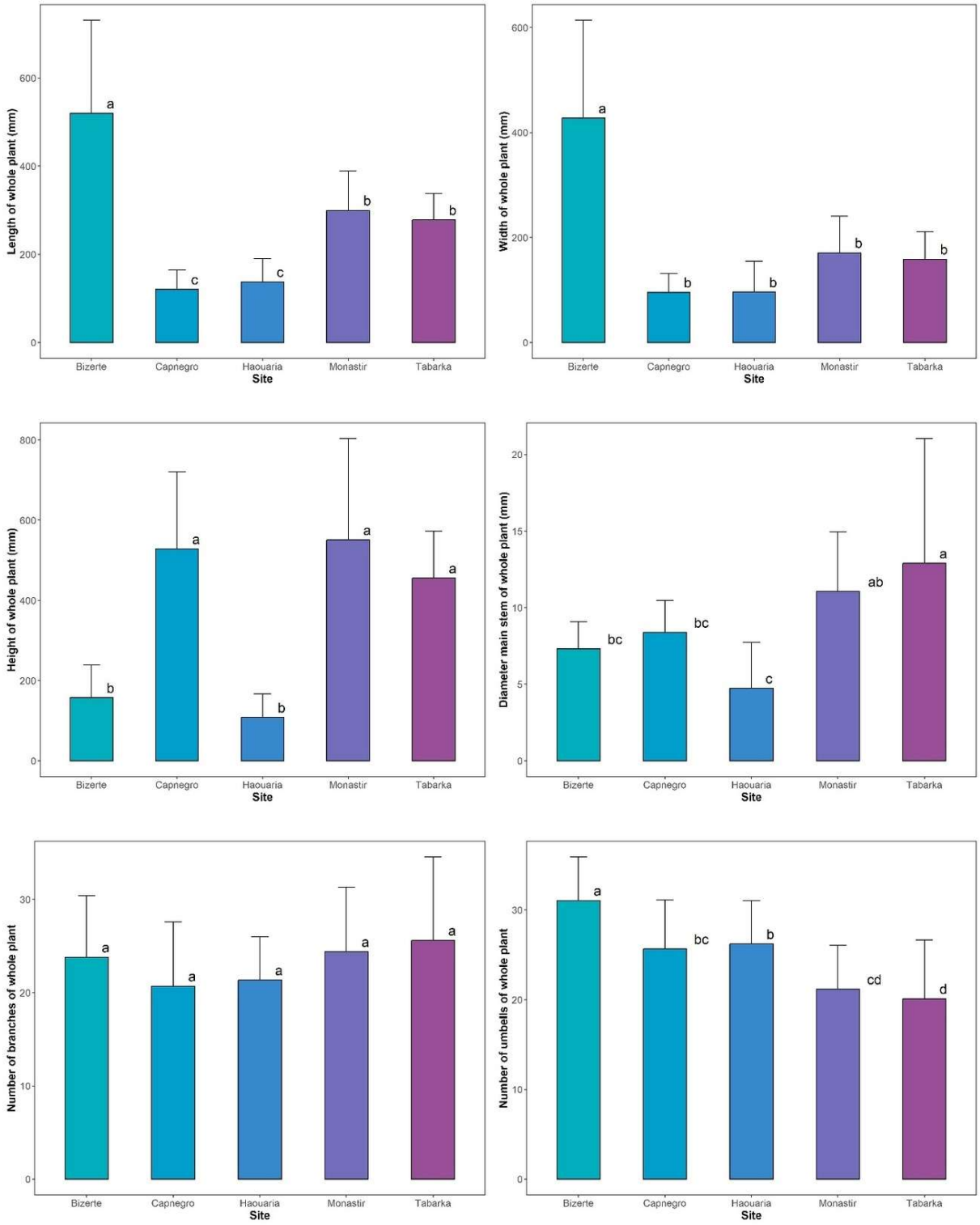


Scanned seeds of sea fennel

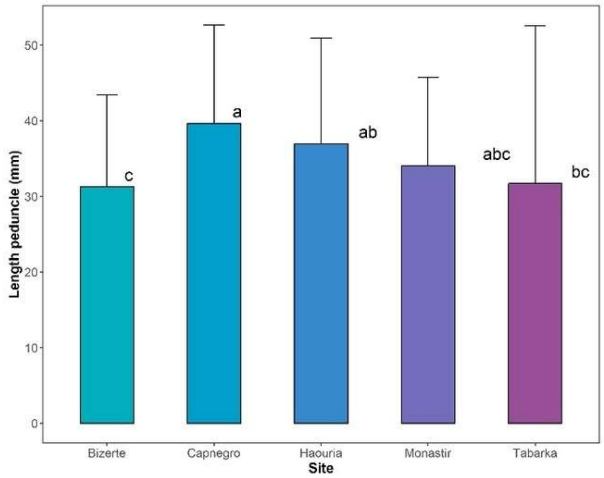
Results

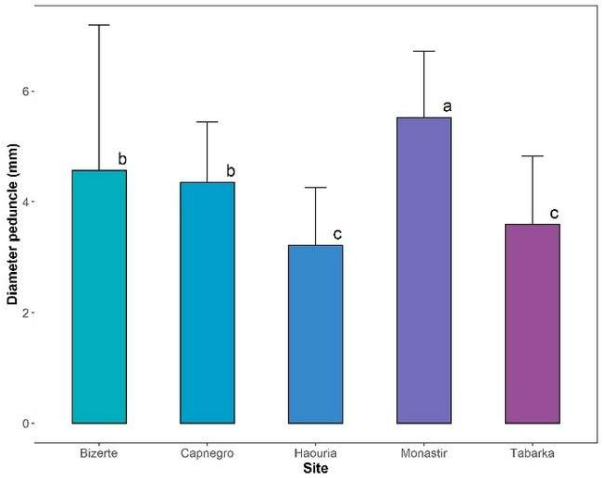
Results of morphological variability of whole plant are summarized in the following graphs:

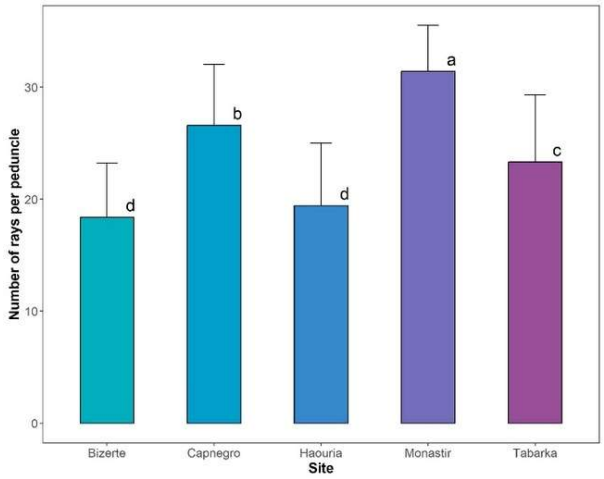
Results show that the length and width of sea fennel plants have the same aspect with the largest plants recorded in Bizerte. While the number of branches doesn't show a significant difference, other characteristics of the plants differ significantly between sites.

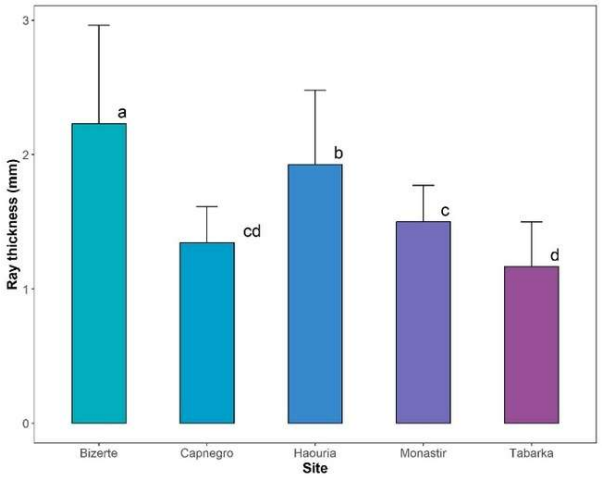


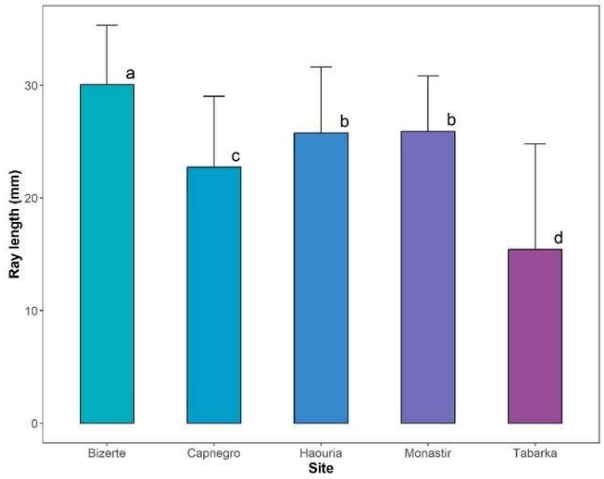
Results of morphological variability of umbells are summarized in the following graphs:
highly significant variability was observed between sites for umbells morphological aspects exept for color where all flowers have white color.

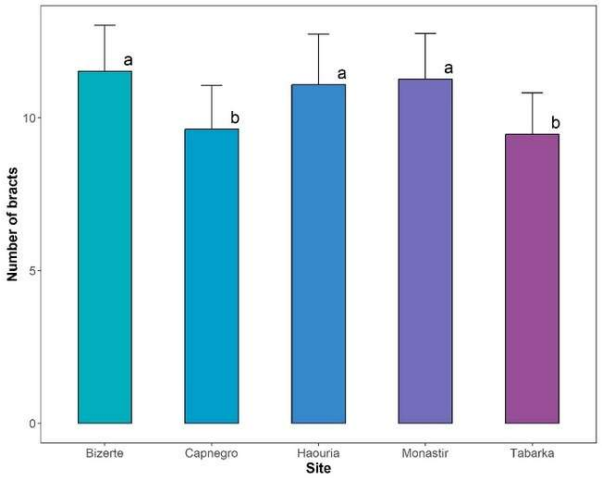

Length peduncle (mm)
0
10
20
30
40
50
c
a
ab
abc
bc
Bizerte
Capnegro
Haouria
Monastir
Tabarka
Site

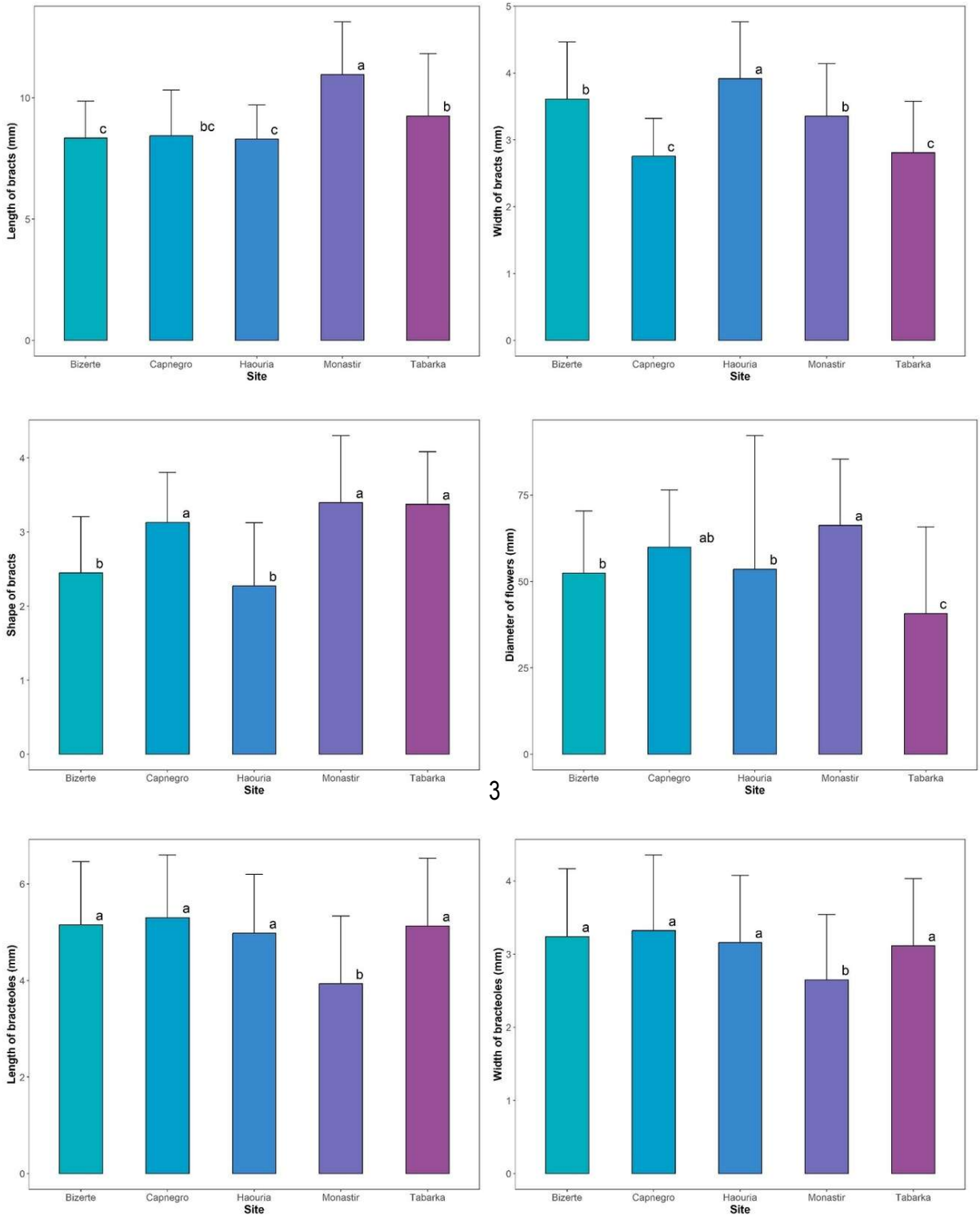

Diameter peduncle (mm)
0
2
4
6
b
b
c
a
c
Bizerte
Capnegro
Haouria
Monastir
Tabarka
Site


Number of rays per peduncle
0
10
20
30
d
b
d
a
c
Bizerte
Capnegro
Haouria
Monastir
Tabarka
Site

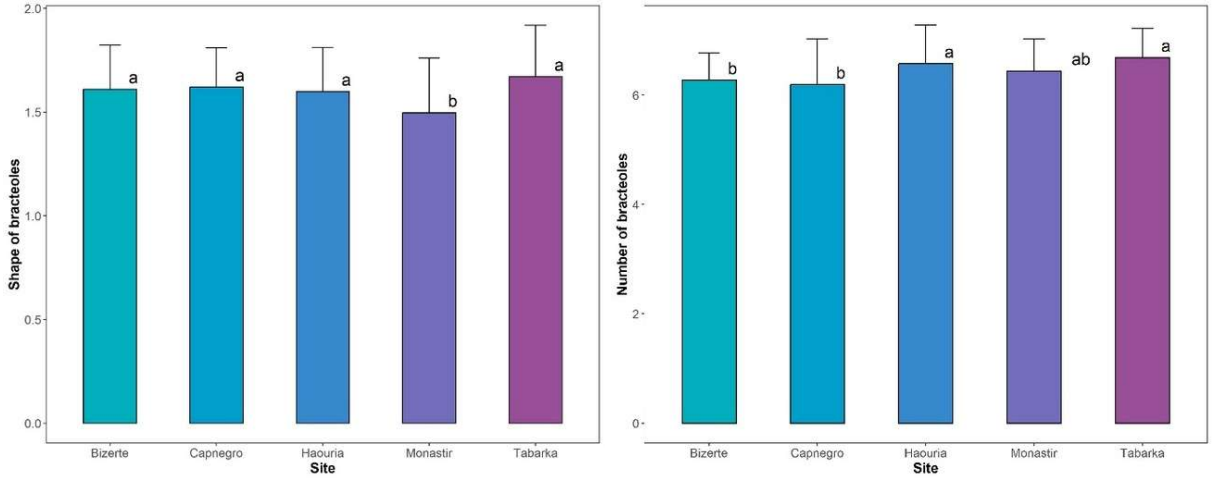

Ray thickness (mm)
0
1
2
3
a
cd
b
c
d
Bizerte
Capnegro
Haouria
Monastir
Tabarka
Site


Ray length (mm)
0
10
20
30
a
c
b
b
d
Bizerte
Capnegro
Haouria
Monastir
Tabarka
Site

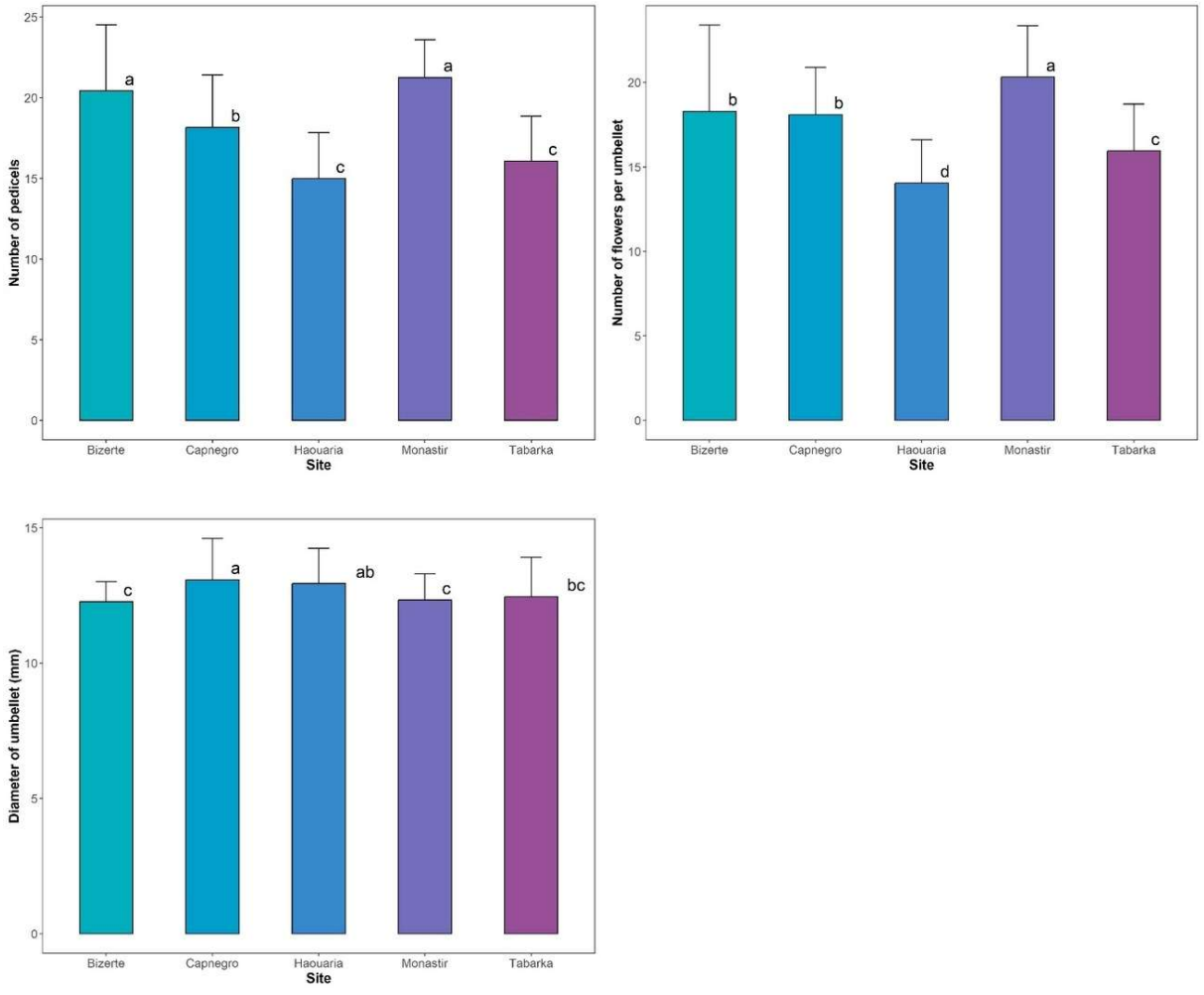

Number of bracts
0
5
10
a
b
a
a
b
Bizerte
Capnegro
Haouria
Monastir
Tabarka
Site


Length of bracts (mm)
Width of bracts (mm)
Shape of bracts
Diameter of flowers (mm)
Length of bracteoles (mm)
Width of bracteoles (mm)
Bizerte
Capnegro
Haouria
Monastir
Tabarka
Site

3

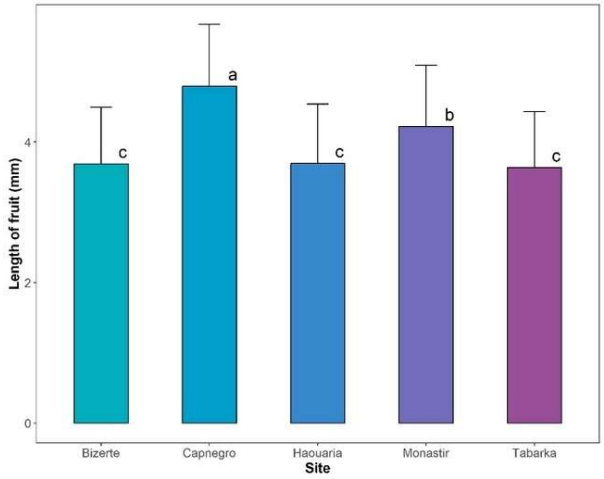


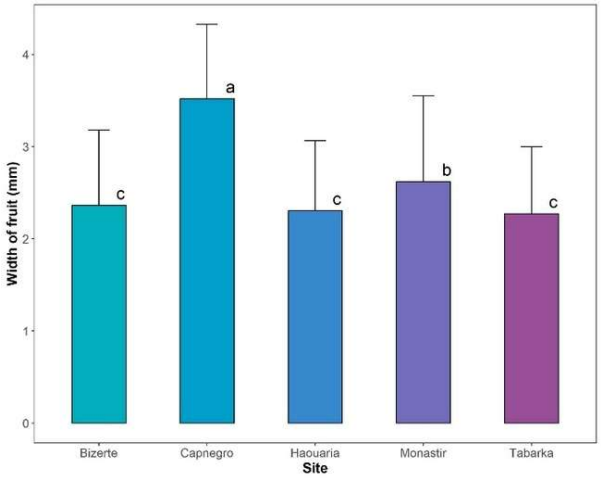
Results of morphological variability of umbellets are summarized in the following graphs:

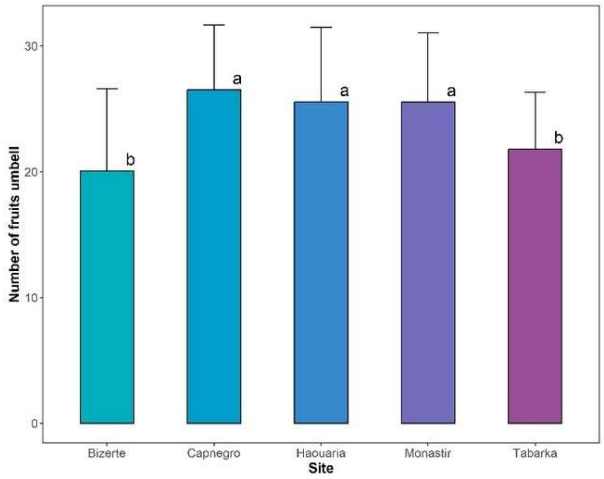


Results of morphological variability of fruits are summarized in the following graphs:

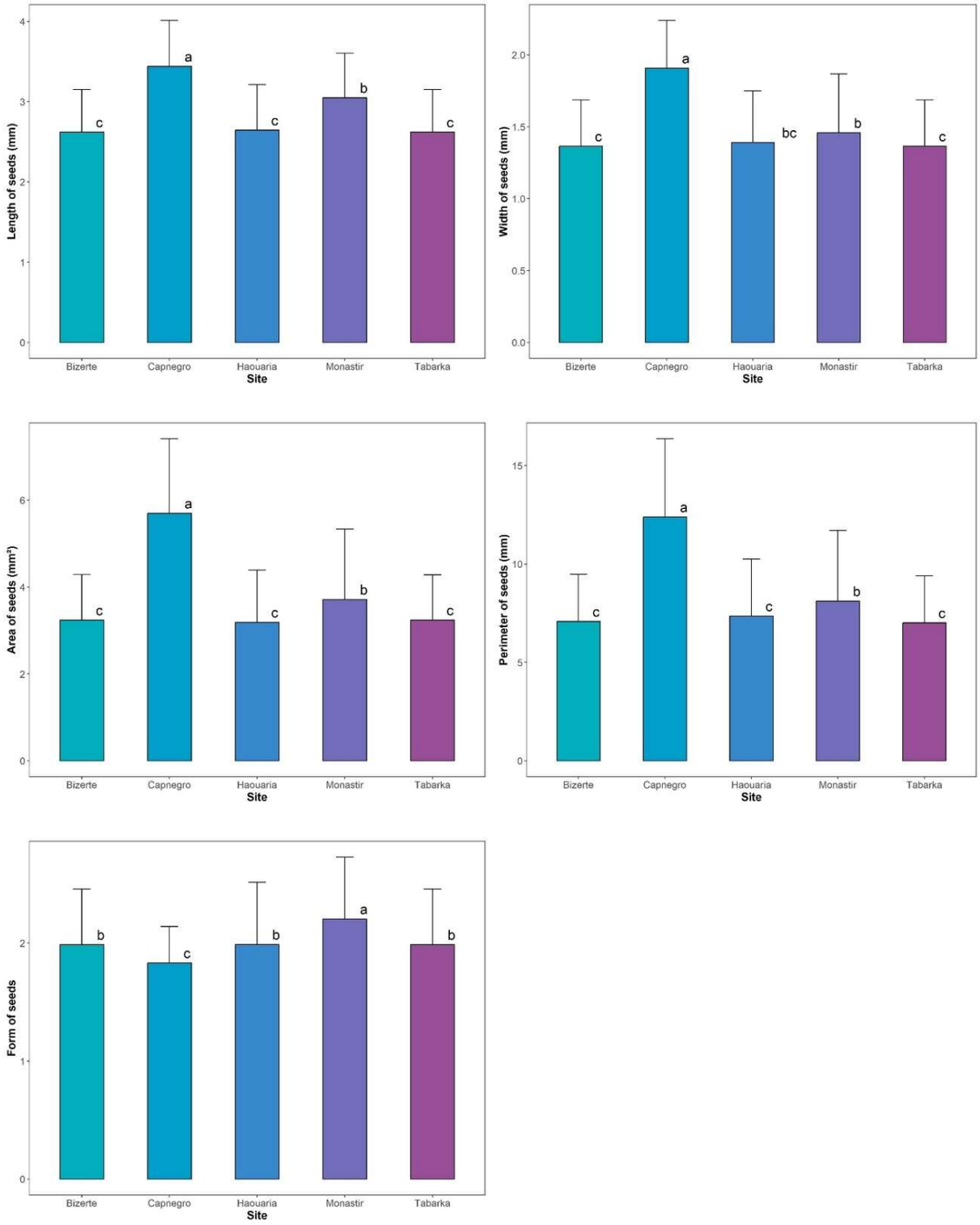
The largest fruits were observed in Capnegro site, followed by monastir ecotype. For the number of fruits in an umbell, the highest values were recorded in Cap Negro, Haouaria and Monastir sites.



