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Volatile Organic Compounds (VOCs) Diversity in the Orchid *Himantoglossum robertianum* (Loisel.) P. Delforge from Sardinia (Italy)

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Abstract: Volatile Organic Compounds (VOCs) are produced by plants to address a variety of physiological and ecological tasks (among others, stress resistance, and pollinator attraction). Genetics is a key factor in determining plants' VOCs content and emission, nevertheless, environment strongly influences VOCs profiles in plants. Orchids are a widespread group of plants that colonize diverse environments and rely on complex and refined pollination mechanisms to reproduce. Orchids VOCs are rarely studied and discussed in relation to growing conditions. In the present study, we compare the volatile profiles of inflorescences of *Himantoglossum robertianum* (Loisel.) P. Delforge sampled in six ecologically diverse populations on Sardinia Island (Italy). The essential oils obtained by steam distillation were characterized by GC-FID and GC-MS analysis. A total of 79 compounds were detected, belonging to the chemical classes of saturated hydrocarbons, esters, alcohols, ketones, unsaturated hydrocarbons, sesquiterpenes, oxygenated terpenes, terpenes, acids, and aldehydes. Multivariate statistics separated *H. robertianum* populations based on their chemical profiles. Differences were positively linked to the distance separating populations and reflected climatological features of the sampling sites. Interestingly, our results differed from those available in the literature, pointing out the high variability of VOCs profiles in this food-deceptive orchid.

Keywords: *Himantoglossum robertianum*; Orchidaceae; Sardinia (Italy); essential oil; VOCs; GC/FID; GC/MS

Citation: De Agostini, A.; Robustelli della Cuna, F.S.; Cortis, P.; Cogoni, A.; Sottani, C.; Soddu, F.; Sanna, C. Volatile Organic Compounds (VOCs) Diversity in the Orchid *Himantoglossum robertianum* (Loisel.) P. Delforge from Sardinia (Italy). *Diversity* **2022**, *14*, 1125. https://doi.org/10.3390/d14121125

Academic Editors: Pavel Kindlmann, Tiiu Kull and Melissa McCormick

Received: 11 November 2022
Accepted: 13 December 2022
Published: 15 December 2022

1. Introduction

Volatile Organic Compounds (VOCs) are low-boiling-point secondary metabolites synthesized by plants to address a variety of ecological and physiological challenges. VOCs are one of the classes of functional and defensive compounds that allow plants to thrive despite suboptimal or even extreme environmental conditions. In particular, VOCs play a key role in protecting plants against biotic and abiotic stressors (e.g., pathogens, drought, herbivores, etc.) [1]. VOCs are also pivotal in pollination strategies acting as olfactory signals that attract pollinators [2]. Depending on their origin, VOCs belong to terpenoids, phenylpropanoids/benzenoids, and fatty acid derivatives categories [3].

Moreover, alkenes can be produced after the desaturation of fatty acids. Although genetics has been known to shape plant VOCs content, blend, and emission, there is growing evidence of a strong influence of the environment in determining plants' VOCs profile [1,4–7]. Orchids are a widespread plant group currently experiencing great and rapid evolutive radiation [8]. Pollination in orchids is probably one of the most refined in the plant kingdom, and in approximately one-third of them it depends on the deception of

pollinators [9]. Deceptive strategies in orchids consist of deceiving pollinators by promising a food reward, a shelter, or even a reproductive act to achieve pollination [10,11].

In orchids, chemical signals consist of the emission of a complex blend of VOCs that has a central role in both the rewarding and deceptive pollination strategies, nevertheless, in the latter, the chemical route is even more crucial [10]. In sex-deceptive orchids, the pollinator female's sex pheromone is finely mimicked to lure naïve males toward the inflorescences, while food-deceptive orchids produce a volatile bouquet similar to that of food-providing plants to attract pollinators in search of food.

Despite the importance of VOCs in the Orchidaceae family, the number of scientific papers focusing on their characterization to date is quite low. Within a project aiming to study VOCs from Sardinian orchids, in the present work, we focused on *Himantoglossum robertianum* (Loisel.) P. Delforge (syn. *Barlia robertiana*) (Figure 1), a food-deceptive orchid widely distributed in the Mediterranean basin, from Portugal to Anatolia [12].

Figure 1. *H. robertianum* in its natural habitat in Sardinia (Italy). Photo courtesy of Vincenzo Rodi.