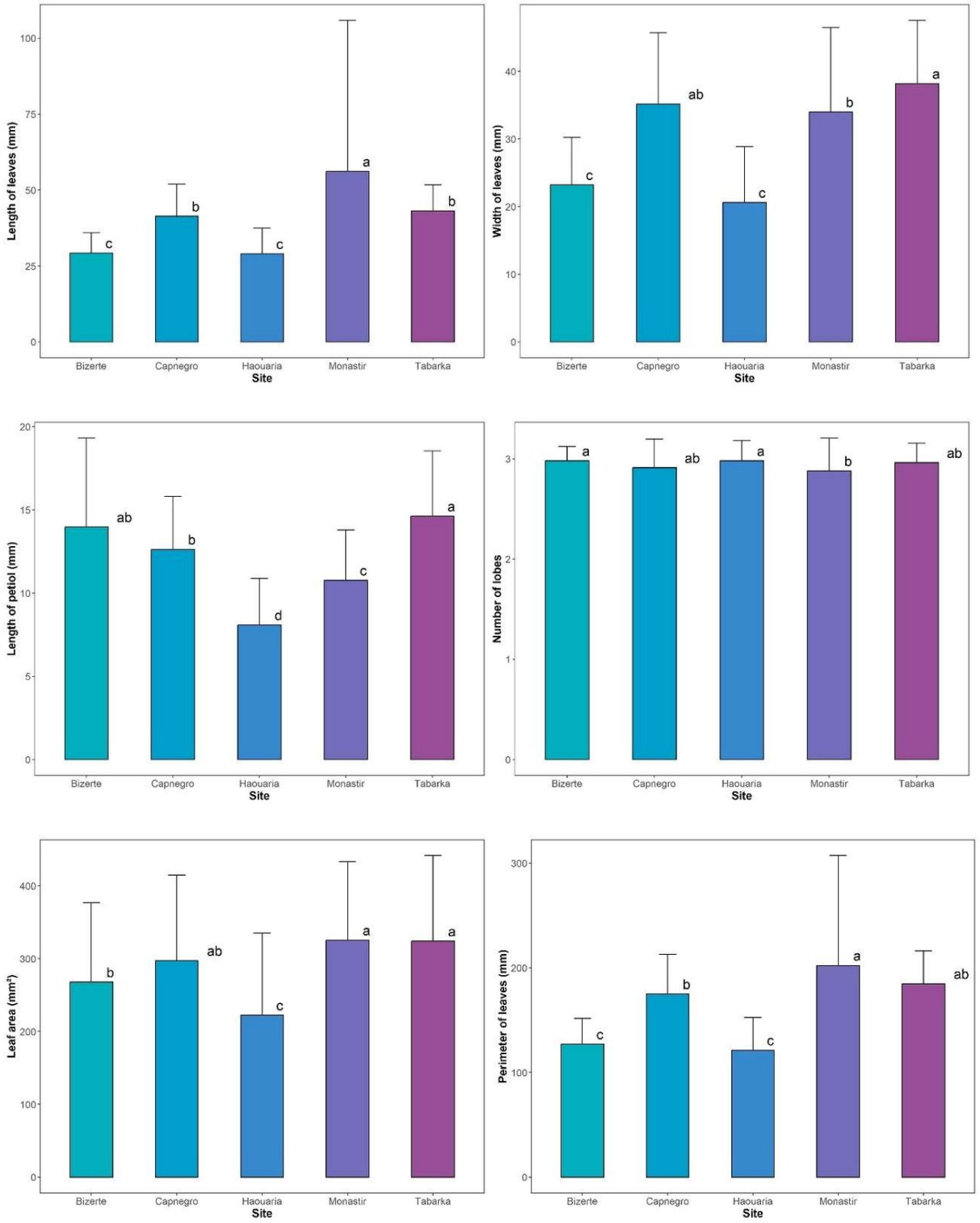




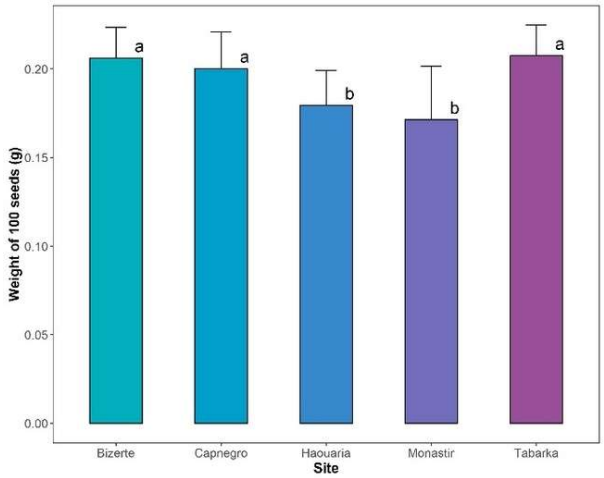
Results of morphological variability of seeds are summarized in the following graphs:
For all the ecotypes, only 2 seeds were found per fruit.
Regarding morphological variability, Cap Negro seeds show a distinctive aspect recording the highest values of length, width, area and perimeter of seeds.



Results of morphological variability of leaves are summarized in the following graphs:
The observed results show that Haouaria is usually characterized by smaller leaves than other sites. Usually, examined leaves contain 3 lobes with the largest leaves observed in Tabarka and Monastir.



Results about the variability of the weight of 100 seeds are summarized in the following graph: Haouaria and monastir seeds are the lightest as shown in the results



Statistical analysis showed highly significant differences between the morphological traits of the five Tunisian populations except for some variables.
(Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05), ns : not significant, NA : not applicable

Plant part	Variables	Significance level at p-value<0.05
Hole plant	Length	***
	Width	***
	Height	***
	Diameter main stem	***
	N°of branches	ns
	N°of umbels	***
Umbell	Length peduncle	***
	Diameter peduncle	***
	Number rays/peducle	***
	Thickness ray	***
	Length ray	***
	Number bract	***
	Length bract	***
	Width bract	***
	Shape bract	***
	Diameter flower	***
	Color	NA
	Length of bracteoles	***
	Width of bracteoles	***
	Shape of bracteoles	***
	N. Of bracteoles	***
Umbellet	Number pedicels	***
	Number flower/umbellet	***
	Diameter of umbellet	***

Plant part	Variables	Significance level at p-value<0.05
Fruits	Length	***
	Width	***
	Number fruits/umbell	***
Seeds	Length	***
	Width	***
	Area	***
	Perimeter	***
	Number of seeds/ fruit	NA
	Form	***
	Thickness	NA
Leaves	Length	***
	Width	***
	Length petiole	***
	Number lobes	**
	Area	ns
	Perimeter	***
weight of 100 seeds	Weight 100 seeds	***

References
VL. Singleton and JA. Rossi (1965). Colorimetry of Total Phenolics with Phosphomolybdic Phosphotungstic Acid Reagents. Am. J. Enol. Vitic. 16, 144-158.
Broadhurst RB, Jones WT. Analysis of condensed tannins using acidified vanillin. Journal of the Science of Food and Agriculture, Volume 29, 1978, Pages 788–794.

Quettier-Deleu C, Gressier B, Vasseur J, Dine T, Brunet J, Luyck M, et al. Phenolic Compounds and antioxidant activities of buckwheat (Fagopyrum esculentum Moench) hulls and flour, Journal of Ethnopharmacology, Volume 72, 2000, Pages 35-40.
W. Brand-Willims, ME. Cuvelier, C. Berset, "Use of a free radical method to evaluate antioxydant activity", Lebensmitel-Wissenschauft and technologie, 28(1995) 25-30.

4.2 Chemical analysis

4.2.1 Carotenoids

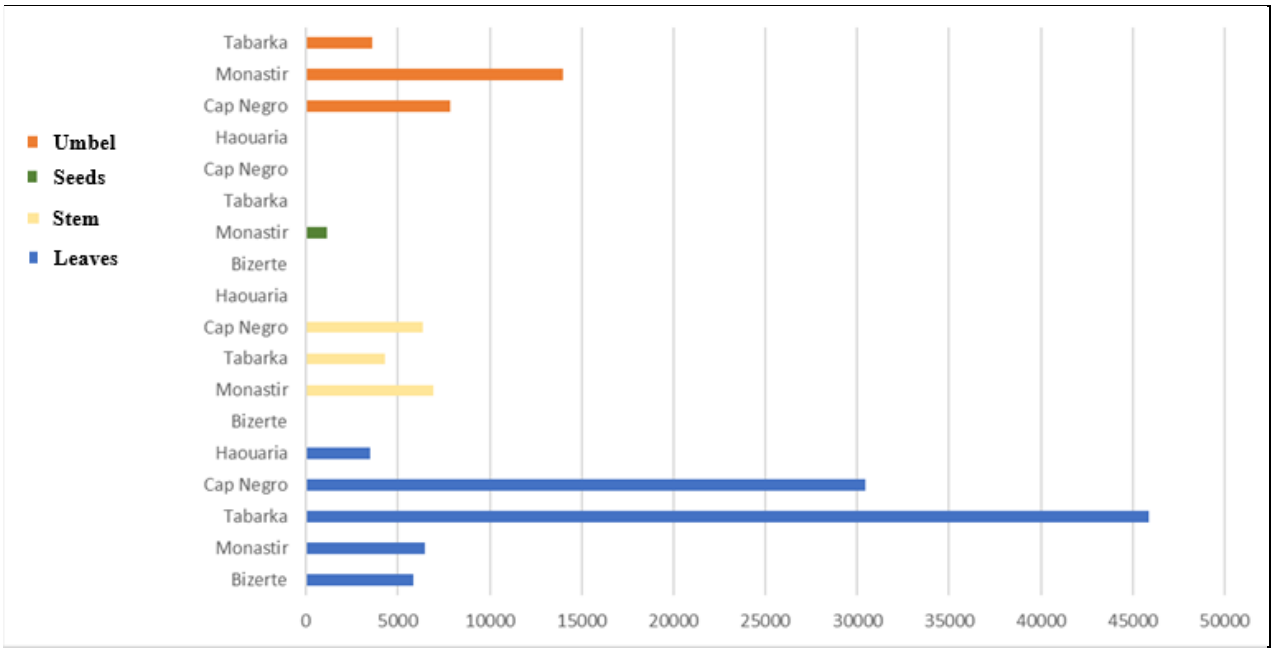
Materials and methods

Carotenoids were extracted from different plant parts according to Nartea et al. (2023). Dried samples (100mg) were mixed with 5ml acetone at 4°C and left at 4°C for 15 min. The mixture was then vortexed for 5min and centrifuged for 10min at 1370rpm. The extraction procedure was performed for a second time and the supernatant was filtered using a 0.45 µm filters. After recuperation, the extract was dried and resuspended in acetone for further analysis.

Chromatographic analysis (HPLC) of β-carotene was performed using a Shimadzu system, consisting of an LC-20AD XR binary pump system, SIL-20AC XR autosampler, CTO-20AC column oven, and DGU-20A 3R degasser (Shimadzu, Kyoto, Japan), equipped with a DiscoVery BIO Wide Pore C18-5 column (Thermo Electron, Dreieich, Germany, 15 cm × 4.6 mm, 5 µm) and a PDA detector (SPD-M20A). The used solvents were: (A) methanol: 1 M ammonium acetate 8:2 and (B) methanol: acetone 8:2. The injection volume was 20 µL and the flow rate 1 mL/min. UV absorbance was settled at 450 nm. The gradient for elution was linear from 0 to 100% B in 20 min; after 5 min, 100% of A was used for a further 5 min. Finally, a linear flow of 100% A for 5 min was used to equilibrate the column. The content beta-carotene was measured and quantified using different concentrations of the standard sample of the two carotenoids (concentration range was from 0.01 to 1 mg/mL beta-carotene).

Results

The analysis of β-carotene content, expressed in parts per million (ppm), reveals marked differences in its accumulation across plant organs and collection sites. Leaves exhibited the highest levels of β-carotene, particularly in samples from Tabarka (45,871.16 ppm) and Cap Negro (30,424.81 ppm), followed by Monastir (6,453.89 ppm), Bizerte (5,833.46 ppm), and Haouaria (3,471.18 ppm). In stems, moderate amounts were detected in Monastir (6,921.77 ppm), Cap Negro (6,330.24 ppm), and Tabarka (4,277.45 ppm), while Bizerte and Haouaria showed no detectable levels. Seeds showed a very low accumulation, with only Monastir presenting a measurable value (1,129.23 ppm), and all other sites recording zero. Regarding umbels, significant amounts were found in Monastir (13,995.79 ppm), Cap Negro (7,832.66 ppm), and Tabarka (3,620.65 ppm). These findings confirm that β-carotene accumulation is highly organ- and site-specific, with leaves being the most enriched, especially in Tabarka, while Monastir stands out for its relatively high levels across multiple plant parts.



Beta carotene content of Sea fennel (ppm)

4.2.2 Tocopherols

Materials and methods

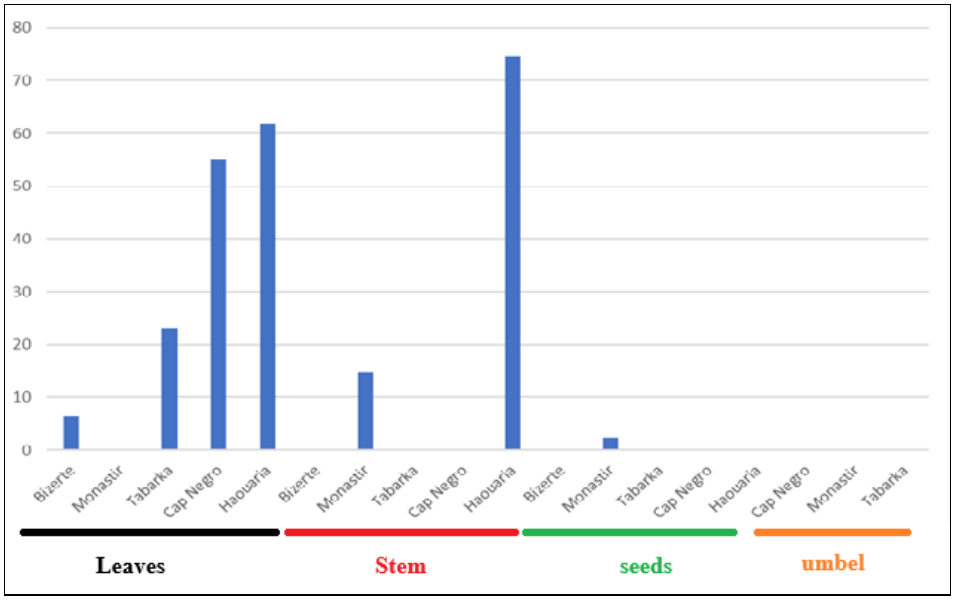
Tocopherols extraction was done simultaneously with carotenoids applying the same extraction protocols. Dried samples (100mg) were mixed with 5ml acetone at 4°C and left at 4°C for 15 min. The mixture was then vortexed for 5min and centrifuged for 10min at 1370rpm. The extraction procedure was performed for a second time and the supernatant was filtered using a 0.45 µm filters. After recuperation, the extract was dried and resuspended in acetone for further analysis.

Analysis was performed using a Shimadzu UFLC XR equipped with a 3µm C18 column (150 mm* 3.0 mm) and a fluorescence detector (RF 20A xs) (excitation wave 295nm and emission wave 330nm). Oven temperature is 30°C. Injection volume is 10µL. LC-20ADXR pumps are used, with a total mobile phase flow rate of 0.4000 ml/min. The mode is isocratic. The mobile phase used is ACN/meOH (50/50). Tocopherol was identified by comparison with the retention time of the standards.

Results

The analysis of α-tocopherol content, expressed in parts per million (ppm), across different plant organs and geographic sites reveals significant variation in its distribution. Leaves are the most enriched in α-tocopherol, with the highest concentrations observed in samples from Haouaria (61.79 ppm) and Cap Negro (54.96 ppm), followed by Tabarka (22.99 ppm) and Bizerte (6.57 ppm), while no α-tocopherol was detected in the leaves from Monastir. In stems, notable levels were found in Haouaria (74.52 ppm) and Monastir (14.80 ppm), whereas all other sites showed no detectable amounts. Seeds exhibited very low or undetectable levels of α-tocopherol, with only Monastir registering a minimal amount (2.34 ppm). Umbels (inflorescences) from all sampled locations (Cap Negro, Monastir, and Tabarka) contained no measurable α-tocopherol. Overall, these findings highlight a clear organ-specific and site-dependent accumulation pattern, with leaves being the

primary site of α-tocopherol storage and Haouaria standing out as the most favorable location for its natural accumulation.



Alpha tocopherol content of Sea fennel (ppm)

4.2.3 Phenolic compounds

Analysis of total phenolic, flavonoids and tannin content were conducted for the five Tunisian populations. The antioxidant activity was determined for all samples studied.

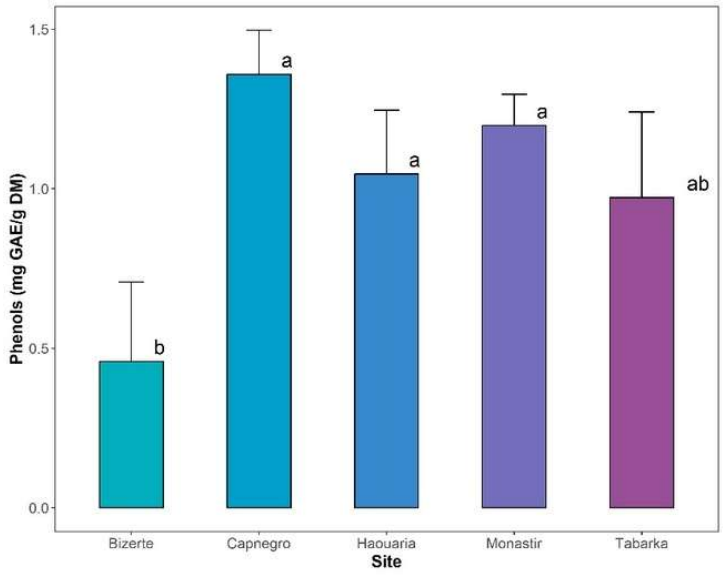
Total phenolic Content (TPC)

Material and methods

Total phenolic content was determined using Folin-Ciocalteu method (1965). 0.5 mL of extract sample was mixed with 2.5 mL of Folin-Ciocalteu reagent (1:10) and 2 mL of 7.5% sodium carbonate. The absorbance was measured at 765 nm after incubation in the dark for 30 min. Gallic acid was used as standard. The total polyphenol content was expressed as milligrams of gallic acid equivalents (GAE) per gram of the sample.

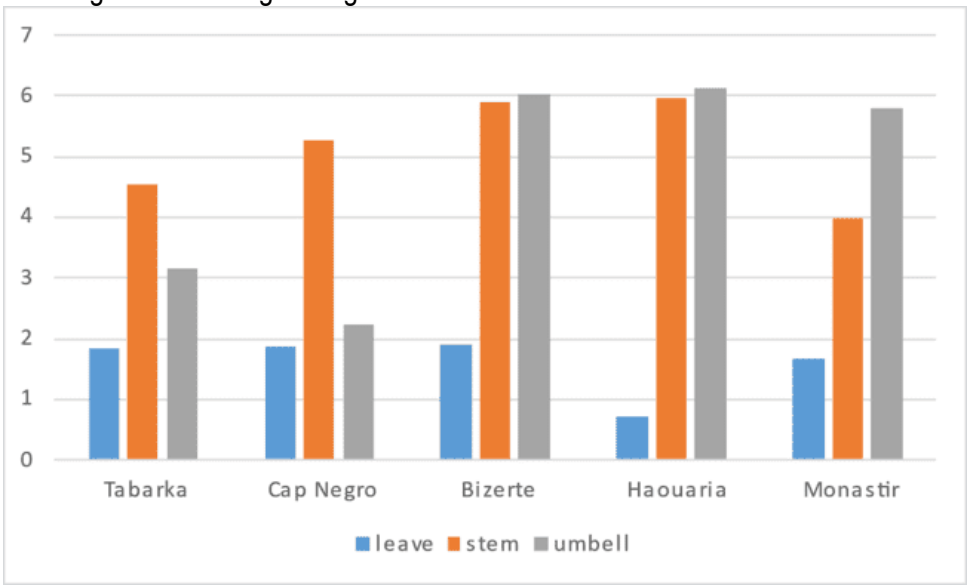
Results

Total polyphenolic content ranged from 0.45 to 1.35 mg GAE/g DM. The highest amount of polyphenols was recorded by plants harvested from Capnegro site although the lowest value was determined for Bizerte site.



Total phenolic content of sea fennel

Phenolic content differs significantly between sites and organs. Leave extracts show the lowest level of phenolic content in all Tunisian sites. The highest levels of phenols are observed in umbel and stem extracts reaching levels of 6 mg EGA/g DM.



Total phenolic content of different organs of sea fennel

Total flavonoids content

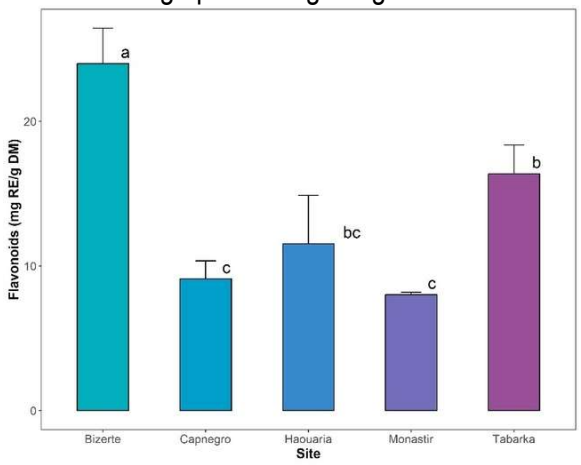
Material and methods

The total flavonoid content of crude extract was determined by the aluminium chloride colorimetric method (Quettier Deleu et al. 2000). 1 ml of sample was mixed with 1 ml of 2% aluminum chloride methanolic solution. The mixture was allowed to stand for 15 min, and absorbance was measured at 430 nm. The total flavonoid

content was calculated from a calibration curve, and the result was expressed as mg rutin equivalent per g of DM (mg RE/g).

Results

Concerning flavonoids, the values vary between 8.01 and 23.98 mg RE/g DM. Bizerte is in the lead with a value reaching up to 26 mg RE/g DM while Monastir and Haouaria hold the lowest value.



Total flavonoids content of sea fennel

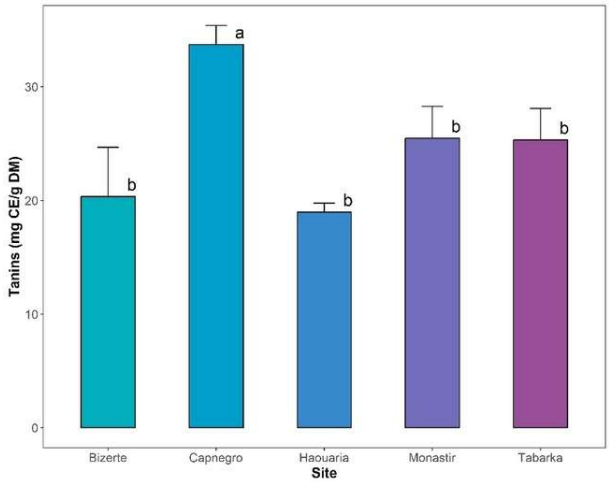
Total condensed tannins

Material and methods

The method described by Broadhurst and Jones (1978) was used to determine the total condensed tannin content in Sea Fennel. 0.5 ml of the extract was mixed with 3 ml of vanillin (4% in methanol) and 1.5 ml of Hydrochloric acid. After incubation for 15 min at 20◦C in the dark, the absorbance was read at 500 nm. The condensed tannin content was calculated from a calibration curve prepared with a solution of catechin (30 ppm). The results were expressed in mg of catechin equivalent per g of DM (mg CE/g).

Results

The determination of tannins allowed to distinguish Capnegro site for its high tannin content with an average of 33.72 mg EC/g DM.



Total condensed tannins content of sea fennel

Antioxidant activity

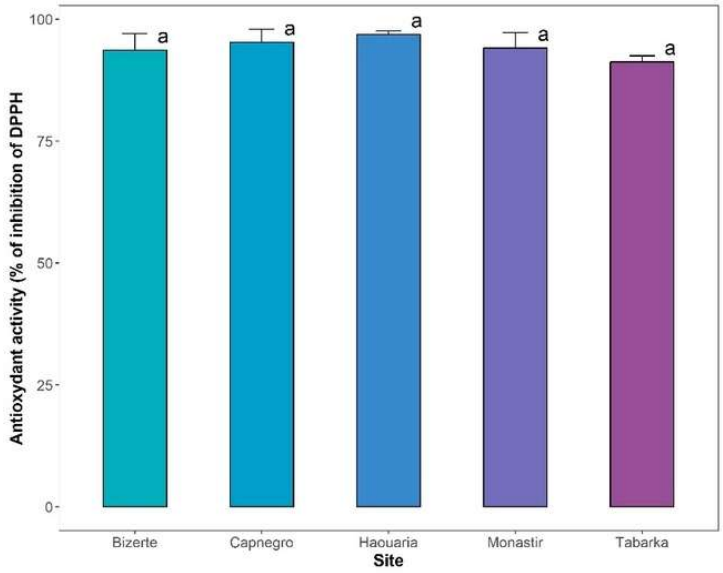
Material and methods

To evaluate the antioxidant activity, we used the DPPH (2.2-diphenyl-1-picrylhydrazyl) method. Briefly, 15 µl of each extract were incubated with 1.5 ml of the solution of DPPH in 0.004% ethanol. In parallel, a negative control is prepared by mixing 15 µl of solvent (water, ethanol or hexane) with 1.5 ml of the DPPH solution. After an incubation period of 30 minutes, absorbances were determined at 517 nm (1995). The percentage inhibition (I%) of the DPPH radical was calculated as follows:

IR = [(DOc – DOe)/DOc]*100
Where:
Doc: control absorbance
DOe: absorbance of the DPPH containing the samples to be tested.

Results

Concerning the antioxidant activity, the overall inhibition of DPPH reaches levels above 90% with no observed difference between sites..



Antioxidant activity of sea fennel

Identification of phenolic compounds in different plant parts

Material and methods

Phenolic compound in different plant parts (leaves, stem, seeds, and flower) collected from the five Tunisian sites (Bizerte, Monastir, Cap-Negro, Tabarka, and Haouaria) were extracted using ethanol (96%). The process was performed by soaking 5 g of plant dried sample in 50ml ethanol and let it agitate for 24 hours at 200rpm. Tha mixture was then filtred, dried and weighted.

In order to identify the phenolic composition, extract was resuspended in ethanol and Analysis was performed using an ultra-fast liquid chromatography system (Shimadzu, Kyoto, Japan) equipped with LC20AD XR binary pump, a SIL-20AC XR auto sampler, a CTO-20AC column oven, a DGU-20AS degasser and a SPD-M20A diode array detector with 13 mm flow cell. Separation was executed using a Discovery Bio C18 column (250 mm × 4.6 mm × 5 µm) (Supelco). The mobile phase was composed of A (0.2% acetic acid in 95% water and 5% methanol) and B (0.2% acetic acid in 50% water and 50% acetonitrile) with a linear gradient elution: 0–45 min, 10–20% B; 45–85 min, 20–55% B, 85–97 min, 55–100% B, 97–110 min, 100% B; the initial conditions were held for 10 min as a reequilibration step. The flow rate of the mobile phase was 0.5 mL/min, the column temperature was maintained at 40 °C and the injection volume was 5 µL. Chromatograms at an absorbance of 280 nm and on-line ultraviolet (UV) spectra from 190 to 800 nm were recorded. The HPLC system was coupled to 2020-MS quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization source (ESI). High purity nitrogen was used as nebulizer and auxiliary gaz. The mass spectrometer (Shimadzu Lab Solutions LC–MS software) was operated in negative ion mode with a capillary voltage of −3.5 V, a nebulizing gas flow of 1.5 L/min, a dry gas flow rate of 12 L/min, a DL (dissolving line) temperature of 250 °C, a block source temperature of 400 °C, a voltage detector of 1.35 V and the full scan spectra from 50 to 2000 Da.

Results

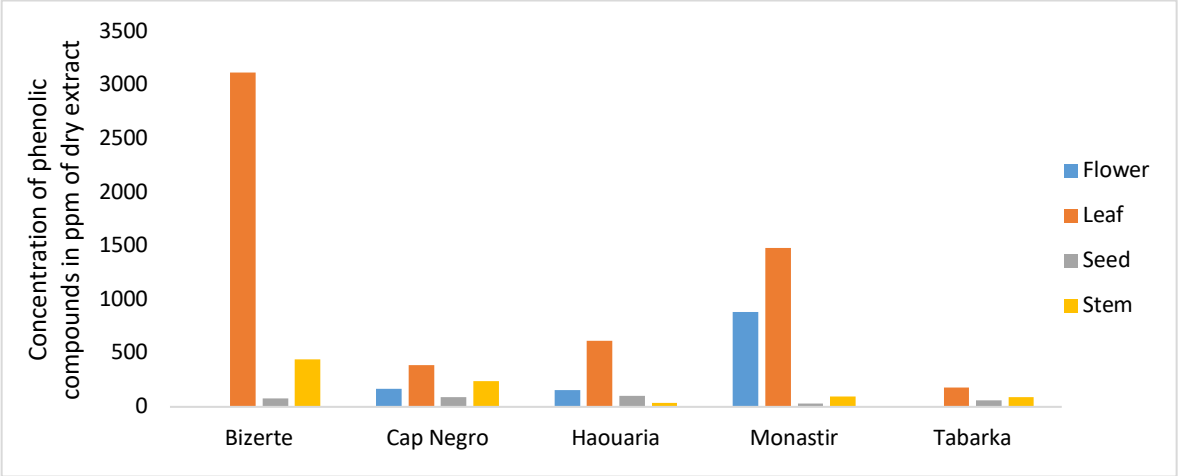
Results illustrate the concentration of phenolic compounds (in ppm of dry extract) in various plant parts of sea fennel plant parts (flowers, leaves, seeds, and stems) collected from five distinct regions in Tunisia: Bizerte, Cap Negro, Haouaria, Monastir, and Tabarka, reveal clear variations in phenolic content depending on both plant part and collection site. Leaves were consistently the richest in phenolic compounds across all regions, with the highest concentration observed in Bizerte, where the leaf content reached approximately 37,500 ppm. This is significantly higher than any other value recorded for any plant part or location. The second highest leaf concentration was found in Monastir, at around 19,000 ppm, followed by Haouaria (~5,500 ppm), Cap Negro (~4,600 ppm), and Tabarka (~2,300 ppm). These figures suggest that environmental factors in Bizerte may particularly enhance phenolic accumulation in sea fennel leaves.

The flowers showed notable phenolic levels only in Monastir, with a concentration close to 14,000 ppm, making it the only site where flower phenolic content approached that of the leaves. In other regions, the flower concentrations were markedly lower: Haouaria (~2,100 ppm), Cap Negro (~1,500 ppm).

Stems, while generally lower in phenolic content, presented some variation. In Bizerte, stem phenols concentration reached about 5,300 ppm, making it the second richest plant part at that site. Other regions had more modest values: Cap Negro (~2,700 ppm), Monastir (~1,100 ppm), Tabarka (~800 ppm), and Haouaria (~500 ppm).

Seeds consistently exhibited the lowest phenolic concentrations, typically ranging between 400 to 1,000 ppm across all sites. The highest seed content was recorded in Haouaria (~1,000 ppm), while other locations such as Bizerte, Cap Negro, and Tabarka showed lower values in the 600–900 ppm range. Monastir had the lowest seed phenolic content, close to 200 ppm.

In conclusion, the phenolic composition of sea fennel varies significantly by plant part and site. Leaves, especially those from Bizerte and Monastir, are the most potent sources of phenolic compounds, followed by flowers in Monastir. Stems and seeds contribute less to the total phenolic profile, but Bizerte again stands out for stem phenolic content. This data underscores the importance of both plant part selection and geographical origin in maximizing the nutritional and medicinal value of sea fennel.



Concentration of phenolic compounds in ppm of dry extract by site and plant part

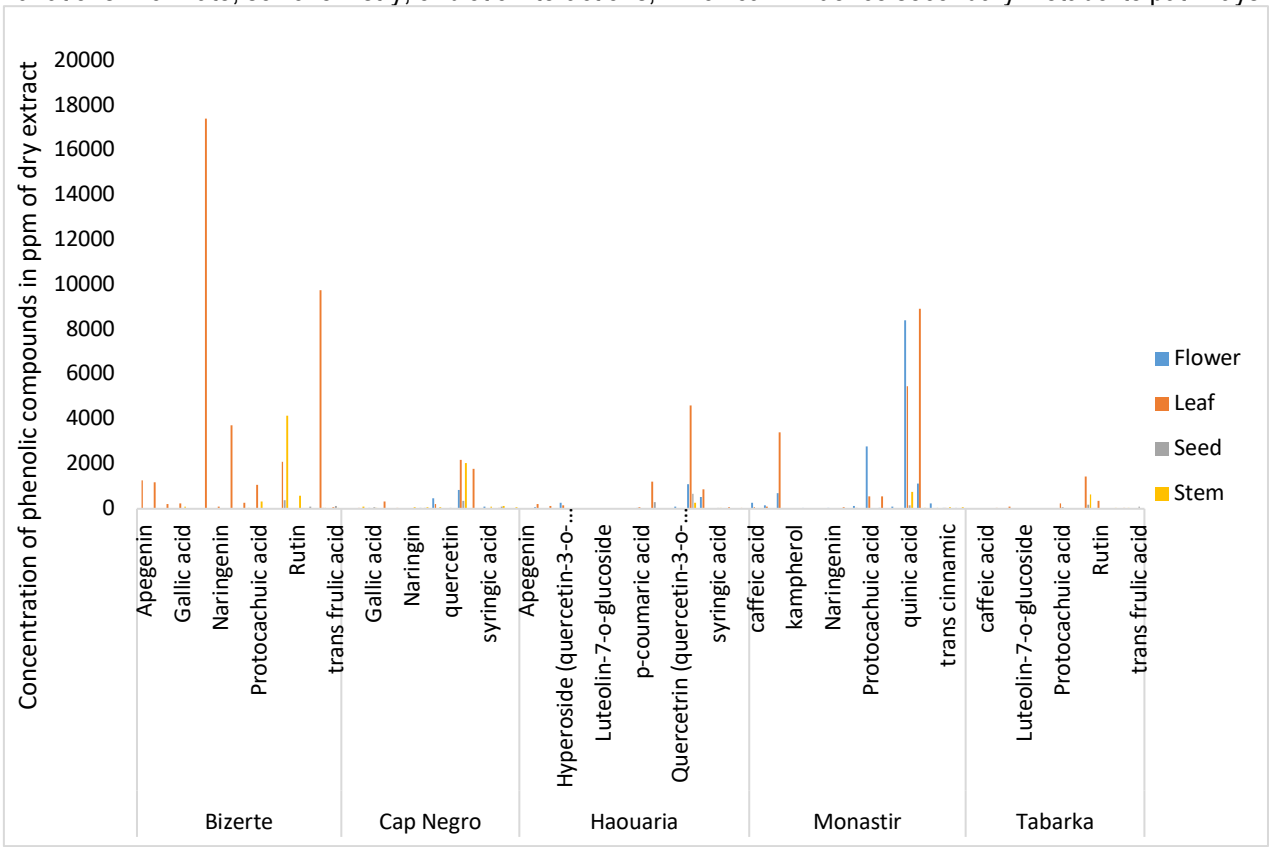
Profiling the phenolic compounds provides a comprehensive quantitative and qualitative overview of phenolic composition in different parts of *Crithmum maritimum* (sea fennel). The analysis includes a wide range of phenolic classes, notably flavonoids (e.g., flavonols, flavones, flavanones), phenolic acids (hydroxybenzoic and hydroxycinnamic acids), and other polyphenolic derivatives. These compounds profiling reveal significant variations in both concentration and composition across sites and tissues.

The analysis of the phenolic composition across different plant parts and geographic locations reveals that phenolic acids are the predominant class of compounds. These include quinic acid, gallic acid, protocatechuic acid, caffeic acid, p-coumaric acid, trans-ferulic acid, and trans-cinnamic acid. Their concentrations are particularly elevated in leaf tissues, which are metabolically active and exposed to environmental stressors. For instance, Bizerte leaves show strikingly high levels of quinic acid (over 2100 ppm), protocatechuic acid (1067 ppm), and trans-cinnamic acid (almost 9800 ppm), indicating robust phenolic acid biosynthesis likely linked to protective functions against UV radiation, herbivory, and oxidative stress. Similarly, flower tissues from Monastir display high phenolic acid concentrations, especially quinic and protocatechuic acids, reflecting their potential role in protecting reproductive organs and supporting pollinator attraction through secondary metabolic signals.

In contrast, flavonoids, including flavonols, flavones, and flavanones, exhibit a more selective distribution, with notable accumulations in leaves and flowers. Bizerte leaves, for example, are especially rich in flavones such as luteolin-7-O-glucoside (17408 ppm) and apigenin-7-O-glucoside (1172 ppm), as well as the flavanone naringin (3726 ppm). This suggests a complex flavonoid network that contributes to antioxidative defense, photoprotection, and possible signaling functions. Monastir flowers, on the other hand, contain high levels of flavonol glycosides like rutin (1129 ppm), hyperoside (707 ppm), and various quercetin derivatives, compounds well known for their roles in color expression and UV protection in floral tissues.

Among less studied organs, stems and seeds show comparatively modest but still significant phenolic content. Stems, particularly from Cap Negro, contain measurable amounts of naringin, quercetin, and luteolin derivatives, indicating ongoing transport or localized accumulation of phenolics possibly linked to structural integrity and defense. Seeds, which are typically rich in storage and protective compounds, show site-specific differences. For instance, Cap Negro and Haouaria seeds have detectable levels of flavonoids like quercetin and naringin and phenolic acids such as syringic and caffeic acids, possibly contributing to seed viability and longevity.

The geographic variation further emphasizes the role of environmental and possibly genetic factors in shaping phenolic profiles. Bizerte samples, especially leaves, demonstrate a rich and diverse phenolic composition, dominated by both acids and flavonoids. Monastir excels in floral phenolic richness, while Haouaria and Cap Negro present more balanced profiles across different organs. These regional differences may reflect variations in climate, soil chemistry, or biotic interactions, which can influence secondary metabolite pathways.



Concentration of different phenols in ppm of dry extract by site and plant part

In summary, phenolic acids are the most widespread and abundant phenolic class across all samples, particularly in leaves and flowers, suggesting a central role in core physiological and protective processes. Flavonoids, while less uniformly distributed, are crucial in specialized functions such as UV screening, pollinator attraction, and antioxidative defense. The data clearly support a complex, tissue- and environment-specific regulation of phenolic metabolism in sea fennel.

Comparative Summary by Phenolic Group

Site	Organ	Key Phenolic Acids	Key Flavonoids	Notable Observations
Bizerte	Leaves	Quinic acid (2132.63 ppm), Trans-cinnamic acid (9799.67 ppm)	Luteolin-7-O-glucoside (17408.12 ppm), Naringin (3726.79 ppm)	Extremely rich in flavonoids and phenolic acids

Site	Organ	Key Phenolic Acids	Key Flavonoids	Notable Observations
	Flowers	Quinic acid (1018.79 ppm), Gallic acid (381.89 ppm)	Rutin, Apigenin derivatives	Moderate phenolics; Rutin and Apigenin present
	Stems	Quinic acid, Caffeic acid	Naringin, Luteolin-7-O-glucoside	Lower overall phenolic levels
Monastir	Flowers	Quinic acid (1282.80 ppm), Protocatechuic acid (434.61 ppm)	Rutin (1129.19 ppm), Hyperoside, Quercetin derivatives	High flavonol content typical of floral tissue
	Leaves	Quinic acid (1093.11 ppm)	Luteolin-7-O-glucoside (4776.04 ppm), Apigenin, Naringin	Good balance of phenolic acids and flavones
	Stems	Quinic acid (911.76 ppm)	Naringin (506.95 ppm), Luteolin-7-O-glucoside	Moderate content in all classes
Haouaria	Seeds	Caffeic acid (252.29 ppm), Syringic acid (56.38 ppm)	Naringin, Quercetin, Luteolin-7-O-glucoside	Key acids with moderate flavonoids
	Stems	Similar to seeds	Similar to seeds	Overall lower concentrations
	Leaves	Quinic acid	Luteolin-7-O-glucoside	Moderate phenolic and flavonoid content
Cap Negro	Seeds	Caffeic acid (240.96 ppm), Quinic acid (221.70 ppm)	Naringin, Quercetin	Balanced phenolic and flavonoid profile in seeds
	Stems	Quinic acid	Naringin, Quercetin, Luteolin-7-O-glucoside	Moderate levels of key flavonoids
	Leaves	Quinic acid	Luteolin-7-O-glucoside (1503.79 ppm)	Luteolin-7-O-glucoside is dominant

To summarize, this detailed phenolic profiling reveals that leaf tissue, especially from Bizerte and Monastir, contains the richest and most diverse array of health-promoting phenolics. Bizerte stands out for its extremely high flavonol content, while Monastir offers a balanced mix of flavonoids and phenolic acids, particularly in flowers. Such data can inform optimized harvesting practices for nutraceutical extraction and functional food production, targeting plant parts and regions with the most valuable phytochemical profiles.

4.2.4 Fatty acids

Material and methods

Lipids were extracted using Chloroform/methanol (2:1 v/v) solution and 1g of plant powder. After the evaporation of solvent fractions, the fatty acids were converted into fatty acid methyl esters (FAMEs) using solution of sodium methoxide (3% in methanol). After neutralization of the reaction mixture with 0.1 mL of H2SO4 and phase separation, the organic phase containing FAMEs was recovered and analyzed by gas phase chromatography with flame ionization detection (GC-FID).
The chromatographic analysis of FAMEs was carried out according to the IUPAC 2.301 (1987) standard method. FAMEs analysis was performed on a Hewlett-Packard HP-6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and a TR-FAMEs polar capillary column (Thermo Fisher Scientific Inc., Bordeaux, France) (60 m × 0.25 mm, film 0.25 m thickness). The column temperature was initially held at 100°C for 5 min, increased to 240°C (4°C/min), then held isothermal for 15 min. The temperature of the injector and the FID detector were maintained at 240°C and 260°C, respectively. The FAMEs were identified by comparing their retention times with those of commercial standards.

Results

Fatty acid composition (in percentage of total identified fatty acids) of different organs (flower and fruit) of sea fennel (*Crithmum maritimum*) collected from five coastal sites in Tunisia: Tabarka, Cap Negro, Bizerte, Monastir, and Haouaria were measured, including saturated fatty acids (SFAs) like palmitic, stearic, arachidic, behenic, lignoceric, cerotic, melissic, and montanic acids, as well as unsaturated fatty acids (UFAs) such as linoleic, oleic, and petroselinic acids.
A distinct pattern is observed between flower and fruit tissues. Fruits consistently exhibit a higher proportion of petroselinic acid, especially in samples from Bizerte (94.44%), Tabarka (89.83%), Monastir (81.39%), Haouaria (71.59%), and Cap Negro (74.7%). This suggests that petroselinic acid is the dominant fatty acid in sea fennel fruits across all sites.
In contrast, flowers display more diverse fatty acid profiles, with a relatively more balanced distribution between saturated and unsaturated fatty acids. For example, the flowers from Cap Negro contain high levels of linoleic acid (73.65%) and moderate levels of several SFAs, including montanic acid (4.94%), melissic acid (3.25%), and cerotic acid (2.32%). Similarly, Monastir flower samples contain notable amounts of oleic acid (27.79%) and linoleic acid (38.55%), alongside a range of SFAs.
Tabarka: Flower samples are rich in linoleic acid (45.49%) and oleic acid (23.32%), whereas the fruit is dominated by petroselinic acid (89.83%), with little presence of other fatty acids.
For Cap Negro, the flower samples exhibit the highest linoleic acid content (73.65%) among all sites and a notable presence of long-chain SFAs (montanic, melissic, and cerotic acids). The fruits have a high petroselinic acid concentration (74.7%) with minor contributions from linoleic and oleic acids.
For Bizerte flowers, they contain a balanced mix, including palmitic (14.87%), linoleic (33.98%), and petroselinic (26.47%) acids, plus a significant presence of long-chain SFAs. Fruits are almost exclusively composed of petroselinic acid (94.44%).
In Monastir flower, samples are rich in oleic (27.79%) and linoleic acids (38.55%) and exhibit a wide range of minor SFAs. Fruit samples, however, are heavily dominated by petroselinic acid (81.39%).
The Haouaria flower samples show a uniquely high palmitic acid concentration (21.78%) and petroselinic acid (33.72%), with a modest amount of linoleic acid (35.46%). The fruit also displays a dominant petroselinic profile (71.59%), with some linoleic and oleic acid contributions.
The results reveal that petroselinic acid is a key biomarker of sea fennel fruits, being overwhelmingly dominant across all regions, suggesting a conserved biochemical profile in reproductive tissues. Conversely, flowers are

chemically more complex, containing a mix of saturated and unsaturated fatty acids, with site-specific differences in the abundance of linoleic, oleic, and long-chain SFAs. This highlights the potential of sea fennel flowers as a more versatile source of fatty acids, while fruits are a concentrated source of petroselinic acid, which has industrial and nutritional relevance.

Fatty acids composition of fixed oil by organ and site in %

Site	Organ	Palmitic acid	Linoleic acid	Petroselinic acid	Oleic acid	Stearic acid	Lignoceric acid	Cerotic acid	Melissic acid	Arachidic acid	Behenic acid	Montanic acid
Tabarka	Flower	17.32	45.49		23.32	4	4.09	3.97	1.81			
Tabarka	Fruit	7.58		89.83		2.6						
Capnegro	Flower	14.24	73.65			3.92	2.62			2.32	3.25	4.94
Capnegro	Fruit	7.73	10.68	74.7	6.9							
Bizerte	Flower	14.87	33.98	26.47		4.06	3.79	4.33	4.51	3.06		
Bizerte	Fruit	5.56			94.44							
Monastir	Flower	16.35	38.55		27.79	2.97	3.05	3	2.87	2.67		2.74
Monastir	Fruit	4.87	13.74	81.39								
Haouaria	Flower	21.78	35.46	33.72		7.49				1.55		
Haouaria	Fruit	6.45	16.74	71.59	5.23							

4.2.5 Volatile organic compounds

Material and methods

Essential oils were extracted from dried sea fennel plants collected from five Tunisian populations. Oil yields were determined. Identification of the essential oils was performed using a Hewlett Packard HP5890 series II GC-MS equipped with an HP5MS column (30 m ×0.25 mm). Helium was used as carrier gas at 1.2mL.min−1. Each sample (1µL) was injected in the split mode (1:20), the program used was isothermal at 70°C, followed by 50 to 240°C at a rate of 5°C.min−1, then held at 240°C for 10 min. The mass spectrometer was an HP 5972. The total electronic impact mode at 70 eV was used. The components were identified by comparing their relative retention times and mass spectra with the data from the library of essential oils constituents, Wiley, Mass Finder, and Adams GC-MS libraries and by comparing their RI calculated with C8 to C40 Alkanes Calibration Standard (40147-U, Supelco, Germany).

Essential oils extraction from Sea Fennel samples

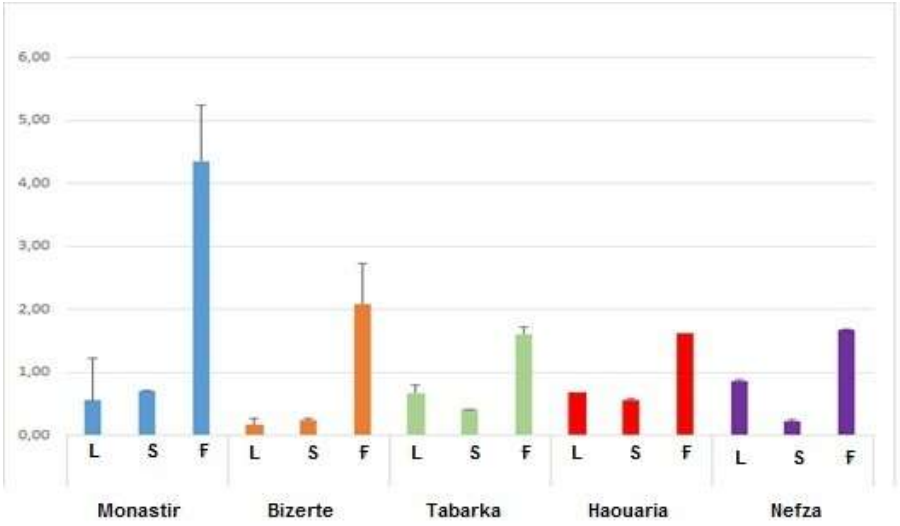
Results

Essential oil from whole plant

The five studied oils showed differences in densities, colors and smells. Oil yield varied from 0.89% and 1.49%. Oil from Monastir showed the highest yield representing about 1.49% of the dried material. The lowest yield was reached by Bizerte locality with 0.89%.

The chemical composition of essential oils extracted from whole plants is summarized in the following table.

The yield of essential oils extracted from the different parts of sea fennel are presented in the following figure.



Significant differences were highlighted between the yields from the different parts of the plants collected from different localities. The highest yield was reached by flowers collected from Monastir site (4.35%) while the lowest yield was determined for flowers from Bizerte (0.18%).
The composition of volatile compounds found in the essential oils of sea fennel (*Crithmum maritimum*) collected from five distinct coastal regions of Tunisia: Monastir (MON), Tabarka (TAB), Cap Negro (CN), Haouaria (HA), and Bizerte (BZ) was assessed via mass spectrometry and retention indices (RI), and their relative abundance (expressed in percentages) illustrates the chemical variability of the essential oils across different geographical sites.
A standout feature across all sites is the prevalence of γ-terpinene, which is the major volatile compound in the essential oils of most locations. It accounts for 63.32% in Cap Negro, 55.43% in Tabarka, 54.95% in Bizerte, and 50.94% in Monastir. These high values suggest that γ-terpinene is a dominant and possibly characteristic component of Tunisian sea fennel essential oil. However, Haouaria diverges from this trend, showing a much lower concentration of γ-terpinene at only 4.41%, indicating a significantly different chemical profile.
In contrast, dillapiole is exceptionally dominant in Haouaria, where it constitutes 88.25% of the essential oil composition. This is remarkably higher than in other locations such as Monastir (11.60%), Tabarka (8.62%), and Bizerte (0.41%), and it is entirely absent in Cap Negro. This stark difference highlights Haouaria's unique chemotype, possibly influenced by distinct environmental conditions or genetic factors.
Another widely present compound is p-cymene, found in considerable amounts at all sites, especially in Bizerte (9.74%) and Cap Negro (7.59%), followed by Monastir (6.52%) and Tabarka (4.51%). Haouaria again has the lowest level of this compound at 0.46%. Anisole also shows fairly consistent levels across the sites, ranging between 28.20% and 31.34%, with Haouaria again being an outlier at 6.72%.
Other minor monoterpenes such as α-pinene, β-myrcene, β-phellandrene, and thymol methyl ether are present in varying low concentrations, reflecting the chemical complexity and diversity of the essential oils. For example, α-pinene is most abundant in Cap Negro (0.57%) and nearly absent in Haouaria (0.01%).

In conclusion, the essential oil profiles of sea fennel vary significantly by site, with γ-terpinene being the principal compound in most locations, whereas Haouaria stands out with dillapiole as its main constituent. These variations suggest potential for regional chemotypes within Tunisia, which may be valuable for both taxonomic studies and applications in food, cosmetic, or pharmaceutical industries.

RI	Identification	Compound	MON	TAB	CN	HA	BZ
928	MS,RI	α-Thujene	0.07	0.06	0.00	0.00	0.08
932	MS,RI	α-Pinene	0.21	0.15	0.57	0.01	0.23
939	MS,RI	Sabinene	0.13	0.14	0.00	0.00	0.42
980	MS,RI	β-Pinene	0.02	0.00	0.00	0.00	0.00
991	MS,RI	β-Myrcene	0.11	0.13	0.00	0.00	0.22
1001	MS,RI	α-Phellandrene	0.00	0.00	0.00	0.00	0.00
1014	MS,RI	α-Terpinene	0.09	0.06	0.00	0.00	0.00
1026	MS,RI	p-Cymene	6.52	4.51	7.59	0.46	9.74
1031	MS,RI	β-Phellandrene	0.12	0.33	0.00	0.04	4.51
1053	MS,RI	γ-Terpinene	50.94	55.43	63.32	4.41	54.95
1235	MS,RI	Thymol methyl ether	0.15	0.23	0.32	0.04	0.30
1327	MS,RI	Anisole	29.91	30.34	28.20	6.72	29.11
1520	MS,RI	β-Sesquiphellandrene	0.01	0.00	0.00	0.07	0.00
1620	MS,RI	Dillapiole	11.60	8.62	0.00	88.25	0.41

Essential oils from the different parts of Sea fennel

4.3 Molecular analyses

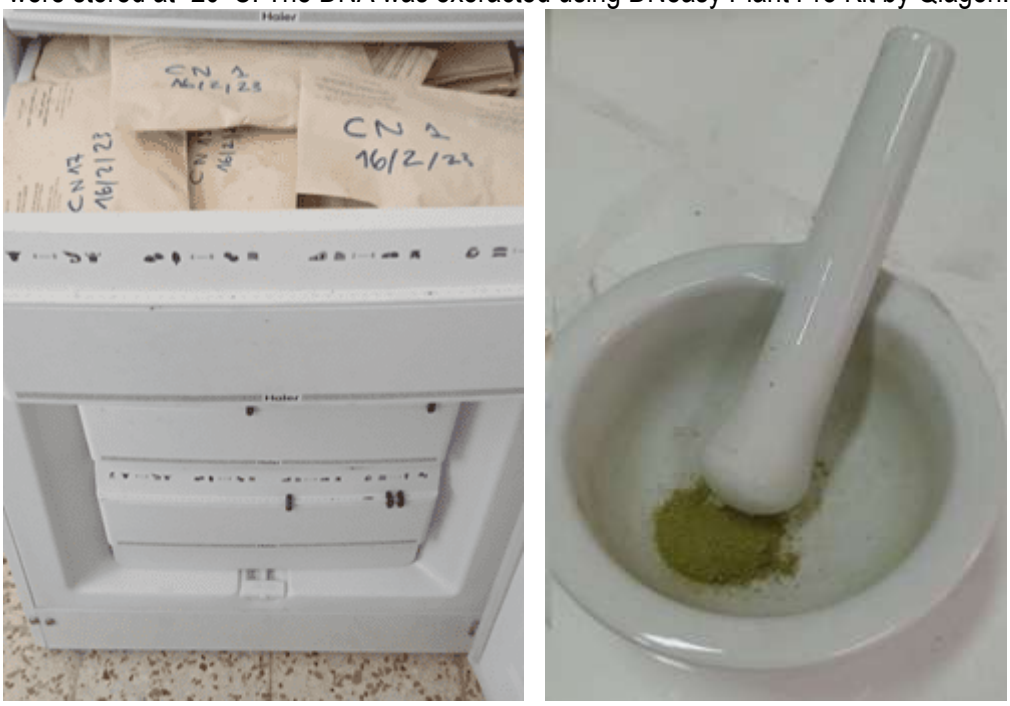
4.3.1 DNA extraction

About 100 gr of leaves were collected from the 5 individuals of each Tunisian population reported below and used for DNA extraction.

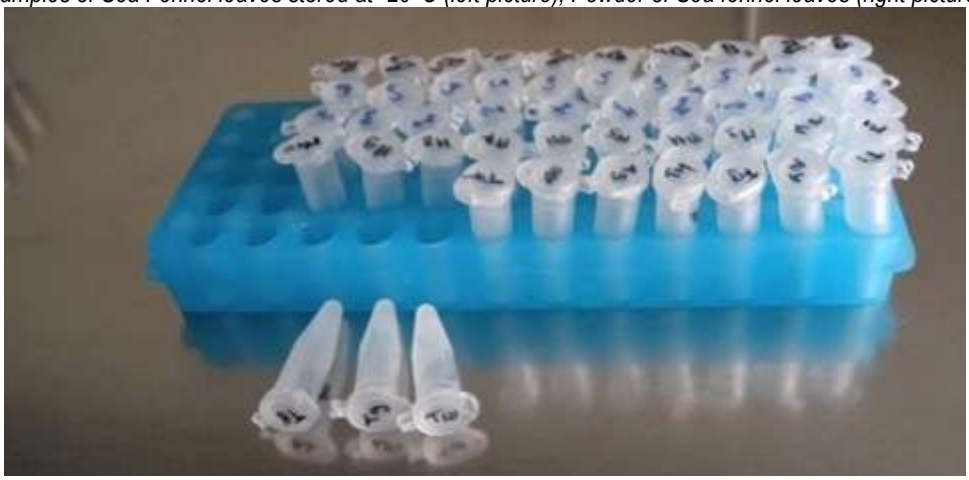
Region	GPS Coordinates	Code
Bizerte	37,25265°N, 9,94500°E	BIZ
Tabarka	36,95951°N, 8,75302°E	TAB
Cap-Negro	37,10278°N, 8,98423°E	CN
Haouaria	37,05143°N, 10,94242°E	HA

Monastir	35,78414°N, 10,83414°E	MON

The leaves were stored at -20°C. The DNA was exeracted using DNeasy Plant Pro Kit by Qiagen.

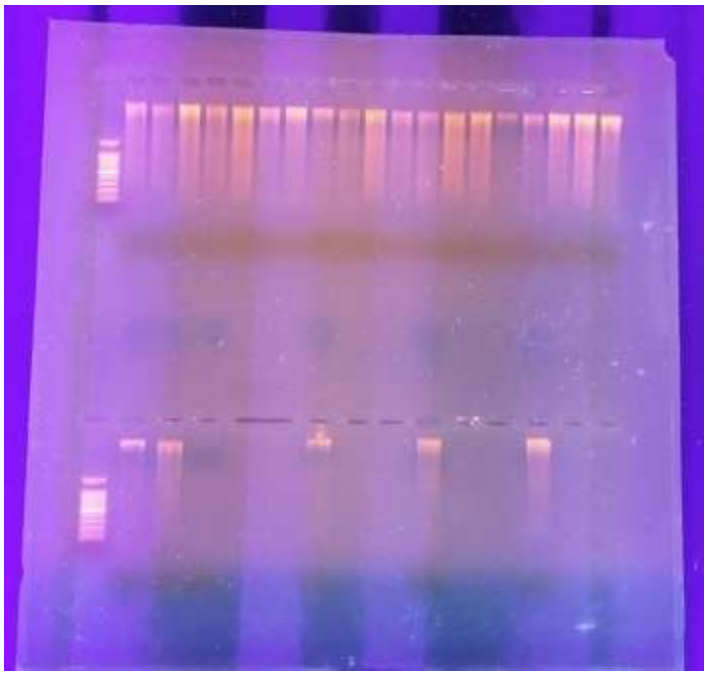


Samples of Sea Fennel leaves stored at -20°C (left picture), Powder of Sea fennel leaves (right picture)



DNA samples with specific codes

Spectrophotometric analysis of DNA purity and quality were performed followed by electrophoresis on agarose analysis in order to check DNA integrity. A digestion with restriction endonuclease HaeIII test was performed in order to characterize the suitability of DNA extracts in terms of ddRAD analysis.



4.3.2 Sequencing

Materials and methods

Sequencing was performed using Specific locus amplified fragment sequencing or SLAF-seq, a method similar to ddRADseq. Analysis was done by Biomarker Technologies (BMK) Company (Munster, Germany). SLAF-seq involve a first digestion using a restriction enzyme HaeIII, followed by heat inactivation and a second digestion with the enzyme Hpy166II. The enzymes and the fragments sizes are optimized with training data. Restriction fragments are selected within a narrow size range to maximize PCR efficiency.