The typical habitat of this species is sunny to mid-shady on alkaline, dry to moist substrates, up to 1700 m asl [13]. The species, commonly called the "giant orchid" because of its large size (25 to 80 cm tall), is featured by thick stems and by a basal rosette of 5 to 10 leaves. Inflorescences are dense, 6–23 cm tall, hosting up to 60 flowers. The blooming stage starts in January and ends in April [13]. *H. robertianum* is one of the 64 wild-occurring orchids in Sardinia (Italy) [14], where it could be easily found in degraded urban lots (even in airport runaways' edges), coastline dunes, mountain roadsides, etc. The widespread distribution of *H. robertianum* in Sardinia, together with its ecological plasticity made it the ideal study species to investigate the variability of VOCs profiles in orchids in relation to different environmental conditions. In particular, we studied the influence of environmental conditions on VOCs profile by comparing essential oils obtained from the inflorescence of *H. robertianum* from six different sites.

2. Materials and Methods

2.1. Chemicals

Octyl octanoate (98%), alkane mix (C6–C35), and anhydrous sodium sulfate were obtained by Sigma-Aldrich, Inc. (St. Louis, MO, USA). Ultrapure water (LC-MS grade) was

produced using Milli Q-Milli RO system, Millipore (Burlington, MA, USA). Diethyl ether was purchased from Carlo Erba reagents (Cornaredo, Italy).

2.2. Plant Material

Himantoglossum robertianum (Loisel.) P. Delforge inflorescences were sampled in six different localities of Sardinia Island (Italy), chosen for the diverse ecological growing conditions (Figure 2).

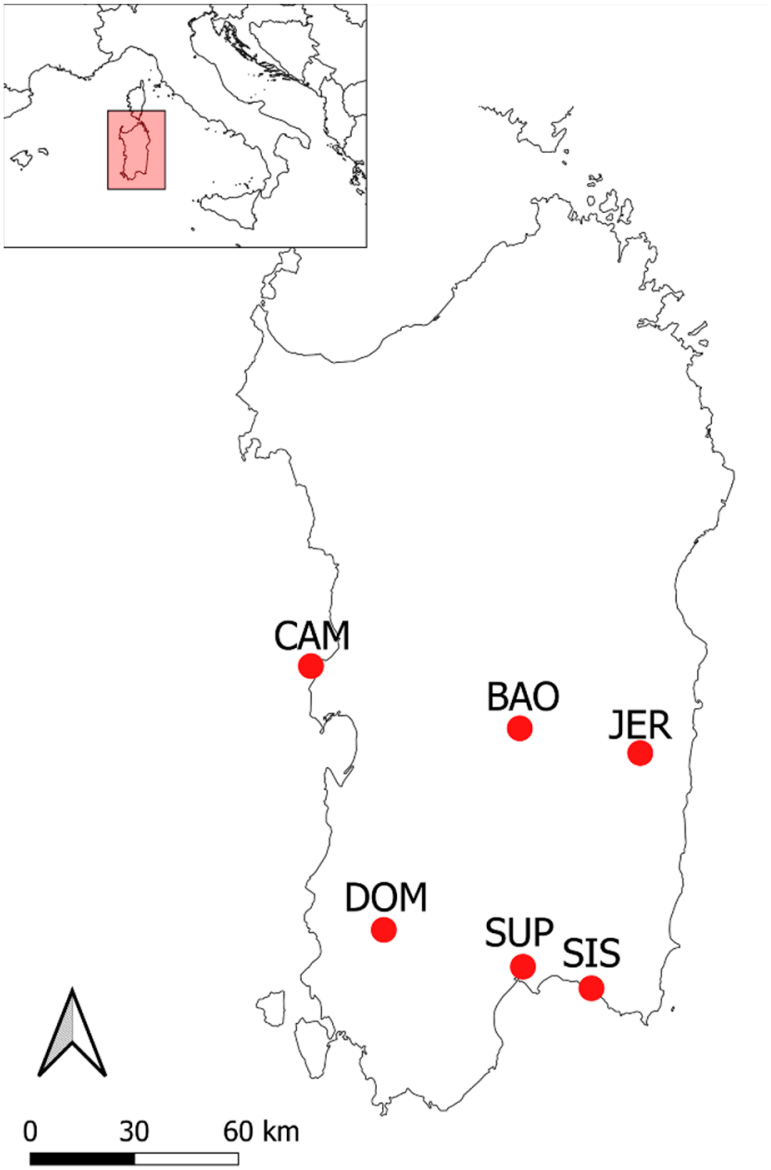

Figure 2. Sampling sites of *H. robertianum* in Sardinia (Italy): BAO = Bau Onu, CAM = Capo Mannu, DOM = Domusnovas, JER = Jerzu, SIS = Sant'Isidoro, SUP = Su Planu.