After digestion, ATP and dual-index sequencing adapter were ligated to the 3' and 5' end of the digested DNA fragments. Fragments were amplified and purified using PCR and E.Z.N.A.H Cycle Pure Kit (Omega). The purified products were mixed and re-incubated with the two restricted enzymes again. After ligation of ATP, and Solexa adapter in the pair-end, the products were purified again using a Quick Spin column (Qiagen, Venlo, Netherlands), and segregated on a 2% agarose gel. Fragments within a desired size range were isolated using a Gel Extraction Kit (Tiangen). These SLAFs were then subjected to PCR amplification to incorporate barcodes. The PCR products were re-purified and then prepared for paired-end sequencing on an Illumina HiSeq sequencing platform (Illumina, San Diego, CA, USA).

Data analysis

The raw sequencing data were processed using the bioinformatics platform BMKCloud (www.biocloud.net).

Quality control

Raw reads (in fastq format) were initially processed with fastp software to remove adapter sequences, poly-N stretches, and low-quality reads. The resulting clean reads were assessed for quality, with Q30 and GC-content values calculated. All downstream analyses were based on these high-quality clean reads.

Reads mapping to the reference genome

After data cleaning, reads were mapped to the reference genome using the BWA software and sequences with adapter contamination or low quality were excluded.

SNP/INDEL Calling

Single nucleotide polymorphisms (SNPs) and insertions/deletions (INDELs) were identified using GATK (v3.8) and SAMtools (v1.9.1). A total of 448,436 SNPs with acceptable minor allele frequency (MAF) and integrity were retained for further analysis.

SNP/INDEL Annotation

SNP annotation was carried out using the snpEff software (version 3.6c). SNPs were categorized based on their location within the genome (intergenic, upstream/downstream, exonic, or intronic). Synonymous and nonsynonymous SNPs in coding exons were further classified, and INDELs in exons were examined for frameshift effects.

Gene functional annotation

Gene function was annotated using several databases:

- Nr (NCBI non-redundant protein sequences)
- Pfam (Protein families)
- KOG/COG (Clusters of Orthologous Groups of proteins)
- Swiss-Prot (A manually curated protein sequence database)
- KO (KEGG Ortholog database)
- GO (Gene Ontology).

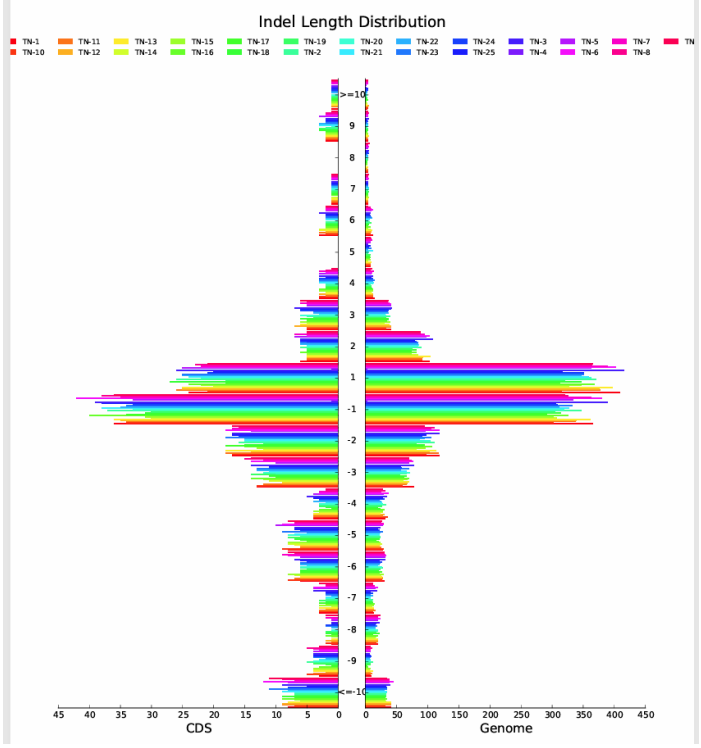
Advantages of this Methodology

This methodology offers several benefits:

- High sequencing depth ensures accurate genotyping.
- A reduced representation strategy minimizes sequencing costs.
- A pre-designed reduced representation scheme optimizes marker efficiency.
- A dual barcode system enables efficient handling of large sample populations.

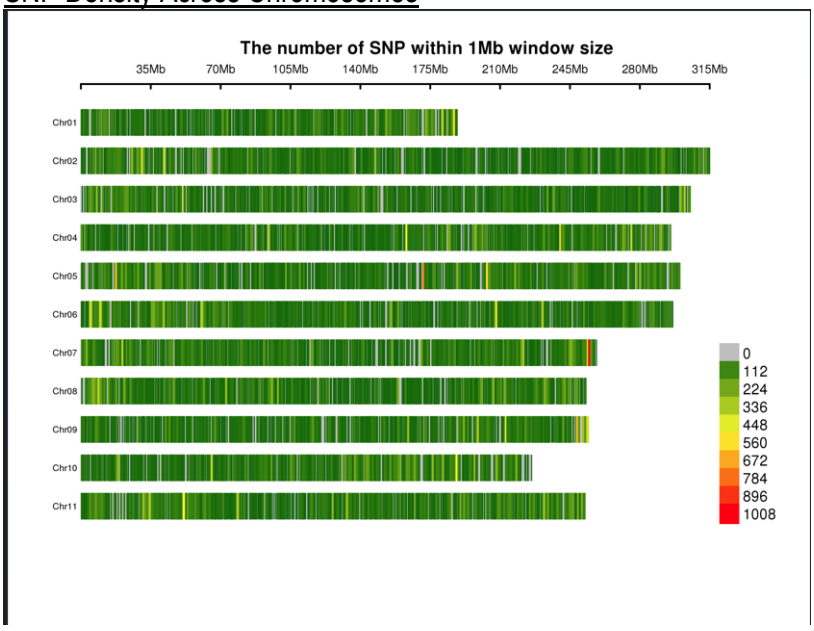
4.3.3 Analysis of sequences

Indel Length Distribution



This graph shows the frequency of insertions and deletions (indels) of different lengths in both coding regions (CDS) and the whole genome. Most of the mutations are short indels, especially those with lengths of ±1 to ±3 base pairs, which are common in natural populations. Longer indels (greater than ±10 bp) are much rarer, especially in coding regions. This is because large indels in genes can disrupt protein function and are often removed by natural selection. The similarity of patterns across all samples (TN-1 to TN-25) suggests that these indels are distributed in a consistent way among the individuals studied.

SNP Density Across Chromosomes



This heatmap represents the number of single nucleotide polymorphisms (SNPs) in 1 Mb windows across each chromosome (Chr01 to Chr11). Most regions are shown in green or yellow, indicating a moderate number of SNPs. Some regions, especially on chromosomes 5 and 7, are colored orange to red, showing high SNP density. These areas may be biologically significant: they could contain genes under selection or regions with high recombination rates. Gray areas with zero SNPs may represent parts of the genome that were not well sequenced or naturally have low variation, such as centromeres or repetitive DNA.

The SNP genotyping data revealed a generally high level of genetic diversity among the 25 samples, with SNP counts ranging from 117,746 to 150,138 and heterozygosity ratios between 20.53% and 27.43%. Most samples showed good data integrity, with over 60% of the total 208,451 SNPs retained per individual. Notably, samples such as TN-6, TN-1, TN-3, and TN-13 exhibited both high SNP integrity (above 69%) and elevated heterozygosity, indicating substantial within-sample genetic variation. In contrast, samples TN-17, TN-19, and TN-14 had relatively lower integrity and heterozygosity, suggesting reduced diversity or potential data gaps. One outlier, TN-4, had extremely poor SNP coverage (only 2,274 SNPs, 1.09% integrity) and low heterozygosity (11.48%), rendering it unsuitable for downstream genetic diversity or structure analyses. Overall, the dataset is of high quality and suitable for reliable assessment of genetic variation, with the exception of TN-4, which should be excluded from further analyses.

4.4 Statistical analysis

Statistical analysis was performed in order to differentiate between-populations differences regarding morphological and biochemical characteristics of sea fennel sampled in Tunisia. Statistical differences

between samples were determined using the analysis of variance (ANOVA coupled with Fisher's significance test with a consideration of a p-value<0.05). Post Hoc Multicomparison test was performed using Tukey test. Statistical analysis were performed using R 4.1.1 software.

5 Turkish sea fennel populations

Flowers, leaves and seeds from the 4 wild sea fennel populations sampled across different regions of İzmir Province listed below have been subjected to morphometric, chemical and molecular analyses. Data were then statistically elaborated.

Location	Çandarlı (Dikili)	Urla	Çeşme	Seferihisar
Geographic latitude	38°55'13.51"N	38°22'3.90"N	38°14'28.14"N	38° 8'14.62"N
Geographic longitude	26°52'47.30"E	26°49'46.50"E	26°22'36.62"E	26°49'48.24"E
Exposure	East	North	South	West
Soil	Andesite rocks formed from extrusive igneous magmatic rocks	Limestone (calcareous rock) on the parent rock	Volcanic tuff/tuffite rocks	Sedimentary sandstone-shale mixed rock
The proximity of the sea	closer than 10 m	closer than 10 m	closer than 10 m	closer than 10 m
Presence of other vegetation	Salsola kali L.	Anthemis tomentosa L. Glacium flavum Crantz, Salsola kali L. Atriplex hastata Geners.	Limonium gmelini (Willd.) Kuntze, Salsola kali L. ,	Glacium flavum Crantz, Centaurea spinosa L. , Salsola kali L., Carlina corymbosa L., Limonium gmelini (Willd.) Kuntze, Trifolium uniflorum L. Malcolmia flexuosa (Sm.) Sm.

5.1 Morphological characterization

Materials and methods

The morphometric characterization of the 4 Turkish wild sea fennel populations was done according to the shared procedure among partners detailed in Annex I (see the end of this deliverable).

Starting from the Northern region of İzmir, the coastal areas were scanned with the mapping method, and 4 different populations were selected for sampling, taking care to have different geological substrates. Sample areas are within the boundaries of Çandarlı (Locality 1), Urla (Locality 2), Çeşme (Locality 3), and Seferihisar (Locality 4). GPS data of each sampling area was recorded. Samples were taken from 20 individuals from each population, paying attention to the fact that there was a distance of 5 meters between individuals. Mature seeds were also sampled, cataloged and stored at +4°C and -20°C. The aerial parts of the sampled individuals were dried in silica gel (as the whole plant and plant parts: leaves, stems, flowers).

Measurements of the whole plant (length, width, height and diameter of the main stem), as well as counting the number of branches and number of umbels were performed in the field. For each of the 20 plants sampled in each location, 5 main umbels and 5 basal leaves were collected for measurements of some traits performed in the laboratory.

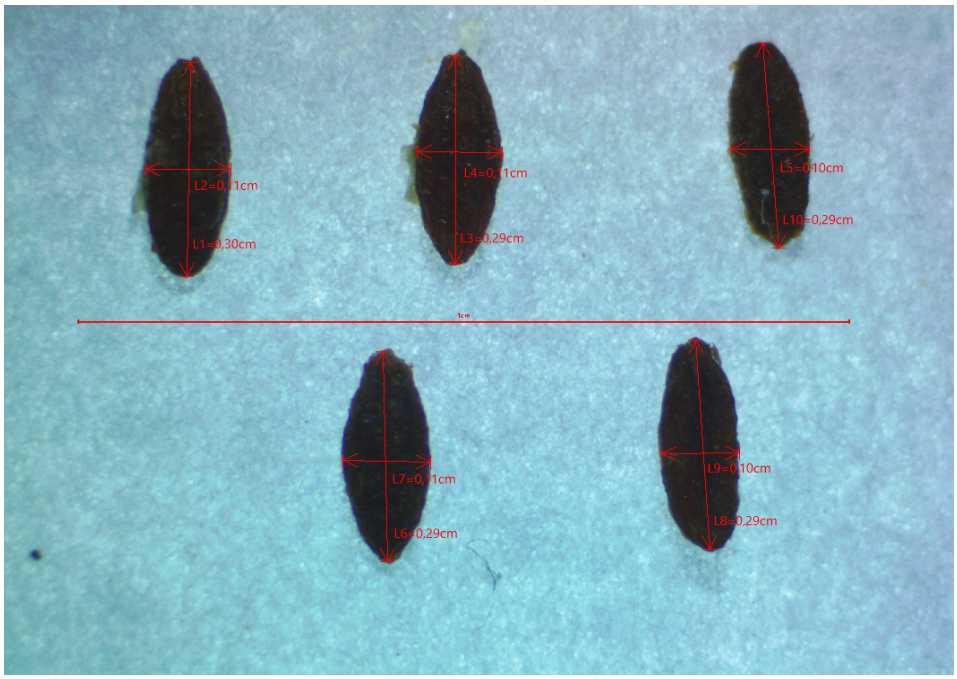
In line with the bedrock and soil characteristics considered in the population determination process; It has been determined that the Çandarlı population spreads on the slopes located on the pink andesite rocks consisting of external igneous rocks. Urla Population Distributes on rocky slopes on limestone bedrock. The Çeşme Population spreads over volcanic tuff/tuffite rocks facing the sea. Seferihisar population spreads on sedimentary sandstone-claystone mixed rocky slopes.

The morphological measurements of each population and seed width and height measurements were ordered in centimeters.

Morphological traits measured/counted on scanned images with the software ImageJ.

Collection of samples and transfer into cilica gel

After the measurements made in the field, the samples were taken into ziplock bags and then brought to the laboratory in an icebox.



Seed measurements

Results

A comprehensive morphological comparison was conducted on four local populations of *Crithmum maritimum* collected from different coastal localities in western Turkey:

In terms of overall plant size, individuals from Urla exhibited the greatest average length (520 mm), followed by Çeşme (436 mm), Çandarlı (422 mm), and Seferihisar (323 mm). The widest plants were recorded in Çandarlı (566 mm), while Urla showed the narrowest width (382.5 mm). Plant height was highest in Çandarlı (279.5 mm) and lowest in Urla (145 mm). Regarding stem diameter, the thickest stems were observed in the Çeşme population (13.00 mm), contrasting with the thinnest stems from Çandarlı (9.80 mm).

One-Way ANOVA (Welch's)

	F	df1	df2	p
Length	4.76	3	40.9	0.006
width	4.86	3	41.6	0.005
height	5.73	3	41.2	0.002
Diameter main stem	8.09	3	41.3	< .001

Tukey Post-Hoc Test – Length

		Dikili	Seferihisar	Urla	Çeşme
Dikili	Mean difference	—	99.0	−98.0	−14.0
	p-value	—	0.322	0.331	0.995
Seferihisar	Mean difference		—	−197.0	−113.0
	p-value		—	0.006	0.212
Urla	Mean difference			—	84.0
	p-value			—	0.468
Çeşme	Mean difference				—
	p-value				—

Tukey Post-Hoc Test – width

		Dikili	Seferihisar	Urla	Çeşme
Dikili	Mean difference	—	139	183.5	151.0
	p-value	—	0.066	0.007	0.038
Seferihisar	Mean difference		—	45.0	12.5
	p-value		—	0.846	0.996
Urla	Mean difference			—	−32.5
	p-value			—	0.935
Çeşme	Mean difference				—
	p-value				—

Tukey Post-Hoc Test – height

		Dikili	Seferihisar	Urla	Çeşme
Dikili	Mean difference	—	59.5	134.5	109.0
	p-value	—	0.305	< .001	0.010
Seferihisar	Mean difference		—	75.0	49.5
	p-value		—	0.130	0.468
Urla	Mean difference			—	−25.5
	p-value			—	0.876
Çeşme	Mean difference				—
	p-value				—

Tukey Post-Hoc Test – Diameter main stem

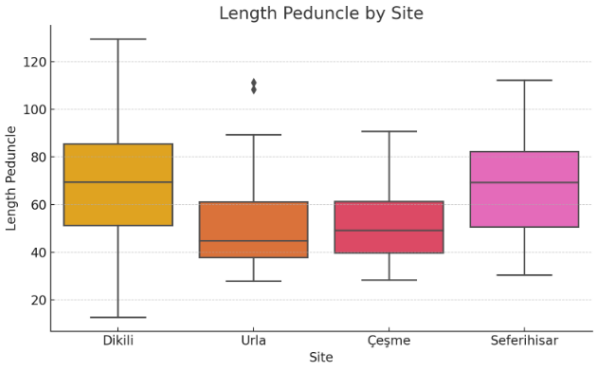
		Dikili	Seferihisar	Urla	Çeşme
Dikili	Mean difference	—	−2.30	−2.150	−3.200
	p-value	—	0.032	0.051	0.001
Seferihisar	Mean difference		—	0.150	−0.900
	p-value		—	0.998	0.693
Urla	Mean difference			—	−1.050
	p-value			—	0.579
Çeşme	Mean difference				—
	p-value				—

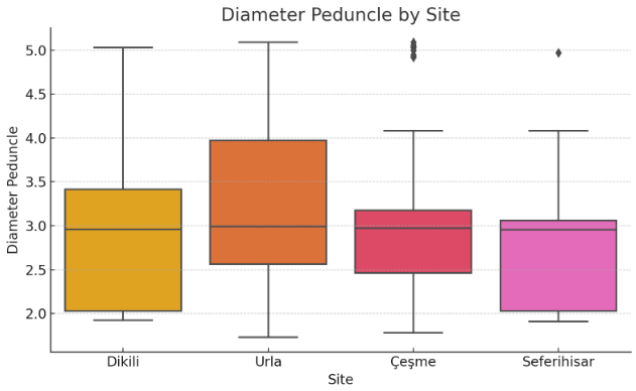
One-way ANOVA (Welch's) on sea fennel plant parts

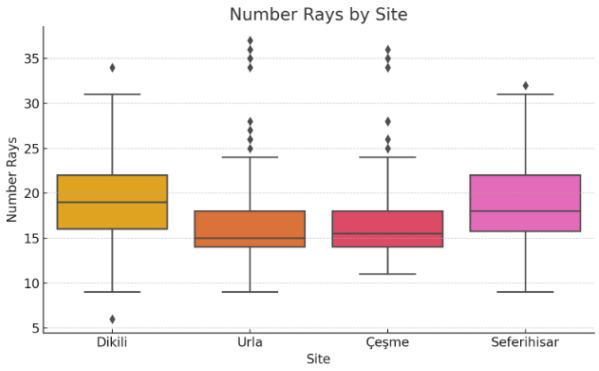
Peduncle length was greatest in Çandarlı (69.39 mm), whereas Urla had the shortest peduncles (50.16 mm). The widest peduncles were measured in Urla (3.24 mm), while Seferihisar had the narrowest (2.71 mm). The number of rays per peduncle was highest in Çandarlı (19.42), followed by Seferihisar (18.84), with Çeşme and Urla displaying slightly lower averages (17.44 and 17.56, respectively). Ray thickness was most pronounced in Çandarlı (0.77 mm), and the slimmest rays were found in Urla (0.61 mm). Interestingly, despite thinner rays, Urla showed the longest ray length (25.24 mm), while Çandarlı had the shortest (22.42 mm).

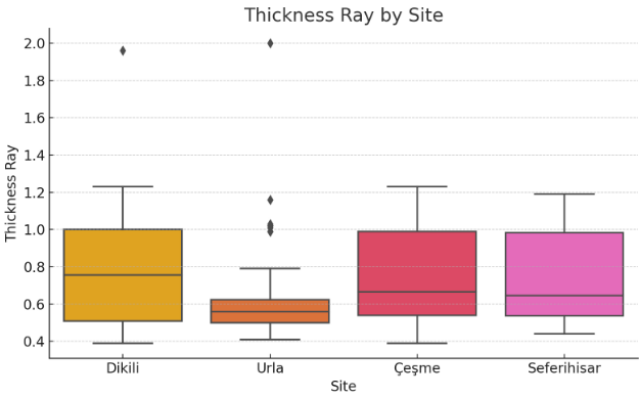
The number of bracts was relatively consistent across populations, with Urla slightly ahead (9.14) and Çandarlı the lowest (8.51). Bract length was longest in Urla (12.93 mm) and shortest in Çandarlı (10.39 mm). With respect to bracteole measurements, Çeşme exhibited the longest bracteoles (4.55 mm), followed by Urla and Seferihisar, while Çandarlı had the shortest (3.67 mm). The widest bracteoles were observed in Urla (1.66 mm), and the narrowest in Seferihisar (1.44 mm). Seferihisar had the highest average number of bracteoles per umbel (8.73), whereas Çeşme had the lowest (7.06).

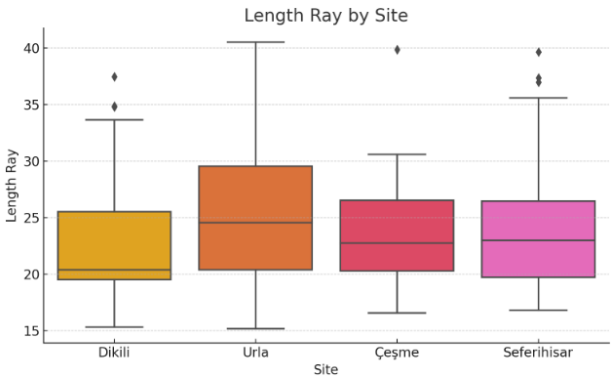
Flower diameter showed relatively limited variation, with the largest average recorded in Urla (55.02 mm) and the smallest in Çandarlı (53.02 mm). The values across all localities were close, suggesting relatively stable floral dimensions despite other morphological fluctuations.

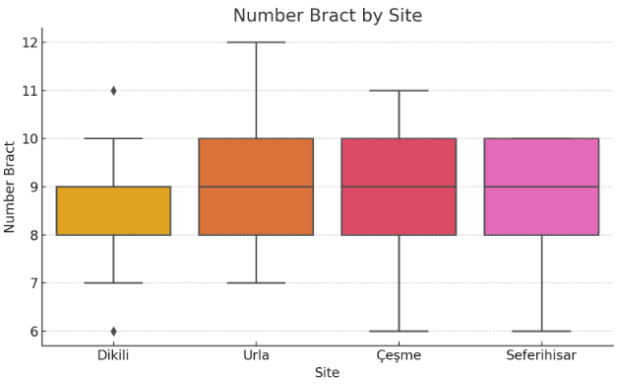

Length Peduncle by Site
Length Peduncle
120
100
80
60
40
20
Dikili
Urla
Çeşme
Seferihisar
Site

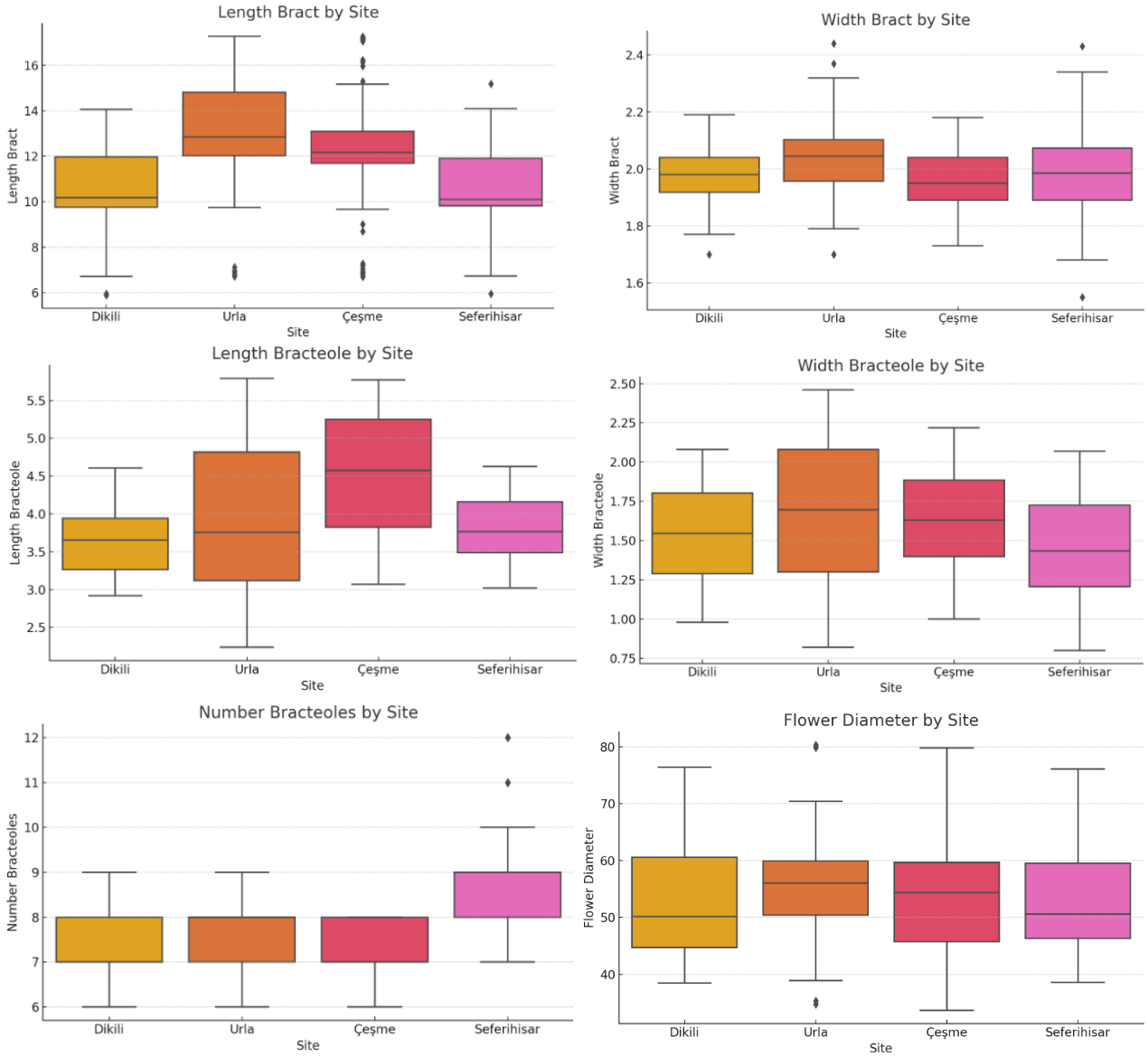

Diameter Peduncle by Site
Diameter Peduncle
5.0
4.5
4.0
3.5
3.0
2.5
2.0
Dikili
Urla
Çeşme
Seferihisar
Site


Number Rays by Site
Number Rays
35
30
25
20
15
10
5
Dikili
Urla
Çeşme
Seferihisar
Site

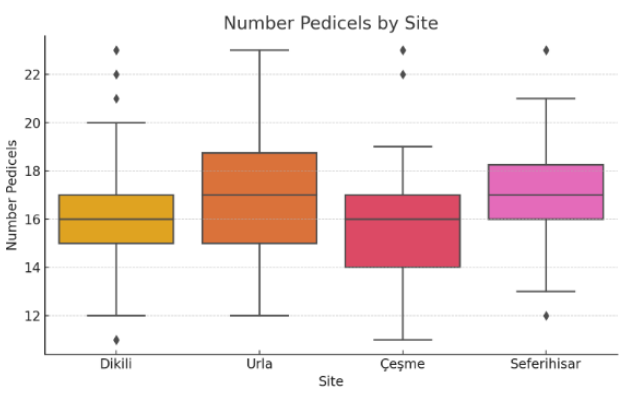

Thickness Ray by Site
Thickness Ray
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
Dikili
Urla
Çeşme
Seferihisar
Site

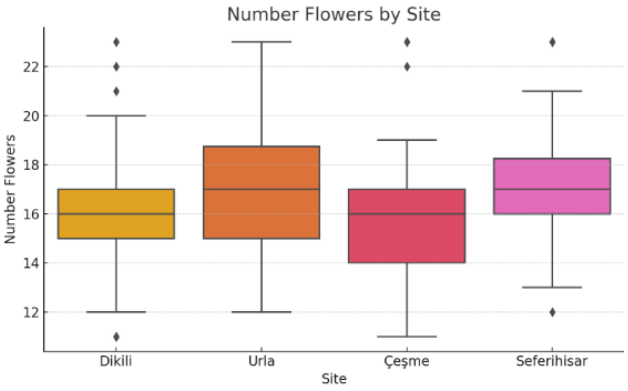

Length Ray by Site
Length Ray
40
35
30
25
20
15
Dikili
Urla
Çeşme
Seferihisar
Site

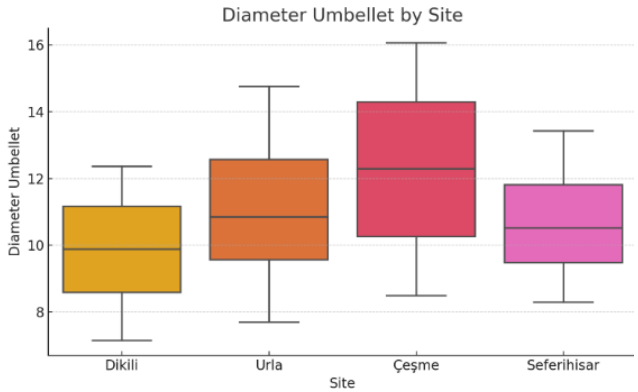

Number Bract by Site
Number Bract
12
11
10
9
8
7
6
Dikili
Urla
Çeşme
Seferihisar
Site



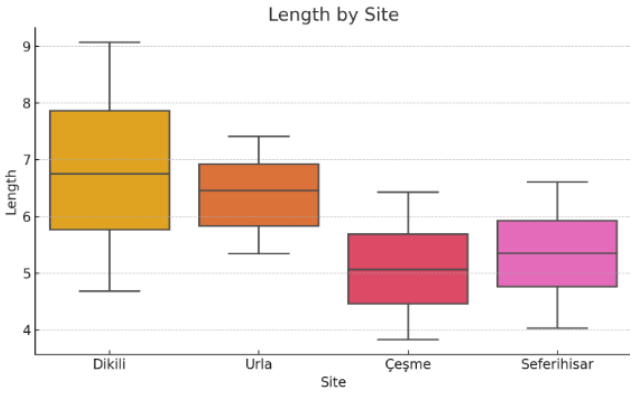
The number of pedicels and number of flowers per umbellet were highest in Seferihisar and Urla (both ~17.18), while Çandarlı (15.93) and Çeşme (15.79) showed slightly lower averages. Interestingly, Çeşme had the widest umbellets (12.33 mm), suggesting a broader floral display despite fewer flowers.

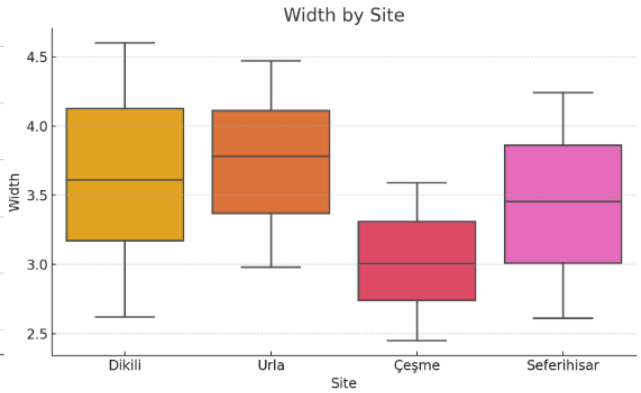


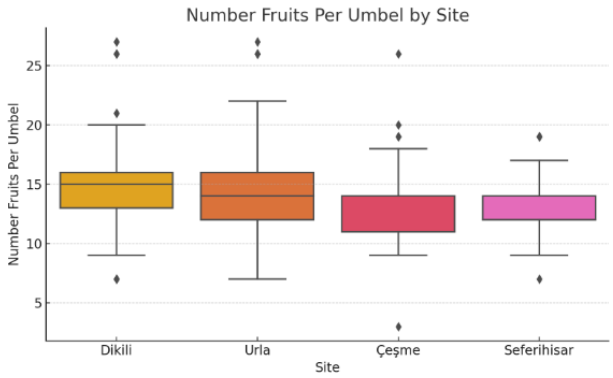




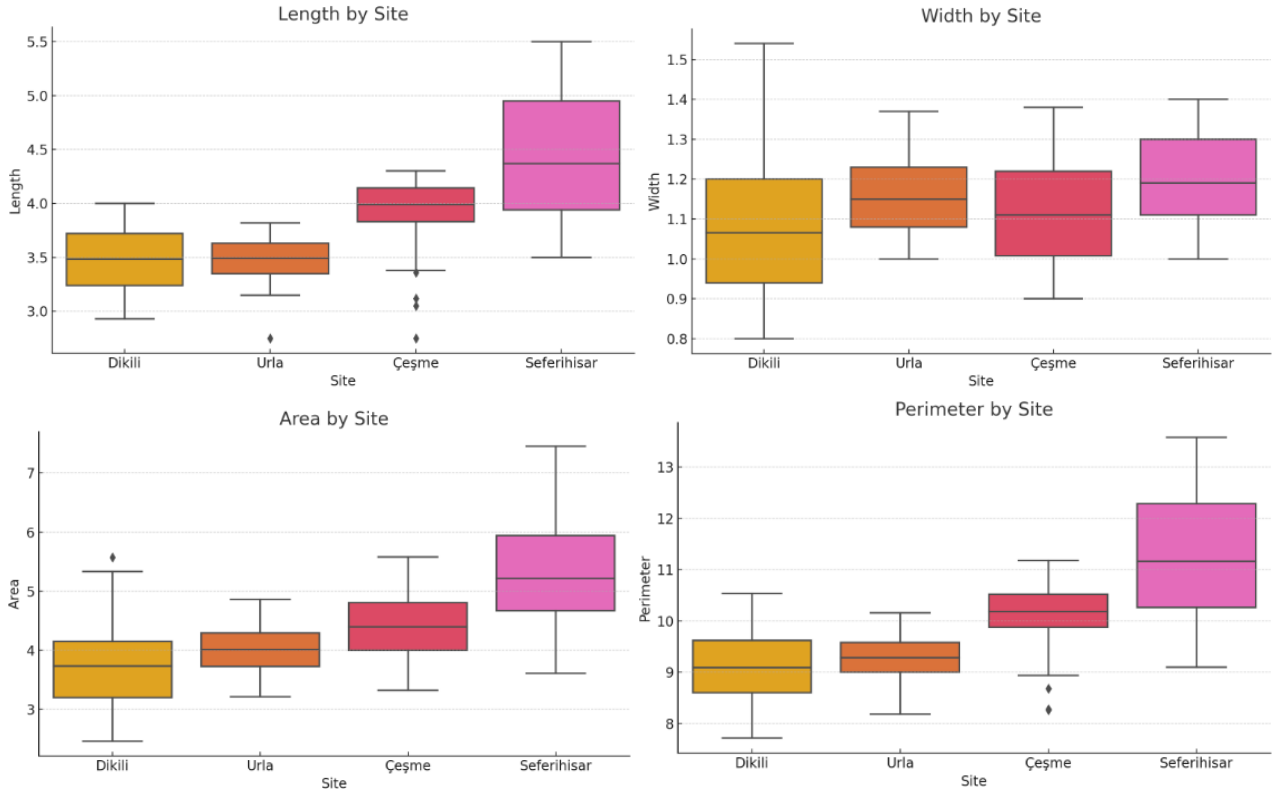
Fruit morphology revealed that the longest and widest fruits were found in Çandarlı (6.82 mm × 3.63 mm), while Seferihisar had the smallest width (3.44 mm). Urla showed the highest number of fruits per umbel (14.19), followed closely by Çandarlı (14.45), with Çeşme again showing the lowest value (12.47).

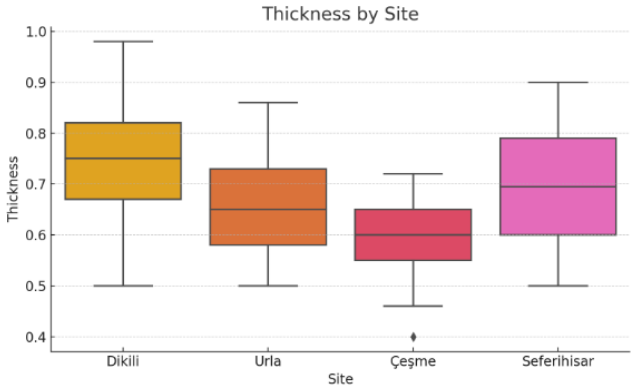




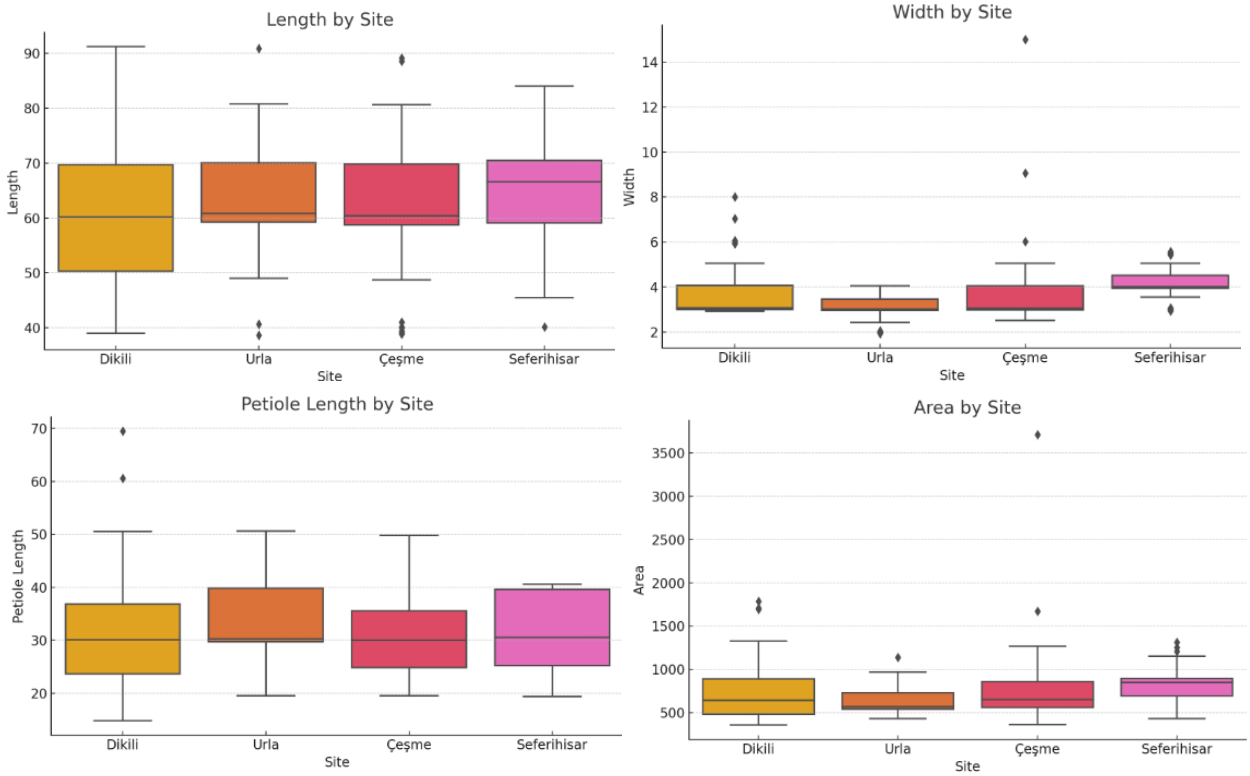


Seed characteristics exhibited considerable variation across sites. Seferihisar had the largest seeds both in length (4.44 mm) and width (1.20 mm), resulting in the highest seed area (5.33 mm²) and perimeter (11.28 mm). In contrast, Çandarlı and Urla had smaller seeds in all dimensions, including seed thickness. All localities showed a consistent number of seeds per fruit (2.0), indicating stability in fecundity per unit fruit.





Leaf traits were also evaluated in terms of size and structure. Seferihisar had the longest leaves (64.35 mm) and the largest leaf area (828.37 mm²), suggesting more photosynthetic surface. Petiole length was longest in Urla (33.83 mm), while other populations averaged around 30–31 mm. The number of lobes per leaf was consistent across all populations (mean ~2.88–2.90), indicating limited variation in leaf segmentation.



In conclusion, while morphological divergence among localities is evident and statistically supported, it does not in itself confirm ecotype formation. Environmental plasticity, phenological shifts, or microhabitat variation may be responsible for the observed patterns. The traits observed may represent adaptive tendencies rather than genetically fixed ecotypic lines.

To distinguish between true ecotypic differentiation and environmentally-induced variation, further evidence—especially from common garden experiments, reciprocal transplant trials, or molecular genetic analyses—is necessary. Until such data are obtained, it is most appropriate to describe these populations as morphologically divergent, with potential for local adaptation, rather than as definitive ecotypes.

5.2 Chemical analyses

Several biochemical properties of sea fennel samples were analyzed using spectrophotometric methods (UV-VIS Cary 50 spectrophotometer), as high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) were not available in the laboratory.

Materials and Methods

5.2.1 Carotenoids

Carotenoid analysis of sea fennel leaves was performed using a solvent extraction method followed by UV-Vis spectrometric detection, according to Biswas et al. (2011). All analyses were conducted using validated spectrophotometric techniques, ensuring reliable quantification of the biochemical parameters assessed in this study.

5.2.2 Total flavonoid

Total flavonoid content was measured using the aluminum chloride colorimetric assay (Zhinsel et al., 1999). Extracts were treated with $NaNO_2$, $AlCl_3$, and NaOH solutions before incubation in darkness for 30 min. Absorbance was measured at 510 nm, with results expressed as mg rutin per gram of dry matter.

5.2.3 Antioxidant activity

Antioxidant activity was determined using the Ferric Reducing Antioxidant Power (FRAP) assay as described by Benzie and Strain (2009). Plant samples (0.5 g) were extracted with 20 mL of 80% (v/v) ethanol and centrifuged for 20 min at 4°C. The reaction was initiated by adding 0.05 mL of the extract to 1.50 mL of FRAP reagent and 0.15 mL of distilled water. Absorbance was measured spectrophotometrically at 593 nm, and results were expressed as µmoles FRAP per gram of dry matter.

5.2.4 Antiradical activity

Antiradical activity was determined following the method of Brand-Williams et al. (1995). A 0.1 mL extract was mixed with 3.9 mL of DPPH• solution, and absorbance at 515 nm was recorded over 10 min. The efficient concentration (EC_{50}) was calculated as the amount of antioxidant required to reduce the initial DPPH• concentration by 50%.

5.2.5 Volatile organic compounds

The chemical composition of essential oils obtained from *Crithmum maritimum* samples was analyzed using Gas Chromatography-Mass Spectrometry (GC-MS). The analysis was performed on an Agilent HP 6890 Series GC system equipped with a mass selective detector. A capillary column with dimensions of 60.0 m × 320 µm × 0.25 µm film thickness was used for compound separation. The injection volume was 1 µL, and the analysis was conducted in split mode to ensure proper resolution of volatile components. The oven temperature was programmed with a maximum of 260 °C. The total run time for each sample was 49.5 minutes. Compound identification was performed by comparing the obtained mass spectra with those in the Wiley 7N library database.

Results

The biochemical composition and antioxidant capacity of sea fennel (*Crithmum maritimum* L.) varied between plant organs (leaves and flowers) and ecotypes (Çandarlı: CAN and Urla: URL), as summarized in the Table below.

Total antioxidant activity, measured by FRAP assay, was substantially higher in flower tissues compared to leaves. The highest activity was recorded in CAN-F (228.2 µmol FRAP/g DW), followed by URL-F (196.3 µmol FRAP/g DW), while leaf tissues showed markedly lower values (CAN-L: 96.4; URL-L: 101.2 µmol FRAP/g DW). This suggests a stronger antioxidant potential in flower tissues regardless of the ecotype.
Total flavonoid content also showed a similar trend, with flower samples accumulating significantly more flavonoids than leaves. The highest value was recorded in URL-F (6.54 mg/g DW), followed by CAN-F (5.22 mg/g DW). Leaf tissues of both ecotypes contained relatively lower levels (URL-L: 4.14; CAN-L: 1.44 mg/g DW), indicating that flavonoid biosynthesis is more pronounced in floral organs, especially in the URL ecotype.
In terms of antiradical activity, assessed by DPPH assay and expressed as IC_{50} values, a wide range was observed among samples. Lower IC_{50} values indicate stronger scavenging capacity. The strongest activity was found in CAN-L (145.7 µg/ml), followed by URL-L (271.5 µg/ml). Particularly, flower samples had higher IC_{50} values (CAN-F: 304.1 µg/ml; URL-F: 392.0 µg/ml), indicating weaker DPPH scavenging activity despite their higher total antioxidant capacity and flavonoid content.

Ecotypes	[1] Total antioxidant activity µmol FRAP/g DW	[2] Total flavonoids mg/g DW	[3] Antiradical activity DPPH as IC50, µg/ml	[4] Total carotenoids mg/g DW	[5] Total phenolic compounds mg/g DW	[6] Total tochoperol mg/kg DW	[7] Vitamin C mg/g DW
CAN-L	96.4 ±14.8	1.44 ±0.24	145.7 ±28,1	52.9 ±13.0	11.4 ±1,09	204.5 ±18.3	3.36 ±0.46
CAN-F	228.2 ±21.0	5.22 ±1.01	304.1 ±35.8	39.4 ±6.3	19.3 ±3.04	103.7 ±11.5	2.92 ±0.80
URL-L	101.2 ±9.8	4.14 ±0.69	271.5 ±30.6	55.3 ±18.4	8.88 ±1.67	181.4 ±10.8	2.23 ±0.73
URL-F	196.3 ±16.3	6.54 ±1.33	392.0 ±51,2	41.5 ±9.7	25.2 ±4.42	112.0 ±10.2	2.71 ±0.20

Total antioxidant activity, total flavonoids, antiradical activity, total carotenoidsi total phenolic compoundsi total tochoperol and vitamin C content of leaves (L) and flowers (F) of two sea fennel (Crithmum maritimum L.) populations (CAN: Çandarlı and URL: Urla) collected from the natural flora of Türkiye.
[1]AO-Benzie and Strain (2009), [2]FLV-Zhishen et al. (1999), [3]AR-Brand-Williams et al. (1995), [4]TC-Arnon and Copper (1949), [5]TPC-Singleton et al.(1999), [6]TTC-Biswas et al (2011), [7]VC-Asghari et al. (2015)

Total carotenoid content was relatively stable across the samples, with leaf samples generally exhibiting slightly higher concentrations. The highest carotenoid level was recorded in URL-L (55.3 mg/g DW), followed by CAN-L (52.9 mg/g DW). Flower samples of both ecotypes had slightly lower levels (URL-F: 41.5; CAN-F: 39.4 mg/g DW).

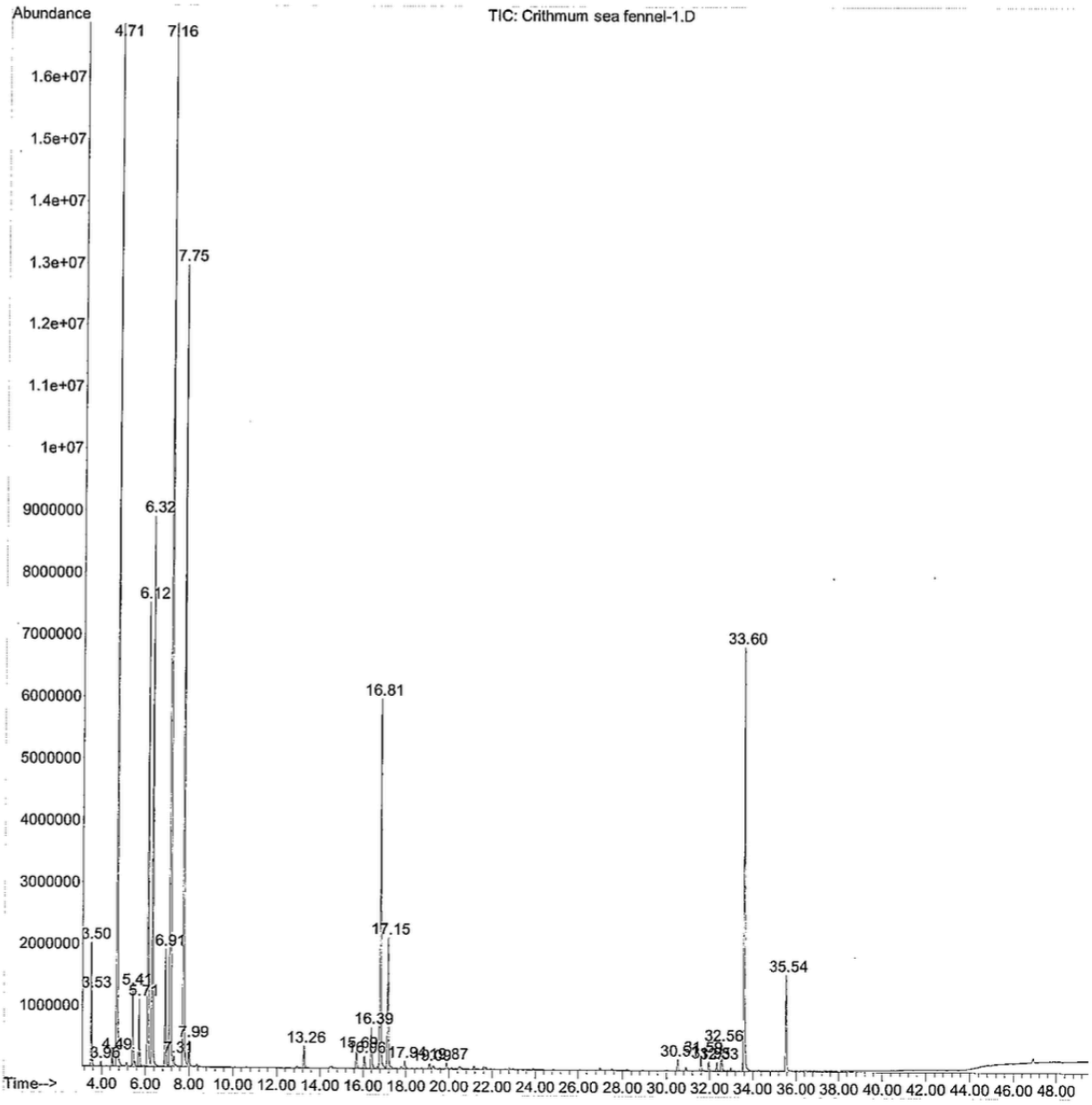
Total phenolic compounds displayed a clear differentiation between organs. Flower tissues of both ecotypes contained significantly more phenolics (URL-F: 25.2; CAN-F: 19.3 mg/g DW) than their respective leaves (URL-L: 8.88; CAN-L: 11.4 DW).

Tocopherol content (vitamin E) was highest in CAN-L (204.5 mg/kg DW) and decreased significantly in flowers, especially in CAN-F (103.7 mg/kg DW). Leaf tissues consistently contained more tocopherols than flowers in both ecotypes, highlighting leaves as a richer source of lipid-soluble antioxidants.

Based on the GC-MS analysis of essential oils extracted from three *Crithmum maritimum* (sea fennel) populations, Atlantis, Çandarlı, and Urla, substantial differences were observed in their chemical compositions, reflecting possible ecological adaptations and genetic variation among the populations.

In the Atlantis population, the essential oil profile was primarily dominated by monoterpene hydrocarbons. The most abundant component was γ-terpinene (22.6%), followed by sabinene (15.9%) and *p*-cymene (14.2%). These compounds are commonly associated with antioxidant, antimicrobial, and aromatic properties, and their abundance in the Atlantis population suggests a chemotype oriented toward volatile monoterpenes. The high content of γ-terpinene and *p*-cymene, both known for their contribution to aroma and bioactivity, indicates potential for culinary and therapeutic uses.

The Çandarlı population, on the other hand, exhibited a more chemically diverse essential oil composition. Dillapole was identified as the most dominant compound, accounting for 18.9% of the oil content. This was followed closely by sabinene (16.8%), γ-terpinene (15.5%), *p*-cymene (12.0%), and thymyl-methyl-ether (11.9%). Dillapole, a phenylpropanoid compound, is particularly noteworthy due to its known insecticidal and antifungal activities, suggesting that the Çandarlı population may have adapted to environmental pressures by increasing its production of secondary metabolites with protective functions. Additionally, the presence of thymyl-methyl-ether, an oxygenated monoterpene with known biological activity, further indicates the pharmacological potential of this population.



Gas chromatography/mass spectrometry (GC/MS) chromatograms of essential oil of sea fennel (Crithmum maritimum) population-Atlantis grown in field conditions

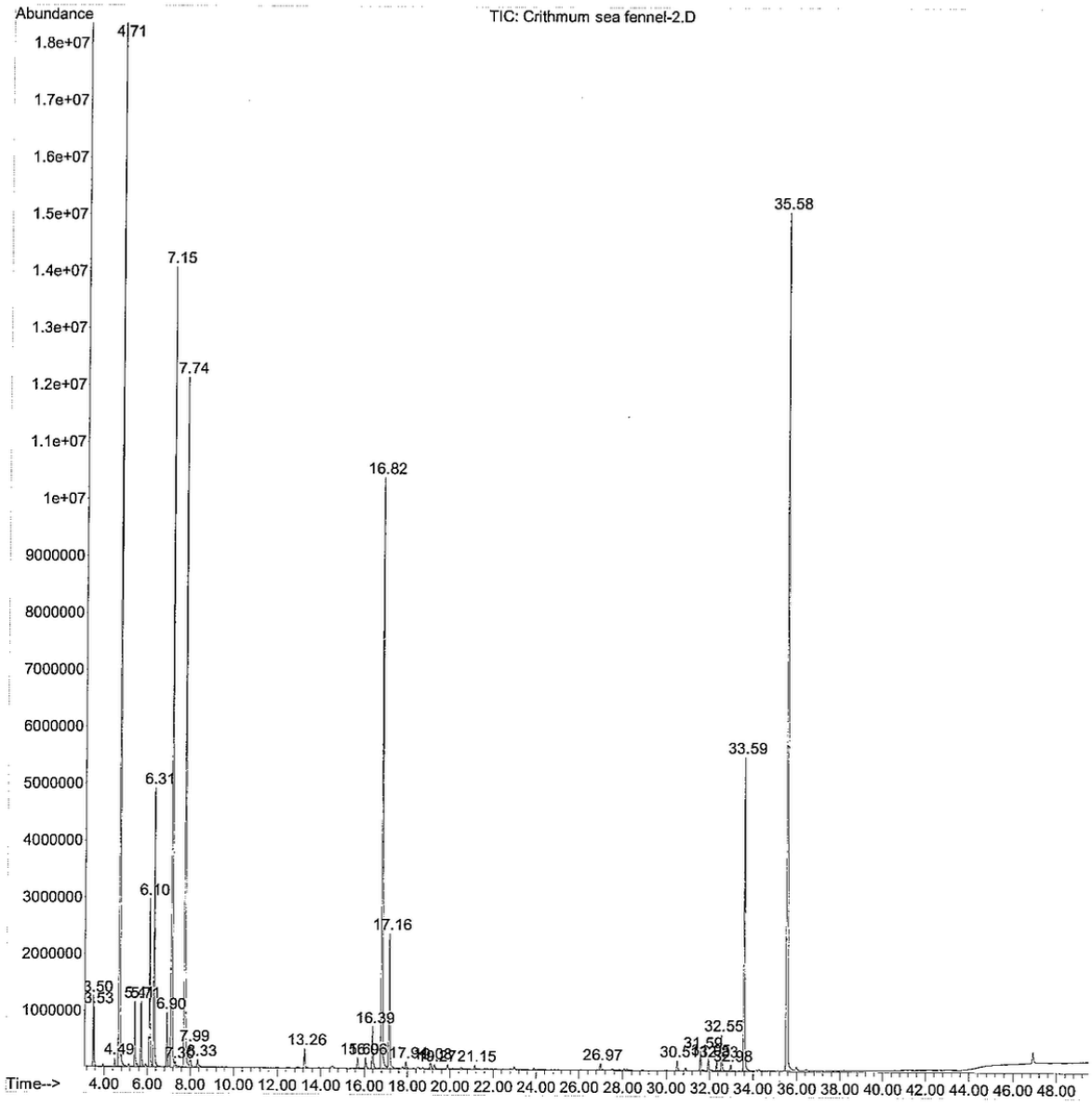
Yapılan Analizler / *Analysis*	Sonuç / *Result (% Area)*
Alpha Pinen	1.29
Alpha Thujene	0.76
Camphene	0.06
Beta Pinene	0.18
Sabinene	15.93
Beta Myrcene	1.16
Alpha Terpinene	1.01
Limonene	7.66
Beta- Phellandrene	8.84
Cis- Ocymene	2.07
Gamma Terpinene	22.63
Delta-3-Caren	0.19
P-Cymene	14.15
Alpha Terpinolene	0.46
Cis- Sabinen Hydrate	0.42
Trans- Sabinen Hydrate	0.36
Carvacrol-Methyl -Ether	0.89
Thymyl-Methyl -Ether	7.14
Terpinen-4-ol	2.54
Cryptone	0.14
Alpha Terpineol	0.13
Spathulenol	0.23
Thymol (Toplam)	0.41
Carvacrol Toplam)	0.65
Myristicin	8.39
Dillapole	1.88
Tanımlanamayan (Toplam)	0.45

Gas chromatography/mass spectrometry (GC/MS) chromatograms of essential oil of sea fennel (Crithmum maritimum) population-Atlantis grown in field conditions

In the Urla population, the essential oil was also rich in dillapole (24.7%), making it the most abundant component among all three populations. This was followed by sabinene (20.7%), thymyl-methyl-ether (14.4%), and γ-terpinene (13.3%). The high dillapole concentration distinguishes the Urla chemotype as one with significant pesticidal and antimicrobial potential. The combined presence of phenylpropanoids and oxygenated monoterpenes may point to this population's use in natural product development, especially in the fields of nutraceuticals and bio-based pest management.

Across all populations, sabinene and γ-terpinene were consistently among the top constituents, indicating their chemotaxonomic relevance in *C. maritimum*. However, the proportion and presence of unique compounds such as dillapole and thymyl-methyl-ether in Çandarlı and Urla populations reflect a notable differentiation, potentially influenced by environmental conditions or genetic divergence.

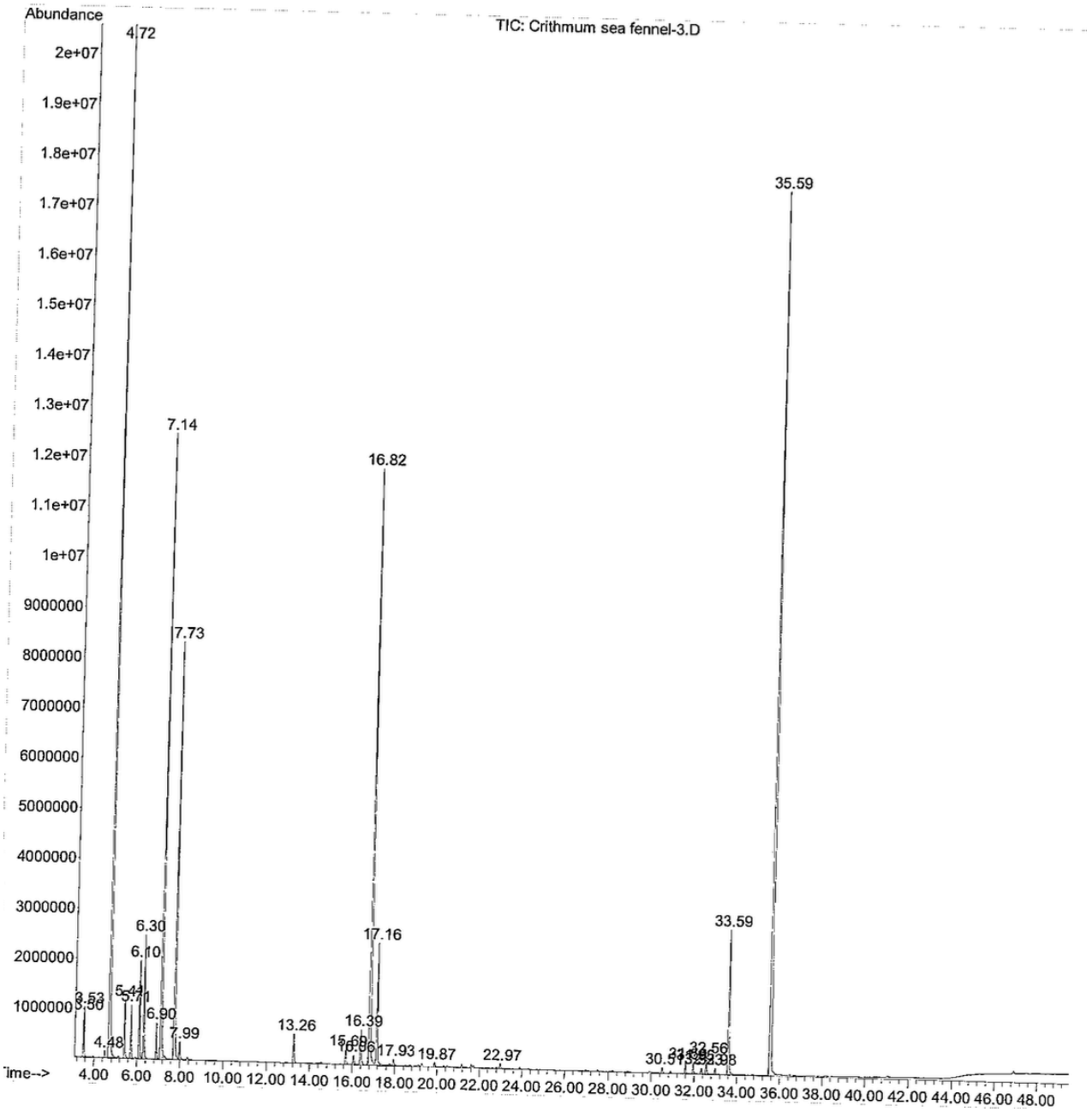
These findings highlight the value of exploring intraspecific diversity within *C. maritimum* for targeted applications. The chemical profiles suggest that different populations may be more suitable for different uses, whether culinary, therapeutic, or industrial, and underline the importance of population-based screening in aromatic and medicinal plant research.



Gas chromatography/mass spectrometry (GC/MS) chromatograms of essential oil of sea fennel (Crithmum maritimum) population-Çandarlı grown in natural flora

Yapılan Analizler / *Analysis*	Sonuç / *Result (% Area)*
Alpha Pinen	0.71
Alpha Thujene	0.64
Beta Pinene	0.12
Sabinene	16.82
Beta Myrcene	0.90
Alpha Terpinene	0.99
Limonene	2.65
Beta- Phellandrene	4.49
Cis- Ocymene	0.93
Gamma Terpinene	15.46
Delta-3-Caren	0.09
P-Cymene	12.02
Alpha Terpinolene	0.39
Octanal	0.15
Cis- Sabinen Hydrate	0.38
Trans- Sabinen Hydrate	0.21
Carvacrol-Methyl -Ether	0.85
Thymyl-Methyl -Ether	11.90
Terpinen-4-ol	2.59
Cryptone	0.18
Piperitol Isomer	0.11
Terpinen-3-ol	0.09
Caryophyllene oxide	0.13
Spathulenol	0.20
Thymol (Toplam)	0.55
Carvacrol Toplam)	0.88
Elemicin	0.12
Myristicin	6.13
Dillapole	18.90
Tanımlanamayan (Toplam)	0.40

Gas chromatography/mass spectrometry (GC/MS) chromatograms of essential oil of sea fennel (Crithmum maritimum) population-Çandarlı grown in natural flora



Gas chromatography/mass spectrometry (GC/MS) chromatograms of essential oil of sea fennel (Crithmum maritimum) population-Urla grown in natural flora

Yapılan Analizler / *Analysis*	Sonuç / *Result (% Area)*
Alpha Pinen	0.52
Alpha Thujene	0.64
Beta Pinene	0.11
Sabinene	20.67
Beta Myrcene	1.03
Alpha Terpinene	0.96
Limonene	1.83
Beta- Phellandrene	2.38
Cis- Ocymene	0.73
Gamma Terpinene	13.26
P-Cymene	8.37
Alpha Terpinolene	0.38
Cis- Sabinen Hydrate	0.64
Trans- Sabinen Hydrate	0.36
Carvacrol-Methyl -Ether	0.88
Thymyl-Methyl -Ether	14.38
Terpinen-4-ol	2.77
Alpha Terpineol	0.13
Germacrene-B	0.12
Spathulenol	0.13
Thymol (Toplam)	0.44
Carvacrol Toplam)	0.54
Elemicin	0.13
Myristicin	3.41
Dillapole	24.73
Tanımlanamayan (Toplam)	0.44

Gas chromatography/mass spectrometry (GC/MS) chromatograms of essential oil of sea fennel (Crithmum maritimum) population-Urla grown in natural flora

5.3 Molecular analyses

Leaf samples of individuals from each population, whose morphological measurements were made, were dried in separate kit bags containing cilica gel. The dried samples were then beaten in a mortar with the help of liquid nitrogen, transferred to Eppendorf tubes, numbered and kept at -20 degrees.


Mortaring of samples and storage in Eppendorf tubes


Preservation of dried leaf samples in silica gel

5.3.1 DNA extraction

DNA Isolation, was successfully performed according to the DNA Isolation Protocol. Targeted concentration of DNA was isolated from 20 individuals, 5 individuals from each population. Qiagen DNA extraction kit was used for isolation. DNA measurements were measured using Qubit 1x dsDNA High Sensitivity. Samples of suitable purity were sent for analysis.

Lokality	Sample Name	Qubit tube conc.	Qubit tube conc. units	Original sample conc.	Original sample conc. Units	Sample Volume (uL)
Seferihisar	4.11a	74.5	ng/mL	7.45	ng/uL	2
Seferihisar	4.20a	34.3	ng/mL	3.43	ng/uL	2
Seferihisar	4.15a	89.6	ng/mL	8.96	ng/uL	2
Seferihisar	4.10a	54.6	ng/mL	5.46	ng/uL	2
Seferihisar	4.5a	77.1	ng/mL	7.71	ng/uL	2
Seferihisar	4.1a	74.6	ng/mL	7.46	ng/uL	2
Çeşme	3.11a	78.6	ng/mL	7.86	ng/uL	2
Çeşme	3.20a	92.1	ng/mL	9.21	ng/uL	2
Çeşme	3.15a	70.4	ng/mL	7.04	ng/uL	2
Çeşme	3.10a	61.2	ng/mL	6.12	ng/uL	2
Çeşme	3.5a	68	ng/mL	6.8	ng/uL	2
Çeşme	3.1a	113	ng/mL	11.3	ng/uL	2
Urla	2.11a	102	ng/mL	10.2	ng/uL	2
Urla	2.20a	115	ng/mL	11.5	ng/uL	2
Urla	2.15a	101	ng/mL	10.1	ng/uL	2
Urla	2.10a	145	ng/mL	14.5	ng/uL	2
Urla	2.5a	60.1	ng/mL	6.01	ng/uL	2
Urla	2.1a	207	ng/mL	20.7	ng/uL	2
Dikili	1.11a	87.2	ng/mL	8.72	ng/uL	2
Dikili	1.20a	186	ng/mL	18.6	ng/uL	2
Dikili	1.15a	217	ng/mL	21.7	ng/uL	2
Dikili	1.10a	55.4	ng/mL	5.54	ng/uL	2
Dikili	1.5a	80.5	ng/mL	8.05	ng/uL	2
Dikili	1.1a	226	ng/mL	22.6	ng/uL	2

DNA measurements

5.3.2 Sequencing

The sequencing was done by Biomarker Technologies (BMK) Company (Munster, Germany), following SLAF-seq methodology.

Specific locus amplified fragment sequencing (SLAF-seq) is an optimized version of ddRADseq, specifically intended for large-scale genotyping experiments. The enzymes and the sizes of the restriction fragments are optimized with training data to ensure even distribution and avoid repeats. The fragments are also selected over a tight range, to optimize the PCR reaction. The protocol is similar to ddRAD, with a first digestion with a restriction enzyme (HaeIII), heat inactivation and a second digestion with an other enzyme (Hpy166II).

Then, the ATP and dual-index sequencing adapter were added at the 3' and 5' end of the digested DNA products, respectively. PCR was performed and the products were purified using E.Z.N.A.H Cycle Pure Kit (Omega). The purified products were mixed and incubated with these two restricted enzymes again. After ligation of ATP, and Solexa adapter in the pair-end, the reaction products were purified using a Quick Spin column (Qiagen, Venlo, Netherlands), and segregated on a 2% agarose gel. Fragments with xx-xx bp were isolated using a Gel Extraction Kit (Tiangen). These SLAFs were subjected to PCR to add barcode. The PCR products were re-purified and then prepared for paired-end sequencing on an Illumina HiSeq sequencing platform (Illumina, San Diego, CA,USA).

Data analysis

The raw reads were further processed with a bioinformatic pipelinetool, BMKCloud(www.biocloud.net) online platform.

Quality control

Raw data (raw reads) of fastq format were firstly processed through fastp software. In this step, clean data(clean reads) were obtained by removing reads containing adapter, reads containing ploy-N and low

quality reads from raw data. At the same time, Q30, GC-content of the clean data were calculated. All the downstream analyses were based on clean data with high quality.

Reads mapping to the reference genome

The adaptor sequences and low-quality sequence reads were removed from the data sets. Raw sequences were transformed into clean reads after data processing. These clean reads were then mapped to the reference genome sequence. bwa soft were used to map with reference genome.

SNP/INDEL Calling

The SNP/INDEL calling was performed using GATK(v3.8 McKenna et al.,2010) and SAMtools packages (Li et al., 2009) (v1.9.1). A total of 448436 SNPs with a minor allele frequency (MAF) and integrity was retained.

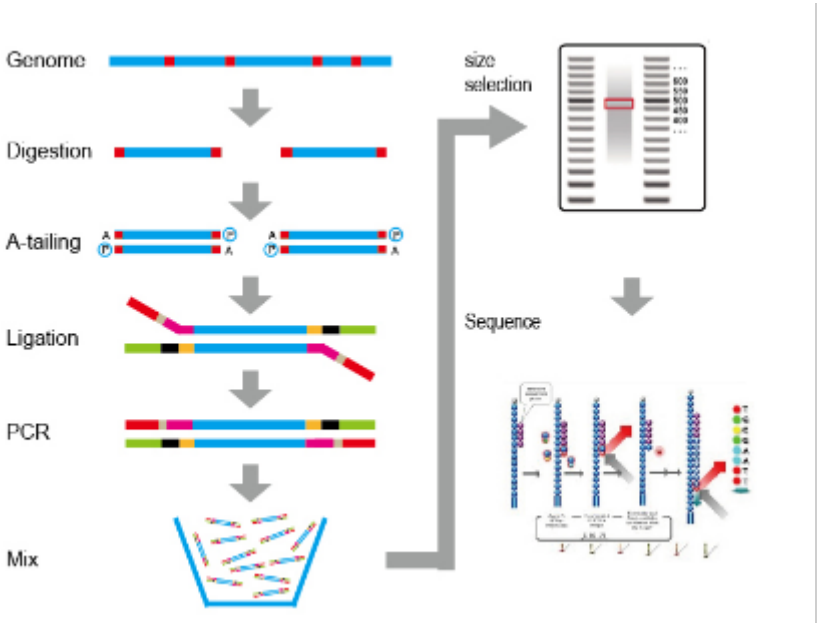
SNP/INDEL Annotation

SNP annotation was performed on the basis of the reference genome using snpEff software(3.6c (build 2014-05-20)) (Cingolani et al., 2012), and SNPs were categorized into intergenic regions, upstream or downstream regions, and exons or introns. SNPs in coding exons were further classified as synonymous SNPs or nonsynonymous SNPs. InDels in exons were grouped according to whether they led to a frameshift.

Gene functional annotation

Gene function was annotated based on the following databases: Nr (NCBI non-redundant protein sequences);Pfam (Protein family); KOG/COG (Clusters of Orthologous Groups of proteins); Swiss-Prot (A manually annotated and reviewed protein sequence database); KO (KEGG Ortholog database); GO (Gene Ontology).

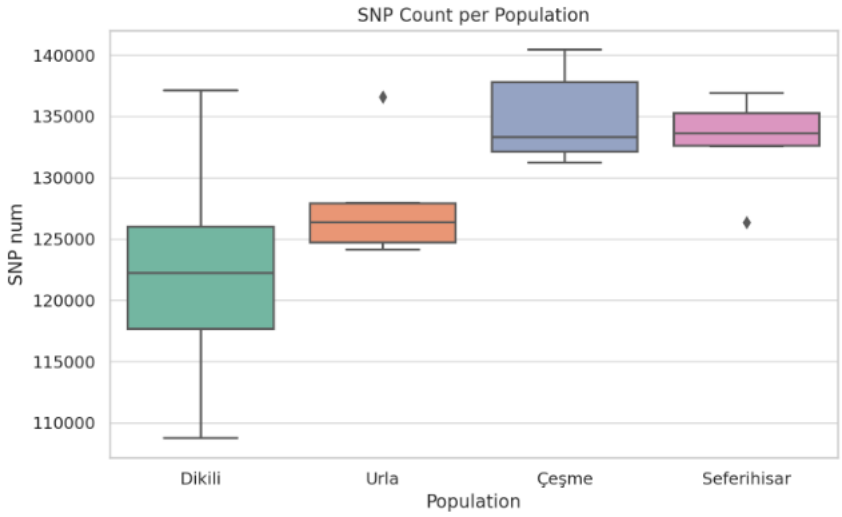
This methodology allows: i) deep sequencing for genotyping accuracy; ii) reduced representation strategy to reduce sequencing costs; iii) pre-designed reduced representation scheme to optimize marker efficiency; iv) double barcode system for large populations.

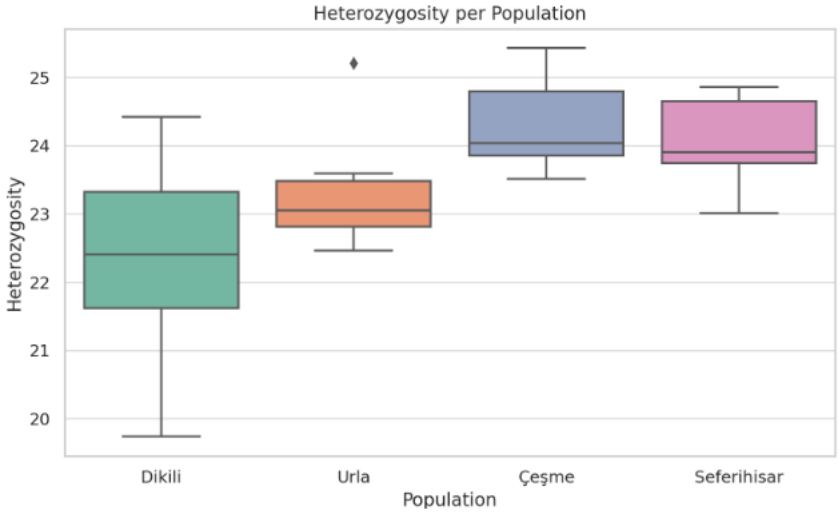


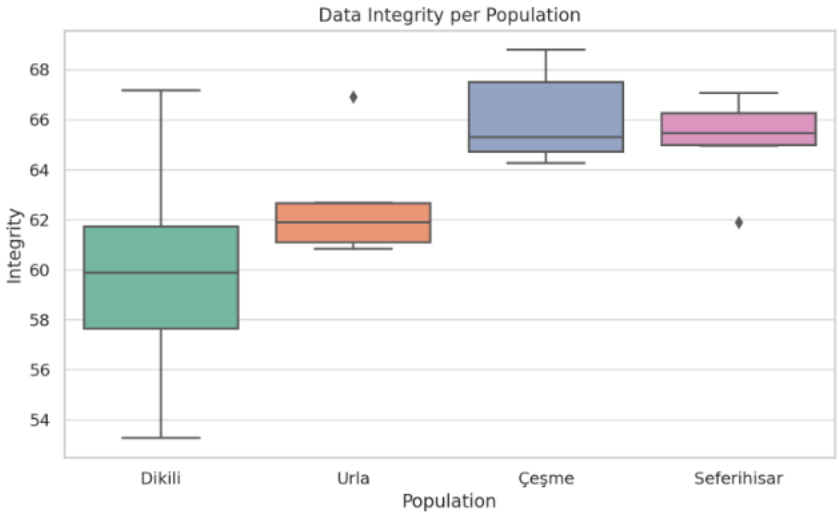
Methodology used for sequencing

5.3.3 Analysis of sequences

The analysis revealed statistically significant differences among populations in terms of SNP count, heterozygosity, and data integrity. SNP count varied notably across populations (ANOVA p = 0.0068), indicating that at least one group exhibits a significantly higher number of variants. Heterozygosity also showed a significant difference (p = 0.0227), suggesting variability in genetic diversity levels among populations. Furthermore, data integrity differed significantly (p = 0.0068), pointing to differences in sequencing quality or completeness. Together, these findings highlight both genetic and technical variation across the studied populations, supporting the presence of underlying genetic structuring.







The results of the statistical analysis revealed significant genetic differences among populations. Notably, the Dikili population showed a significantly higher SNP count and data integrity compared to Seferihisar and Çeşme ($p < 0.05$, Tukey HSD). These findings suggest that Dikili individuals may possess greater genomic variability or higher sequencing coverage. However, heterozygosity levels did not differ significantly among populations, indicating similar levels of within-population genetic diversity. Overall, these results provide preliminary evidence for genetic divergence, potentially reflecting ecotypic differentiation.

5.4 Statistical elaboration of data

In line with the bedrock and soil characteristics considered in the population determination process; It has been determined that the Çandarlı population spreads on the slopes located on the pink andesite rocks consisting of external igneous rocks. Urla Population Distributes on rocky slopes on limestone bedrock. The Çeşme Population spreads over volcanic tuff/tuffite rocks facing the sea. Seferihisar population spreads on sedimentary sandstone-claystone mixed rocky slopes.

The morphological measurements of each population were ordered in centimeters, and the arithmetic mean and standard deviations were calculated and tabulated for each data group.

In addition, comparative data graphs of the populations for each morphology trait are given below.

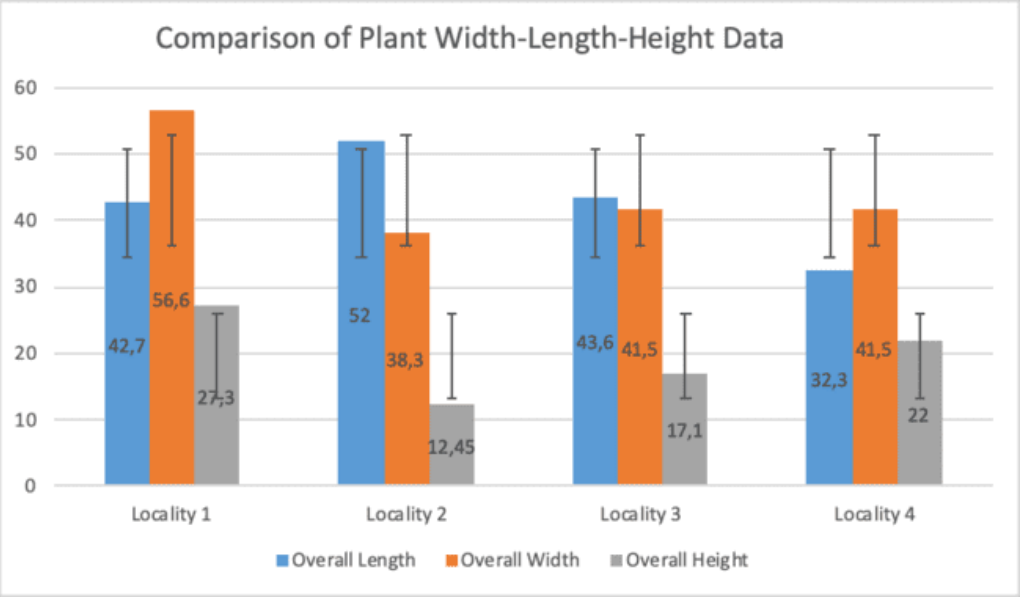
Seed width and height measurements were ordered in centimeters, and the arithmetic mean and standard deviations were calculated for each population and tabulated.

In the calculations made after the seed measurements, the largest population of seed width was determined as Urla Population, and the largest seed length was determined as Çandarlı population. The population with the lowest seed width and length was identified as Çeşme population.

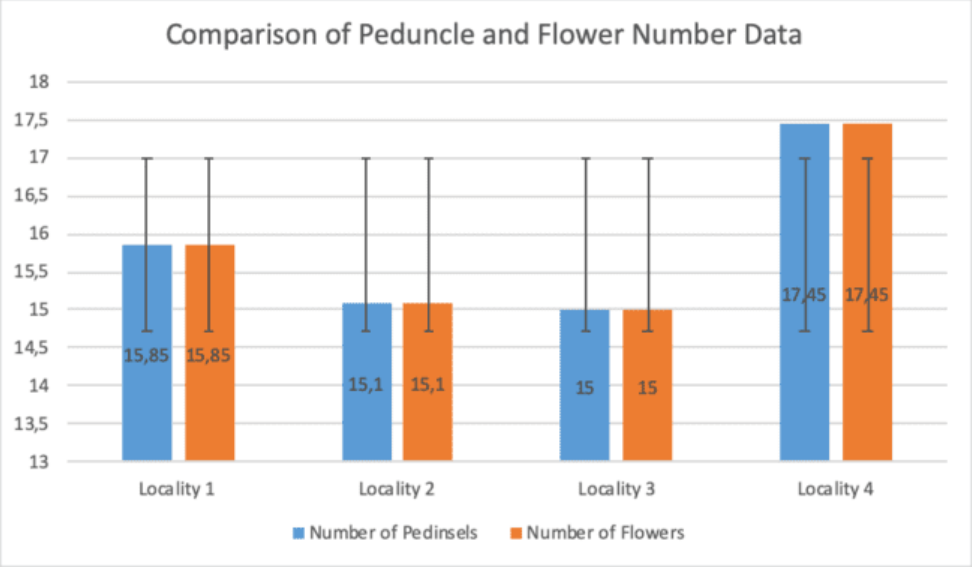
By taking the arithmetic mean of the measurement data of 20 individuals measured in all localities;

-General plant width, highest population Locality 1.
-General plant height, highest population Locality 2.
-General plant height, highest population Locality 1.
-Umbel width, highest population Locality 1.
-Umbel length, the highest population is Locality 2.
-Peduncle length, the highest population is Locality 4.
-Peduncle diameter, highest population Locality 3.

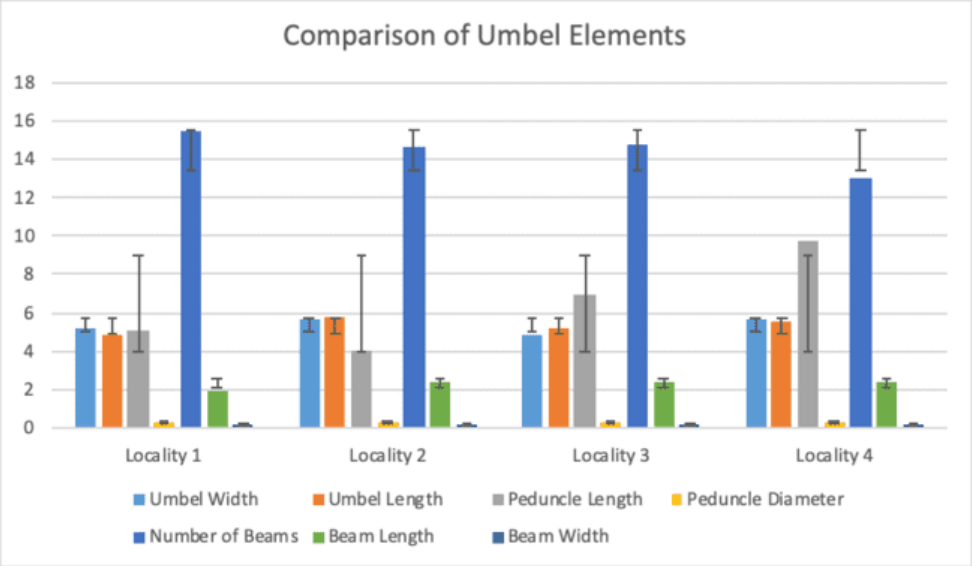
-Beam number, highest population Locality 1.
-Beam length, highest population Locality 3 and 4.
-The beam width is the highest population Locality 3.
-The number of pedicels and the number of flowers are equal, and the highest population is Locality 4.
-Leaf size, the highest population is Locality 4.
-Leaf width, highest population Locality 3.
-Petiol size, highest population Locality 1.
-The number of leaf lobes is the highest population Locality 1.
Comparative analyzes of measurement data are listed below and statistical analyzes are ongoing.



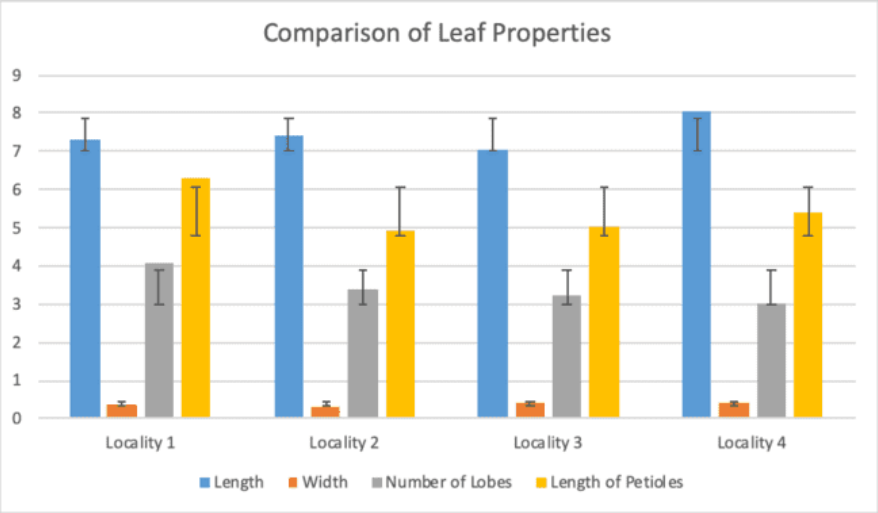
Comparison of Plant Width-Length-Height Data



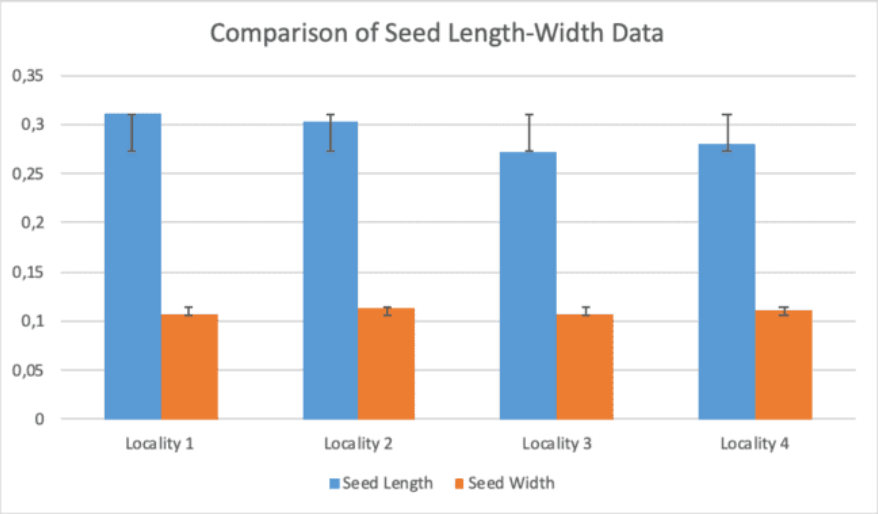
Comparison of Peduncle and Flower Number Data



Comparison of Umbel Elements



Comparison of Leaf Properties



Comparison of Seed Length-Width Data

Seed Width Ar. Ort. / L3<L1<L4<L2
Seed Length Ar.Ort. / L3<L4<L2<L1
Seed Width St. Sap. / L4<L1<L3<L2
Seed Length St. Sap. / L4<L1<L2<L3

	LOCALITY 1 / İZMİR-ÇANDARLI-BADEMLİ YOLU, LİMAN ÇIKIŞI, SAHİL YOLU, EYKO PLAJI YANI, KAYALIKLAR / 13.09.2022 SALI																
	GENERAL (cm)			UMBEL (cm)								UMBELLULA		LEAF (cm)			
	WIDTH	LENGTH	HEIGHT	WIDTH	LENGTH	HEIGHT	PEDINCULE LENGTH	PEDINCULE DIAMETER	NUMBER OF BEAM	BEAM LENGTH	BEAM WIDTH	NUMBER OF PEDINSELS	NUMBER OF FLOWERS	LENGTH	WIDTH	PETIOL LENGTH	NUMBER OF LOBES
Loc.1.1	55	30	85	11	8,5	-	11	0,35	27	3,50	0,15	23	23	14	0,4	7	5
Loc.1.2	30	55	20	5,5	5	-	6	0,2	15	1,70	0,10	14	14	7	0,4	1	5
Loc.1.3	40	34	12	5	3,5	-	3	0,3	10	1,40	0,20	11	11	11	0,5	1,5	4
Loc.1.4	60	50	45	5	3,5	-	4,5	0,4	26	2,50	0,20	15	15	9	0,3	4	3
Loc.1.5	70	60	30	5	5,5	-	5	0,3	20	2,00	0,20	16	16	12	0,5	4,5	3
Loc.1.6	80	60	55	5,5	5	-	6	0,3	15	2,80	0,20	16	16	8	0,3	4	5
Loc.1.7	47	30	12	4	4	-	2	0,3	7	1,70	0,20	11	11	5	0,3	2	5
Loc.1.8	70	20	20	4	3	-	4	0,4	15	1,50	0,20	16	16	6	0,3	7	9
Loc.1.9	35	35	30	5	5,5	-	5	0,3	20	2	0,20	17	17	7	0,4	4	12
Loc.1.10	20	100	20	5,5	5	-	5	0,3	10	2	0,2	16	16	7	0,4	4	4
Loc.1.11	50	30	15	4	4	-	4	0,3	9	1,5	0,2	15	15	5	0,3	3	7
Loc.1.12	60	40	27	6	6	-	6,5	0,3	15	2,8	0,15	17	17	5	0,3	3	5
Loc.1.13	90	80	30	4,5	5	-	4	0,3	9	1,5	0,15	17	17	6	0,4	3	5
Loc.1.14	50	25	15	3,5	3,5	-	3	0,2	10	1,7	0,1	13	13	9	0,4	3	9
Loc.1.15	55	30	15	6	6,5	-	4,5	0,4	16	1,3	0,2	18	18	6	0,3	4	3
Loc.1.16	60	55	15	4,5	4,5	-	3,5	0,3	17	1,7	0,2	18	18	6,5	0,3	3,5	9
Loc.1.17	70	20	15	4	4,5	-	4	0,35	15	1,8	0,15	17	17	4	0,5	15	5
Loc.1.18	70	40	45	8	7,5	-	13	0,4	20	3	0,2	19	19	8	0,3	4	8
Loc.1.19	70	30	20	3,5	3	-	4	0,3	16	1,3	0,1	15	15	5	0,3	2	7
Loc.1.20	50	30	20	4	3,5	-	3	0,3	18	1,5	0,15	13	13	6	0,3	2	13
Art.Ort.	56,6	42,7	27,3	5,18	4,825	-	5,05	0,315	15,5	1,96	0,17	15,85	15,85	7,325	0,36	4,075	6,3
St.Sapma	17,2	20,63	18,12	1,73	1,471	-	2,645253943	0,056428809	5,43381227	0,627778	0,0379577	2,75824124	2,75824124	2,587	0,075	2,99681849	2,885535616

Measurement data of population number one

	LOCALITY 2 / İZMİR-URLA-ASULAR ÇEŞMESİ- KAYALIK YAMAÇLAR (KALKER ANAKAYA) / 14.09.2022 ÇARŞAMBA																
	GENERAL (cm)			UMBEL (cm)								UMBELLULA		LEAF (cm)			
	WIDTH	LENGTH	HEIGHT	WIDTH	LENGTH	HEIGHT	PEDINCULE LENGTH	PEDINCULE DIAMETER	NUMBER OF BEAM	BEAM LENGTH	BEAM WIDTH	NUMBER OF PEDINSELS	NUMBER OF FLOWERS	LENGTH	WIDTH	PETIOL LENGTH	NUMBER OF LOBES
Loc.2.1	50	100	10	4	5	-	5,5	0,25	17	2,20	0,15	17	17	8	0,3	3	5
Loc.2.2	60	80	15	4,5	5,5	-	6	0,3	15	2,00	0,12	15	15	9	0,3	4	5
Loc.2.3	30	25	20	5,5	6	-	4	0,3	13	2,00	0,10	17	17	6	0,25	3	4
Loc.2.4	30	50	10	6	5,5	-	3	0,3	13	2,20	0,20	15	15	6	0,3	2,5	5
Loc.2.5	30	50	7	3,5	5,5	-	3	0,3	12	1,00	0,10	15	15	9	0,4	3	5
Loc.2.6	40	90	10	7	6,5	-	4	0,25	15	2,70	0,10	12	12	10	0,4	4	5
Loc.2.7	45	70	13	4,5	5,5	-	5	0,25	15	2,50	0,12	15	15	7	0,35	2,5	5
Loc.2.8	35	40	10	5,5	5	-	4	0,3	14	2,50	0,17	17	17	8	0,25	4	5
Loc.2.9	20	25	10	6	7	-	5	0,25	16	3	0,20	15	15	7	0,25	2,5	5
Loc.2.10	30	45	10	6	6	-	3	0,2	14	3	0,15	13	13	8	0,3	3	5
Loc.2.11	40	30	12	6	6	-	4	0,25	14	2,5	0,15	16	16	6	0,3	3	5
Loc.2.12	25	20	12	7	5,5	-	3	0,45	14	3	0,2	17	17	9	0,35	4	5
Loc.2.13	45	35	12	5	4	-	3	0,35	15	1,5	0,12	17	17	6	0,3	3	4
Loc.2.14	50	90	12	6	7	-	5	0,18	16	2	0,2	15	15	7	0,3	3	4
Loc.2.15	30	40	14	8	7	-	3,5	0,3	15	3	0,12	16	26	8	0,27	4	5
Loc.2.16	20	30	10	6	6	-	2,7	0,5	10	1,8	0,15	15	15	6	0,25	3	5
Loc.2.17	50	70	15	5,5	6	-	5	0,4	14	2,00	0,15	14	14	7	0,2	4	5
Loc.2.18	30	45	15	5	4	-	5	0,2	16	3	0,15	11	11	9	0,4	5	7
Loc.2.19	35	55	17	5	6	-	3,5	0,35	17	4	0,2	15	15	5,5	0,3	3	5
Loc.2.20	70	50	15	6	5,5	-	3,5	0,4	17	2	0,15	15	15	7	0,2	4	5
Art.Ort.	38,3	52	12,45	5,6	5,725	-	4,035	0,304	14,6	2,40	0,15	15,1	15,6	7,425	0,299	3,375	4,95
St.Sapma	13,1	23,81	3,069	1,06	0,835	-	0,99115828	0,084005012	1,759186415	0,6684428	0,0355409	1,651155895	2,945111918	1,311	0,059	0,68585329	0,604805319

Measurement data of population number two

	LOCALITY 3 / İZMİR-ÇEŞME-ALAÇATI-GÜVERCİN KAYA YAKINI DENİZE BAKAN TÜF KAYALIKLAR / TARİH BİLGİSİ YOK																
	GENERAL (cm)			UMBEL (cm)								UMBELLULA		LEAF (cm)			
	WIDTH	LENGTH	HEIGHT	WIDTH	LENGTH	HEIGHT	PEDINCULE LENGTH	PEDINCULE DIAMETER	NUMBER OF BEAM	BEAM LENGTH	BEAM WIDTH	NUMBER OF PEDINSELS	NUMBER OF FLOWERS	LENGTH	WIDTH	PETIOL LENGTH	NUMBER OF LOBES
Loc.3.1	20	32	16	3	3,5	-	5	0,35	11	1,50	0,20	13	13	5	0,3	2	5
Loc.3.2	20	35	15	4,5	5	-	5	0,35	13	2,20	0,20	17	17	6	0,35	3	4
Loc.3.3	60	45	15	4	5	-	8	0,4	13	2,50	0,22	15	15	6	0,4	3,5	5
Loc.3.4	25	20	11	5	5	-	6	0,28	17	3,50	0,20	13	13	7	0,45	3	5
Loc.3.5	25	40	15	5	4,5	-	8	0,3	13	2,00	0,21	17	17	7	0,4	3,5	5
Loc.3.6	40	45	15	5	6	-	7	0,3	11	2,50	0,22	14	14	7	0,25	5	4
Loc.3.7	70	50	17	4,5	5	-	6	0,35	17	3,00	0,20	12	12	7	0,45	4	5
Loc.3.8	40	45	15	5,5	6	-	9	0,27	12	4,20	0,17	12	12	6	0,4	3,5	6
Loc.3.9	80	80	10	5	4,5	-	7	0,3	7	1,5	0,12	15	15	8,5	0,35	4	4
Loc.3.10	30	40	12	5,5	6	-	6	0,3	11	2,7	0,15	15	15	9	0,3	4	5
Loc.3.11	50	45	25	6	4,5	-	7	0,4	19	2,2	0,2	14	14	7	0,35	2	5
Loc.3.12	25	15	10	5	6	-	7	0,25	15	3,2	0,22	15	15	7	0,5	3	5
Loc.3.13	40	45	20	4,5	5	-	8	0,3	15	2,00	0,22	15	15	9	0,4	3,5	7
Loc.3.14	70	50	22	4	5	-	9	0,35	17	1,5	0,2	17	17	5	0,25	2	4
Loc.3.15	30	25	12	5	5,5	-	5	0,35	11	2,00	0,2	17	17	6	0,5	3	7
Loc.3.16	45	70	15	5,5	6	-	6	0,3	17	3,00	0,22	17	17	6	0,55	2,5	5
Loc.3.17	60	70	30	5	5	-	9	0,5	21	2,00	0,3	20	20	13	1,5	5	7
Loc.3.18	35	35	10	4	4,5	-	5	0,3	15	2,00	0,22	12	12	4	0,25	2	5
Loc.3.19	25	40	27	5,5	6	-	9	0,25	21	2,5	0,2	15	15	8	0,5	4	5
Loc.3.20	40	45	30	6	6,5	-	7	0,35	19	2,5	0,2	15	15	7	0,35	2,5	3
Art.Ort.	41,5	43,6	17,1	4,88	5,225	-	6,95	0,3275	14,75	2,43	0,20	15	15	7,025	0,44	3,25	5,05
St.Sapma	18,1	15,98	6,464	0,74	0,752	-	1,431782106	0,059016055	3,753945293	0,6972691	0,0340704	2,077447827	2,077447827	1,909	0,265	0,92480439	1,050062655

Measurement data of population number three

	LOCALITY 4 / İZMİR-SEFERİHİSAR-HARİTACILAR SİTESİ KUMTAŞI, KİL TAŞI KARIŞIK KAYALIKLI YAMAÇLAR / TARİH BİLGİSİ YOK																
	GENERAL (cm)			UMBEL (cm)								UMBELLULA		LEAF (cm)			
	WIDTH	LENGTH	HEIGHT	WIDTH	LENGTH	HEIGHT	PEDINCULE LENGTH	PEDINCULE DIAMETER	NUMBER OF BEAM	BEAM LENGTH	BEAM WIDTH	NUMBER OF PEDINSELS	NUMBER OF FLOWERS	LENGTH	WIDTH	PETIOL LENGTH	NUMBER OF LOBES
Loc.4.1	20	50	25	6	6,5	-	9	0,35	12	2,30	0,20	18	18	7	0,4	1,8	5
Loc.4.2	40	40	17	5	5	-	8	0,3	12	2,00	0,20	17	17	9	0,3	2,4	5
Loc.4.3	30	20	10	4,5	5	-	7	0,25	15	3,50	0,20	16	16	8	0,5	4	5
Loc.4.4	60	50	25	7	5,5	-	12	0,2	12	1,80	0,10	18	18	10	0,4	3	5
Loc.4.5	90	45	19	5	5,5	-	11	0,4	12	2,50	0,20	21	21	8	0,45	2	5
Loc.4.6	25	30	20	5	6	-	9	0,35	12	2,50	0,20	19	19	9	0,4	2,5	5
Loc.4.7	25	35	18	5	5,5	-	9	0,28	16	3,00	0,22	15	15	7	0,45	3	6
Loc.4.8	40	55	20	6	6	-	11	0,25	15	2,10	0,20	16	16	9	0,4	4	5
Loc.4.9	90	30	30	6	6	-	8	0,35	14	2,3	0,20	23	23	9	0,4	3	7
Loc.4.10	30	20	22	6	7	-	11	0,4	13	2,3	0,2	20	20	8	0,45	2	6
Loc.4.11	30	30	15	5,5	5	-	6	0,23	14	3,2	0,2	17	17	8,5	0,5	3,5	6
Loc.4.12	30	40	45	8	6	-	10	0,2	10	2,00	0,2	15	15	7	0,3	3	4
Loc.4.13	40	20	25	5,5	6	-	10	0,25	13	2,00	0,25	17	17	7	0,35	2,5	7
Loc.4.14	20	20	25	7	5	-	10	0,35	14	2,00	0,15	17	17	9	0,5	3	7
Loc.4.15	40	30	17	6	5	-	7	0,2	14	3,00	0,19	15	15	9	0,55	2,5	6
Loc.4.16	40	30	20	6	5,5	-	9	0,3	14	2,50	0,1	19	19	7	0,3	4	5
Loc.4.17	30	16	28	5	5,5	-	16	0,4	13	1,80	0,15	16	16	8,5	0,5	2,4	5
Loc.4.18	30	25	19	5	5,15	-	9	0,3	12	2,00	0,2	18	18	7	0,4	3,4	5
Loc.4.19	50	20	15	5	4,5	-	8	0,25	12	3,5	0,21	16	16	6	0,4	4	4
Loc.4.20	70	40	25	5,5	5	-	14	0,2	12	2,2	0,1	16	16	8	0,3	4	5
Art.Ort.	41,5	32,3	22	5,7	5,533	-	9,7	0,2905	13,05	2,43	0,18	17,45	17,45	8,05	0,413	3	5,4
St.Sapma	20,8	11,68	7,298	0,86	0,611	-	2,386364776	0,070148339	1,431782106	0,5369259	0,041457	2,114486375	2,114486375	1,037	0,076	0,73699247	0,88257995

Measurement data of population number four

LOCALITY 1	WIDTH(cm)	LENGTH(cm)
SEED 1	0,11	0,31
SEED 2	0,11	0,27
SEED 3	0,11	0,36
SEED 4	0,09	0,3
SEED 5	0,13	0,39
SEED 6	0,11	0,3
SEED 7	0,11	0,29
SEED 8	0,1	0,29
SEED 9	0,11	0,29
SEED 10	0,1	0,29
SEED 11	0,12	0,36
SEED 12	0,11	0,31
SEED 13	0,12	0,41
SEED 14	0,12	0,32
SEED 15	0,11	0,29
SEED 16	0,11	0,33
SEED 17	0,11	0,39
SEED 18	0,1	0,27
SEED 19	0,1	0,28
SEED 20	0,1	0,31
SEED 21	0,11	0,34
SEED 22	0,1	0,29
SEED 23	0,12	0,33
SEED 24	0,1	0,33
SEED 25	0,1	0,3
SEED 26	0,12	0,4
SEED 27	0,1	0,35
SEED 28	0,09	0,31
SEED 29	0,1	0,27
SEED 30	0,12	0,33
SEED 31	0,1	0,32
SEED 32	0,09	0,25
SEED 33	0,1	0,31
SEED 34	0,12	0,36
SEED 35	0,1	0,33
SEED 36	0,11	0,26
SEED 37	0,1	0,31
SEED 38	0,12	0,28
SEED 39	0,11	0,3
SEED 40	0,1	0,26
SEED 41	0,13	0,27
SEED 42	0,12	0,28
SEED 43	0,11	0,29
SEED 44	0,12	0,27
SEED 45	0,1	0,23
SEED 46	0,09	0,33
SEED 47	0,11	0,27
SEED 48	0,11	0,29
SEED 49	0,1	0,33
SEED 50	0,12	0,36
Art.Ort.	0,108	0,3102
St.Sapma	0,0101015	0,03971788

LOCALITY 2	WIDTH(cm)	LENGTH(cm)
SEED 1	0,12	0,29
SEED 2	0,14	0,38
SEED 3	0,13	0,39
SEED 4	0,12	0,33
SEED 5	0,1	0,27
SEED 6	0,12	0,29
SEED 7	0,13	0,34
SEED 8	0,11	0,28
SEED 9	0,1	0,25
SEED 10	0,11	0,3
SEED 11	0,12	0,31
SEED 12	0,12	0,29
SEED 13	0,12	0,32
SEED 14	0,12	0,3
SEED 15	0,11	0,32
SEED 16	0,12	0,33
SEED 17	0,11	0,33
SEED 18	0,12	0,37
SEED 19	0,12	0,32
SEED 20	0,12	0,37
SEED 21	0,12	0,34
SEED 22	0,1	0,25
SEED 23	0,11	0,31
SEED 24	0,11	0,33
SEED 25	0,09	0,28
SEED 26	0,12	0,28
SEED 27	0,1	0,26
SEED 28	0,1	0,25
SEED 29	0,13	0,32
SEED 30	0,11	0,24
SEED 31	0,12	0,31
SEED 32	0,13	0,36
SEED 33	0,11	0,29
SEED 34	0,1	0,25
SEED 35	0,12	0,29
SEED 36	0,11	0,39
SEED 37	0,11	0,26
SEED 38	0,08	0,24
SEED 39	0,12	0,3
SEED 40	0,12	0,31
SEED 41	0,11	0,28
SEED 42	0,12	0,29
SEED 43	0,12	0,28
SEED 44	0,1	0,25
SEED 45	0,11	0,26
SEED 46	0,12	0,29
SEED 47	0,12	0,28
SEED 48	0,1	0,35
SEED 49	0,12	0,31
SEED 50	0,11	0,35
Art.Ort.	0,114	0,3036
St.Sapma	0,01106567	0,03983435

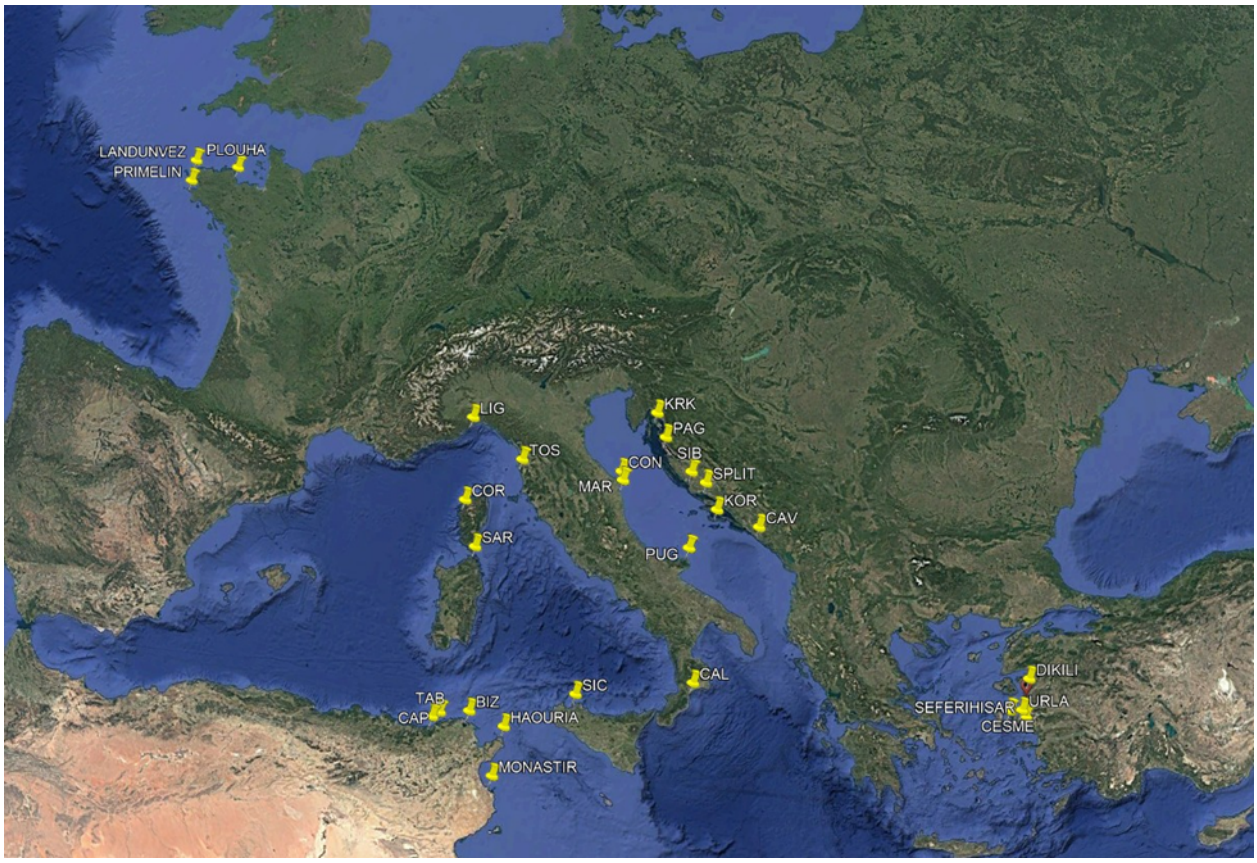
LOCALITY 3	WIDTH(cm)	LENGTH(cm)
SEED 1	0,11	0,4
SEED 2	0,1	0,28
SEED 3	0,11	0,3
SEED 4	0,11	0,27
SEED 5	0,1	0,24
SEED 6	0,12	0,29
SEED 7	0,1	0,24
SEED 8	0,12	0,28
SEED 9	0,12	0,27
SEED 10	0,11	0,27
SEED 11	0,1	0,24
SEED 12	0,11	0,26
SEED 13	0,09	0,22
SEED 14	0,13	0,29
SEED 15	0,1	0,27
SEED 16	0,1	0,28
SEED 17	0,1	0,25
SEED 18	0,11	0,27
SEED 19	0,1	0,26
SEED 20	0,1	0,25
SEED 21	0,09	0,31
SEED 22	0,12	0,26
SEED 23	0,12	0,45
SEED 24	0,11	0,26
SEED 25	0,11	0,24
SEED 26	0,13	0,28
SEED 27	0,1	0,23
SEED 28	0,1	0,25
SEED 29	0,09	0,26
SEED 30	0,12	0,27
SEED 31	0,12	0,26
SEED 32	0,08	0,37
SEED 33	0,1	0,25
SEED 34	0,1	0,27
SEED 35	0,1	0,37
SEED 36	0,11	0,27
SEED 37	0,11	0,25
SEED 38	0,1	0,24
SEED 39	0,1	0,22
SEED 40	0,09	0,27
SEED 41	0,11	0,29
SEED 42	0,1	0,23
SEED 43	0,1	0,25
SEED 44	0,11	0,27
SEED 45	0,1	0,25
SEED 46	0,11	0,25
SEED 47	0,1	0,23
SEED 48	0,12	0,26
SEED 49	0,12	0,27
SEED 50	0,1	0,27
Art.Ort.	0,1062	0,2716
St.Sapma	0,0106694	0,0432062

LOCALITY 4	WIDTH(cm)	LENGTH(cm)
SEED 1	0,09	0,28
SEED 2	0,12	0,28
SEED 3	0,12	0,27
SEED 4	0,13	0,29
SEED 5	0,12	0,28
SEED 6	0,11	0,32
SEED 7	0,12	0,27
SEED 8	0,1	0,29
SEED 9	0,11	0,25
SEED 10	0,12	0,29
SEED 11	0,11	0,32
SEED 12	0,1	0,26
SEED 13	0,12	0,26
SEED 14	0,11	0,28
SEED 15	0,11	0,28
SEED 16	0,09	0,3
SEED 17	0,1	0,28
SEED 18	0,11	0,3
SEED 19	0,11	0,28
SEED 20	0,12	0,28
SEED 21	0,1	0,24
SEED 22	0,13	0,29
SEED 23	0,11	0,26
SEED 24	0,11	0,32
SEED 25	0,11	0,24
SEED 26	0,13	0,33
SEED 27	0,11	0,26
SEED 28	0,1	0,25
SEED 29	0,11	0,29
SEED 30	0,12	0,32
SEED 31	0,11	0,27
SEED 32	0,1	0,34
SEED 33	0,1	0,28
SEED 34	0,1	0,26
SEED 35	0,12	0,31
SEED 36	0,12	0,31
SEED 37	0,11	0,24
SEED 38	0,11	0,29
SEED 39	0,12	0,21
SEED 40	0,11	0,26
SEED 41	0,11	0,27
SEED 42	0,11	0,23
SEED 43	0,12	0,26
SEED 44	0,1	0,27
SEED 45	0,11	0,26
SEED 46	0,13	0,33
SEED 47	0,12	0,27
SEED 48	0,11	0,31
SEED 49	0,12	0,28
SEED 50	0,11	0,27
Art.Ort.	0,1118	0,2796
St.Sapma	0,0096235	0,02747615

Dimensions of the seeds and their calculations

6 Overall statistical analyses for comparison of sampled Mediterranean sea fennel populations

In total, 31 wild populations of *Crithmum maritimum* were analyzed in order to verify morphological and genetic differences between the different areas of the Mediterranean and to identify possible ecotypes or geographical forms.



Distribution of the Sea Fennel wild populations under research: Italy (8 populations), Croatia (10 populations), France (4 populations), Tunisia (5 populations), Türkiye (4 populations).

Materials and Methods

According to the shared protocol, minimum 4 populations for each country, with a minimum distance of about 100 km between populations have been sampled. For each population, the following field activities have been donen on 20 individuals per population:

- Measurement of some traits of the whole plant;
- Sampling of 5 umbels and 5 leaves for each individual;
- Aerial parts of sampled sea fennel individuals will be scanned and then pressed using an herbarium press in order to store them for a long time;
- Sampling of mature seeds from each individual, then catalogued according to international protocols (ENSCONET, 2009; BUREAU OF LAND MANAGEMENT, 2018; ISTA, 2018) and stored at +4°C and at -20°C for long-term conservation;
- Sampling of 5 gr of fresh leaves for each individual, dried on silica gel according to the tea box method (Wilkie et al., 2013).

In the filed and later on at the laboratory, the following morphological traits have been measured:
•Plant : Height, Width, Diameter of main stem, number of branches, number of umbels,
•Umbell: Lenght of penduncle, Diametre of penducle, Number of rays per penduncle, Thickness of rays, Length of rays, Number of bracts, Length and Width of bracts, Shape of bracts, (5 umbels/individual counting the main umbell)

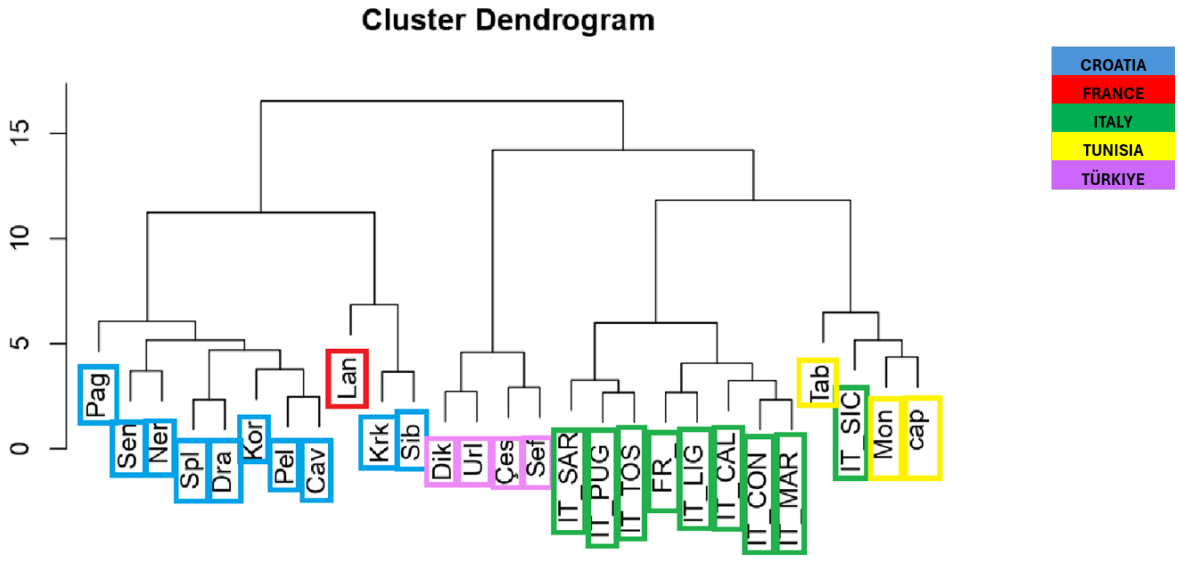
•Umbellet: Number of pedicels per umbellet, Number of flowers per umbellet, (all umbellets from 5 umbells), number of bracteoles, length and width of bracteoles, shape of bracteoles
•Leaves: Length , Width, Leaf area, Length petiol, Number of leaflets (lobes) (5 basal leaves/individual – it's possible to scan leaves and to make the necessary measurements directly on the scanned image),
•Flower: Diameter, Color (for the 5 umbels/individual),
•Fruit: Length, Width, Number of fruits per umbel (20 fruits/individual),
•Seed: Length, Width, Form, Number of seeds per fruit, Weight of 100 seeds, Thickness, Perimeter (scanned seed), Area (scanned seed), J-index (20 seeds/individual).
Italy, Tunisia and Türkiye performed all measurements correctly, following properly the protocol and respecting all the expected repetitions; France and Croatia performed only a few measurements relating to a few characters (Croatia), a few populations (only 1 complete for France) and very few repetitions.
Unfortunately, this compromised the results of the research which was aimed at identifying different ecotypes in the Mediterranean range.

For genetic analysis, high molecular weight genomic DNA was isolated with commercial kits (Qiagen DNeasy® Plant Pro Kit) followed by Next Generation Sequencing.-Sequencing has been done by Biomarker Tecnologies (Muster, Germany) following the SLAF-Seq methodology. Specific locus amplified fragment sequencing (SLAF-seq) is an optimized version of ddRADseq, specifically intended for large-scale genotyping experiments.

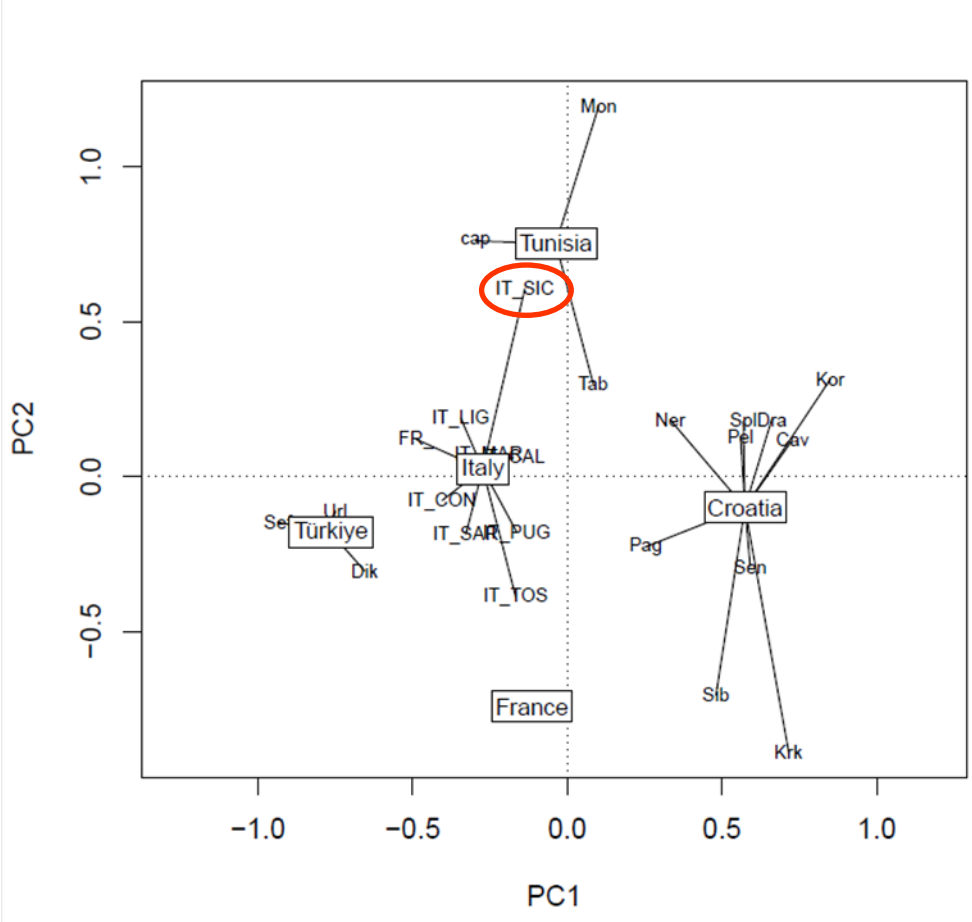
Results

6.1.1 Morphological analysis results

It is not possible to compare all the populations with each other because data from France (except Corsica which population has been properly analysed by the Italian partner) and Croatia are not complete. Therefore, Croatian and French populations are completely separate from the others and form a separate cluster.
Nevertheless, it is possible to recognize at least three morphotypes: a Turkish morphotype, a North African morphotype formed by the Tunisian populations and the population of Sicily, and an Italian morphotype, including Italian population (except for Sicily) and the Corsican population.



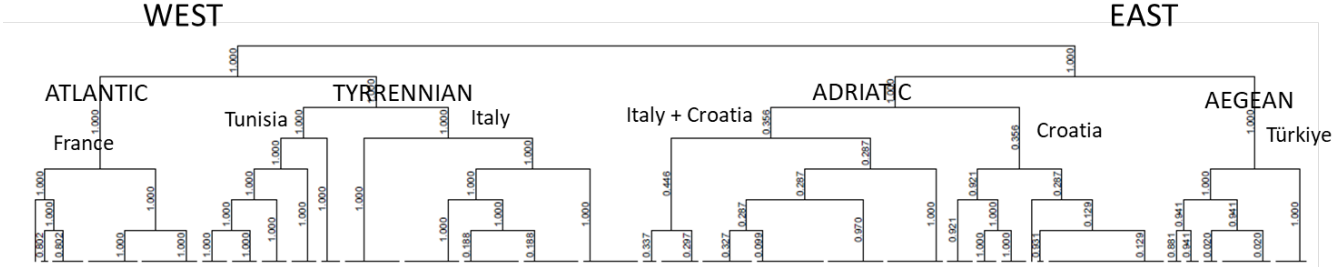
Cluster dendrogram. The French (except Corsican) and Croatian populations are completely separate due to the lack of data for many of the morphological characters considered.



PCA elaboration showing the three different morphotypes: a north-African morphotype (Tunisia + Sicily), a central Mediterranean morphotype (Italy) and an Aegean morphotype (Turkiye).

6.1.2 Genetic analysis results

All the populations show a well genetic structure.
The statistical analysis confirms the occurrence of different groups.



Cluster dendrogram grouping the different populations.

It is possible to recognize two main groups:
1. A Western group formed by Atlantic (France) and Tyrrhenian populations (Tunisia and western Italy)

2. A Eastern group formed by Turkish and Adriatic populations (Croatia and eastern Italy)
In the western group, there are the Atlantic population (France) and the Tyrrhenian populations (Tunisia, Corsica and western Italian population that are Sicily, Sardinia, Tuscany, Liguria).
In the Eastern group, there are the Adriatic populations (Croatia and the eastern Italian populations that are Conero, Porto Potenza, Puglia and Calabria) and the Aegean population) populations of Turkiye).

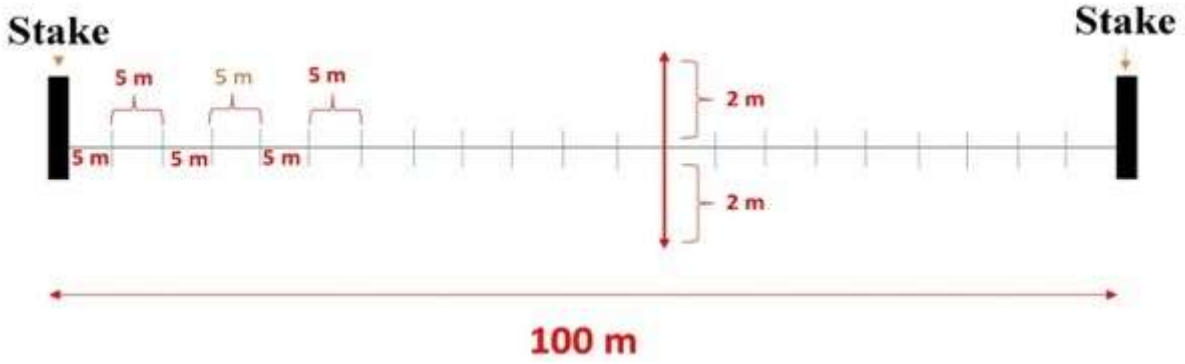
Conclusion

The morphological characterization has highlighted some important and interesting differences that distinguish the single populations even if some populations have not been analyzed in sufficient depth. It is however possible to identify some important characteristics that concern above all the seeds, the fruits and the parts that make up the inflorescence for which it can be said that three different morphological types exist: a North African morphotype (including Sicily), a Central Mediterranean morphotype (Italy) and an Eastern Mediterranean morphotype (Türkiye).
The genetic characterization carried out on 27 populations has highlighted a good genetic structuring of the populations. It is evident that the best structured populations are 4: an Atlantic population (France), a large Tyrrhenian population (which includes Tunisia, Corsica and the Italian populations of the Tyrrhenian coasts), a large Adriatic population (which includes Croatia, the Italian populations of the Adriatic coast and the population of Ionian coast) and an Aegean population (Turkiye).

7 ANNEX I

Proposed protocol for sampling and morphological characterization of wild sea fennel populations

Given that Crithmum maritimum L. is a sparse plant, the sampling on transects method is more appropriate (Wahid et al., 2018).
The plots or transects represent the "sampling units". To mark out the transect and survey the plants, proceed as follows:
• A 100 m rope is stretched, with marks every 5 m, along the length of the transect, marking the start and end with 2 stakes.
• The width of the transect is delimited by a 2 m stake. The stake is perpendicular to the transect strip, which divides the transect into two equal longitudinal halves. Counting is done as a "sweep" along the transect, from the start of the ribbon, marked by one of the stakes, to the end, marked by the next stake.



Specific cases

To facilitate field work it is possible to:

- Install the transect in parallel to the beach or by choosing the direction that contains the most individuals.
- If the number of individuals requested (20 individuals) has already been collected over a distance less than that of the rope (100 m) it is not necessary to cover the rest of the transect.
- If ever over the 100 m of the transect it was impossible to collect the 10 individuals in question, it would be possible to extend the transect until the requested number of individuals was collected.

N. of harvests

A first harvest in August/September 2022 to collect leaves/flowers/roots; a second harvest in September/October 2022 to collect the seeds
Number of populations per country: minimum 4 populations for each of the following countries: ITALY, FRANCE, CROATIA, TUNISIA, TURKEY, with a minimum distance of about 100 km between populations
WARINING FOR FRANCE: AT LEAST 1 ATLANTIC POPULATION SHOULD BE INCLUDED IN THE SAMPLING CAMPAIGN FOR COMPARISON WITH MEDITERRANEAN POPULATIONS!
The quantity of vegetable mass for molecular analysis is 5 g per individual.

Sampling of individuals
Number of individuals per population: 20 individuals.

For each population, individuals will be sampled into separate bags to avoid cross contamination. Aerial parts of sampled sea fennel individuals will be scanned and then pressed using a herbarium press in order to store them for a long time.

Sampling of seeds
Mature seeds will be also sampled, catalogued according to international protocols (ENSCONET, 2009; BUREAU OF LAND MANAGEMENT, 2018; ISTA, 2018), and stored at +4°C and at -20°C for long-term conservation.

Sampling of leaves

WARINING: THE LEAVES FROM THE 20 SAMPLED INDIVIDUALS HAVE TO BE STORED IN SEPARATED BAGS and coded with an unique alphanumerical code!

From each wild population selected for sampling (in view of the genetic and morphological characterization), at least 500 g of fresh young leaves/sprouts have to be sampled (by mixing leaves/sprouts from different individuals of the same population); the sampled leaves should be stored in a plastic sterile bag, transported to the laboratory under refrigerated conditions and subjected to freezing (-20 °C) as soon as possible prior to freeze drying, for long term storage. An aliquot (100 g) of fresh leaves/sprouts per population will be stored at +4°C and subjected immediately after sampling to the analysis of vitamin C.

The Partners involved in the chemical analyses of the sampled sea fennel populations are detailed as follows:

Sampling of flowers

From each wild population selected for sampling (in view of the genetic and morphological characterization), at least 500 g of fresh flowers have to be sampled (by mixing flowers from different individuals of the same population); the sampled flowers should be stored in a plastic sterile bag, transported to the laboratory under refrigerated conditions and subjected to freezing (-20 °C) as soon as possible prior to freeze drying, for long term storage. An aliquot (100 g) of fresh flowers per population will be stored at +4°C and subjected immediately after sampling to the analysis of vitamin C.

Methodology for morphological characterization of wild sea fennel populations

Morphological quantitative and qualitative traits will be analysed by adapting the International Plant Genetic Resources Institute (IPGRI) descriptors (1998) and a common field sheet (see for reference the fac simile proposed at the end of this document as an ANNEX)

The proposed morphological traits are:

- Plant : Height, Width, Diameter of main stem, number of branches, number of umbels,
- Umbell: Lenght of penduncle, Diametre of penducle, Number of rays per penduncle, Thickness of rays, Length of rays, Number of bracts, Length and Width of bracts, Shape of bracts, (5 umbels/individual counting the main umbell)
- Umbellet: Number of pedicels per umbellet, Number of flowers per umbellet, (all umbellets from 5 umbells), number of bracteoles, length and width of bracteoles, shape of bracteoles
- Leaves: Length , Width, Leaf area, Length petiol, Number of leaflets (lobes) (5 basal leaves/individual – it's possible to scan leaves and to make the necessary measurements directly on the scanned image),
- Flower: Diameter, Color (for the 5 umbels/individual),
- Fruit: Length, Width, Number of fruits per umbel (20 fruits/individual),
- Seed: Length, Width, Form, Number of seeds per fruit, Weight of 100 seeds, Thickness, Perimeter (scanned seed), Area (scanned seed), J-index (20 seeds/individual).

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Field sheet

Sampling of plant material

Type : □ Whole plant □ Flower □ Seeds □ Root □ Leaves □ Other _____

PROVIDER OF SAMPLES :

Partner (organization name) :_____Name (operator) : _____

GENERAL SITE INFORMATIONS

SITE NAME : _____ GPS COORDINATES : _____ UTM/WGS84

Site ID Number : _____ Altitude : _____

Country : _____ Topographic exposure : _____

Date : _____/_____/20___ Time : _____h_____

SITE CHARACTERIZATION

SOIL TYPE :Sandy Solid Rock Gravelly Other_____

SURROUNDING PLANTS (scientific name of indicator species) : _____

BIOCLIMATE : Classification by aridity index: Arid Sub-Arid Humid Sub-Humid Other_____

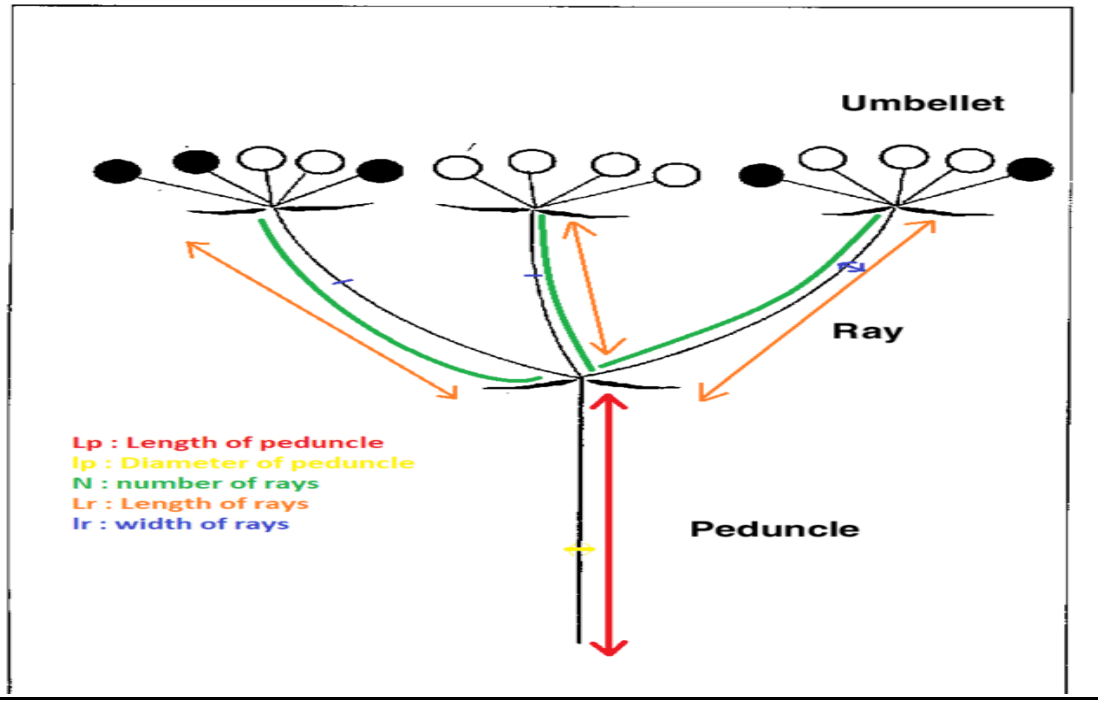
Or köppen classification

GUIDE

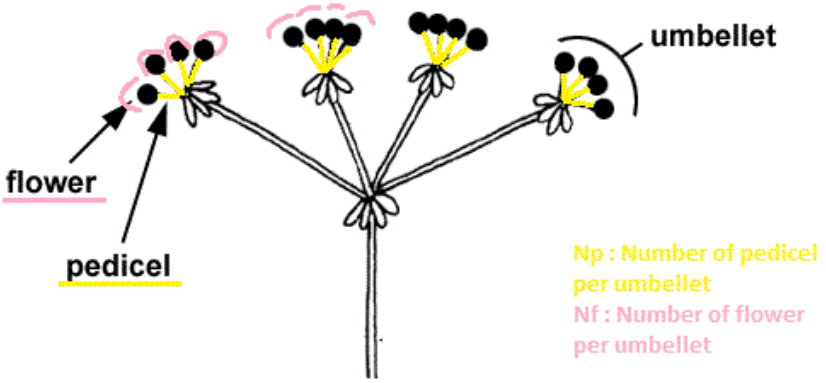
PLANT MEASUREMENT

Front view | Seen from above

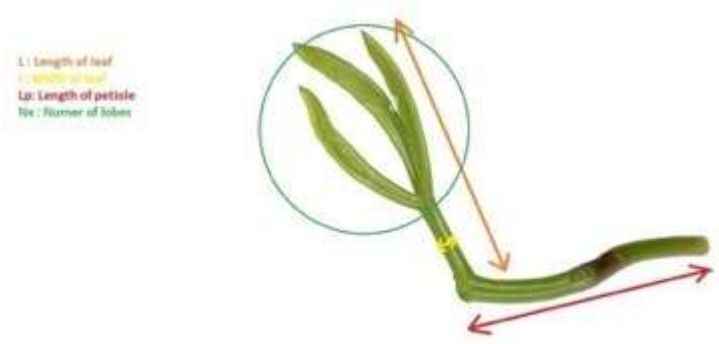
UMBELL MEASUREMENT



UMBELLET MEASUREMENT

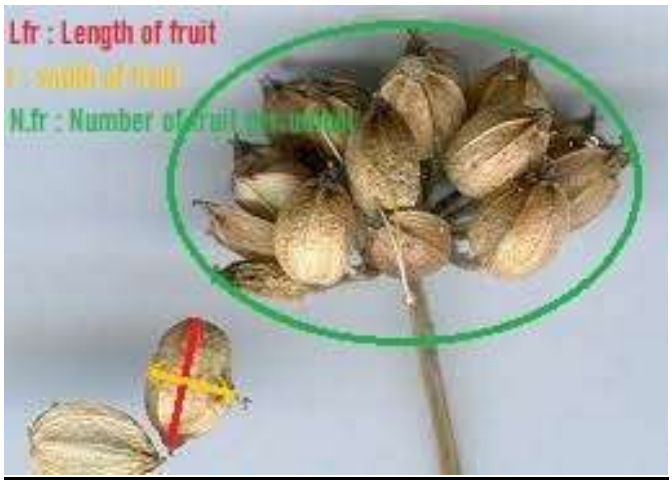


LEAVES MEASUREMENT



FLOWER MEASUREMENT

FRUIT MEASUREMENT



SEED MEASUREMENT

WARNING:

- ✓ Measurement of length and width must be done with Vernier caliper
- ✓ Unit of measure must be in centimeter (cm)
- ✓ Please carefully fill in and verify the details that are required

Morphological description

PLANT MATERIAL	DESCRIPTORS	MEASUREMENTS
Plant	-Height	_____ (cm)
	-Width	_____(cm)
	- Diameter of mean stem	_____(cm)
Umbell	-Lenght of Penduncle	_____ (cm)
	-Diametre of Penducle	_____ (cm)
	-Number of rays per penduncle	_____ / penducle
	-Thickness of rays	_____(cm)
	- length of rays	_____(cm)
	- Number of bracts	__ _____
	- Length of bract	_____(cm)
	- Width of bract	_____(cm)
	- Shape of bract	_____
Umbellet	-Number of pedicels per umbellet	_____/ umbellet
	- Number of flowers per umbellet	_____/ umbellet
LEAVES	-Lenght	_____(cm)
	-Width	_____(cm)
	-Leaf area	_____(cm^2)
	-Length petiol	_____(cm)
	-Number of leaflets (lobes)	_____
FLOWER	-Diameter	_____ (cm)
	-Color	☐ White ☐ Yellow Other (specify)
FRUIT	-Lenght	_____(cm)
	-Width	_____(cm)
	-Number of fruits per umbel	_____ /umbel
SEED	-Lenght	_____ (cm)
	-Widht	_____ (cm)

- Form	☐ OVOID ☐ OBLONG
-Number of seeds per fruit	_____/ fruit
-Weight of 100 seeds	_____ (g)
-Thickness	_____ (cm)
- Perimeter (scanned seed)	_____ (cm)
- Area (scanned seed)	_____ (cm^2)
- J-index	_____

Bibliography

1. Bacchetta, G., Fenu, G., Mattana, E., Piotto, B., &Virevaire, M. (2007). Manuel pour la récolte l'étude, la conservation et la gestion ex situ du matériel végétal.
2. Bureau of Land Management (2018) Seeds of success. Technical protocol for the collection, study, and conservation of seeds from native plant species. Restoration Ecology 28:S285–S302
3. Cervantes E, Martín J J , Saadaoui E (2016) Updated Methods for Seed Shape Analysis. Hindawi Publishing Corporation Scientifica Volume 2016, Article ID 5691825, 10 pages http://dx.doi.org/10.1155/2016/5691825
4. ENSCONET. 2009. Seed collecting manual for wild species. https://www.luomus.fi/sites/default/files/files/collecting_protocol_english.pdf
5. Hernandez, J.A., Almansa, M.S., Short-term effects of salt stress on antioxidant systems and leaf water relations of pea leaves, Physiol. Plant 115 (2002) 251–257
6. I.A. Ungar, Ecophysiology of Vascular Halophytes, CRC Press, Boca Raton, 1991.
7. IPGRI. 1998. Descriptors for wild and cultivated carrots (Daucus carota L.) International plant genetic resources institute, Rome, Italy. ISBN 92-9043-392-2.
8. ISTA. 2018. International Seed Testing Association. Seedling evaluation.

9. Karkanis, A., Polyzos, N., Kompocholi, M., &Petropoulos, S. A. (2022). Rock Samphire, a Candidate Crop for Saline Agriculture: Cropping Practices, Chemical Composition and Health Effects. Applied Sciences, 12(2), 737.
10. Katsouri, E., Demetzos, C., Perdetzoglou, D., &Loukis, A. (2001). An interpopulation study of the essential oils of various parts of Crithmum maritimum L. growing in Amorgos Island, Greece. Journal of Essential Oil Research, 13(5), 303-308.
11. Kostoula, O. K., Dimou, D., Yfanti, P., Douma, D., Karipidis, C., Kritsimas, A., &Patakioutas, G. (2016). Morphological and physiological aspects of Crithmum maritimum L.(Sea Fennel, Apiaceae) symbiosis with Glomus intraradices and Bacillus amyloliquefaciens FZB42. Fresenius Environmental Bulletin, 25(5), 1702-1714.
12. MAGINI, E. (1962): Le traitement des graines forestières, équipement et méthodes. II. Traitement, conservation, essai et transport des graines. Unasylva 16(1) 20–35.
13. Marchioni-Ortu, A., & Bocchieri, E. (1984). A study of the germination responses of a Sardinian population of sea fennel (Crithmum maritimum). Canadian journal of botany, 62(9), 1832-1835.
14. Nabet, N., Boudries, H., Chougui, N., Loupassaki, S., Souagui, S., Burló, F., ... &Larbat, R. (2017). Biological activities and secondary compound composition from Crithmum maritimum aerial parts. International Journal of Food Properties, 20(8), 1843-1855.
15. NIENSTAEDT, H. and SNYDER, E.B. (1974): Principles of genetic improvement of seed. In Seeds of woody plants in the United States. Agric. Handbook No 450, Forest Service, USDA, Washington D.C.
16. NIGOVI, B. (2003). Quantitative analysis of the polyphenols of the aerial parts of rock samphire–Crithmum maritimum L. Acta Pharm, 53, 139-144.
17. NIGOVI, B. (2003). Quantitative analysis of the polyphenols of the aerial parts of rock samphire–Crithmum maritimum L. Acta Pharm, 53, 139-144.
18. Rao, N. K., Hanson, J., Dulloo, M. E., Ghosh, K., & Nowell, A. (2006). Manuel de manipulation des semences dans les banques de gènes-Manuels pour les banques de gènes No. 8. Bioversity International.
19. Ravindran, P. N. (2017). The encyclopedia of herbs and spices. CABI.
20. Rozema,J., Van Diggelen J., A comparative study of growth and photosynthesis of four halophytes in response to salinity, Acta ¨col. 12 (1991) 673–681.
21. Souid, A., Croce, C. M. D., Pozzo, L., Ciardi, M., Giorgetti, L., Gervasi, P. G., ... & Longo, V. (2020). Antioxidant properties and hepatoprotective effect of the edible halophyte Crithmum maritimum L. against carbon tetrachloride-induced liver injury in rats. European Food Research and Technology, 246(7), 1393-1403.
22. Wahid N, Aabdousse J, Chkhichekh A, El Aboudi A, Lamhamedi MS, Bakry M (2018) Prospection et modalités d'échantillonnage pour capturer la diversité génétique des populations naturelles: cas du Myrtus communis L. au Maroc / Prospecting and sampling procedures to capture the genetic diversity of natural populations: case of Myrtus communis L. in Morocco. EcologiaMediterranea 44: 85-96.
23. Wilkie P, Dalberg Poulsen A, Harris D, Forrest LL (2013) The collection and storage of plant material for DNA extraction: The Teabag Method. Gardens' Bulletin Singapore 65(2): 231–234.
24. Zarrouk, M., El Almi, H., Ben Youssef, N., Sleimi, N., Ben Miled, D., Smaoui, A., Abdelly,C., Lipid composition of seeds of local halophyte species: Cakile maritima, Zygophyllum album and Crithmum maritimum, in: H. Lieth (Ed.), Cash Crop Halophytes: Recent Studies, Kluwer Academic Publishers Group, 2004, pp. 121–126.
25. Zenobi, S., Fiorentini, M., Zitti, S., Aquilanti, L., Foligni, R., Mannozzi, C., ... & Orsini, R. (2021). Crithmum maritimum L.: First results on phenological development and biomass production

26. Martins-Noguerol, R.; Matías, L.; Pérez-Ramos, I.M.; Moreira, X.; Muñoz-Vallés, S.; Mancilla-Leytón, J.M.; Francisco, M.; García-González, A.; DeAndrés-Gil, C.; Martínez-Force, E.; et al. Differences in Nutrient Composition of Sea Fennel (Crithmum Maritimum) Grown in Different Habitats and Optimally Controlled Growing Conditions. Journal of Food Composition and Analysis 2022, 106, 104266, doi:10.1016/J.JFCA.2021.104266.
27. Nartea, A.; Orhotohwo, O.L.; Fanesi, B.; Lucci, P.; Loizzo, M.R.; Tundis, R.; Aquilanti, L.; Casavecchia, S.; Quattrini, G.; Pacetti, D. Sea Fennel (Crithmum Maritimum L.) Leaves and Flowers: Bioactive Compounds, Antioxidant Activity and Hypoglycaemic Potential. Food Biosci 2023, 56, 103417, doi:10.1016/J.FBIO.2023.103417.