The studied populations ranged in altitude from ca 40 m up to ca 700 m asl. Moreover, they grew on diverse lithology and were included in different types of vegetation. Information on sampling sites is reported in Table 1.

Table 1. Details of collection sites of the six Sardinian populations of *H. robertianum*.

Label [a]	Site [b]	Municipality [c]	Coord. [d]	Alt. [e]	Date [f]	Voucher [g]
BAO	Rio Bau Onu	Laconi	39°52′31.36″ N 9°5′36.38″ E	719 m	16 March 2021	CAG1305/V1e
CAM	Capo Mannu	San Vero Milis	40° 2′35.60″ N 8°23′15.46″ E	48 m	23 February 2021	CAG1305/V1f
DOM	Domusnovas	Domusnovas	39°21′30.13″ N 8°37′4.20″ E	258 m	2 March 2021	CAG1305/V1c
JER	Jerzu	Jerzu	39°48′20.6″ N 9°30′46.3″ E	550 m	25 February 2021	CAG1305/V1b
SIS	Sant'Isidoro	Quartucciu	39°14′43.03″ N 9°18′3.31″ E	54 m	1 March 2021	CAG1305/V1d
SUP	Su Planu	Selargius	39°15′27.62″ N 9°6′28.60″ E	41 m	17 February 2021	CAG1305/V1a

[a] labels used to refer to the studied populations; [b] collection sites; [c] municipality; [d] sampling sites coordinates; [e] altitude (m asl); [f] sampling date; [g] specimen voucher.

Further environmental data (climatological, lithological and vegetational) of sampling sites can be found in Table S1. Climatological data used in the present study were obtained from the database of the climatic monitoring authority of Sardinia [15] and include: mean value of the daily minimum temperature (°C); mean value of the daily maximum temperature (°C); monthly precipitations (mm); solar radiation (MJ/m^2); precipitation to temperature ratio. As regards climatological data, in the present study we chose to consider the mean value of the two months (January and February 2021) preceding the collection of the plant material because they represented the climate features of each population during the inflorescence development. Lithological frameworks were obtained from [16,17], while vegetation data consisted of personal annotations. The sampling of plant material provided to collect mature inflorescences presenting well-developed flowers with no signs of pollination (i.e., the presence of swollen ovaries or fruits). Three individuals per site were sampled. Plants were transported zip-locked in polyethylene bags in dark and cool conditions, and once in the laboratory flowers were separated from the inflorescence, weighted, and immediately stored at −80 °C until steam distillation. A specimen voucher for each population was deposited at the General Herbarium (Herbarium CAG) of the Department of Life and Environmental Sciences, University of Cagliari (see Table 1 for specimens' vouchers).

2.3. Extraction of Essential Oils

Frozen inflorescences of *H. robertianum* (SUP 19.7 g, JER 20.61 g, DOM 24.83 g, SIS 20.59 g, BAO 21.6 g, CAM 23.18 g) to which octyl octanoate (0.25 mg) was added as internal standard were subjected to steam distillation with distilled water for 3 h according to Robustelli della Cuna et al. [18] with slight modifications to the protocol. Diethyl ether (3 × 100 mL) was used as solvent to separate organic and aqueous phases. The former was dried with anhydrous sodium sulfate and submitted to rotary evaporation at 30 °C to yield pure essential oil. The yield was calculated as the ratio between the mass of the extracted oil (g) and the initial plant biomass (g). The essential oils were stored at −20 °C until GC/FID and GC/MS analyses.

2.4. GC-FID and GC-Ms Analysis

The analyses (performed in triplicate) of essential oils were carried out using a Hewlett Packard model 5980 GC, equipped with Elite-5MS (5% phenyl methyl polysiloxane) capillary column of (30 m × 0.32 mm i.d.) and film 0.32 µm thick. The carrier gas was He at a flow of 1 mL/min. In split mode (30:1), 1 µL of essential oil was manually injected. The oven temperature program included an initial isotherm of 40 °C for 5 min, followed by a temperature ramp to 260 °C at 4 °C/min, and a final isotherm at this temperature for

10 min. Injector and detector temperatures were set at 250 and 280 °C, respectively. The relative amount of each component was calculated based on the corresponding FID peak area without response factor correction. The GC-MS analyses were carried out using a GC Model 6890 N, coupled to a benchtop MS Agilent 5973 Network, equipped with the same capillary column and following the same chromatographic conditions used for the GC/FID analyses. The carrier gas was He at a constant flow of 1.0 mL/min. The essential oils were diluted before analysis and 1.0 µL was manually injected into the GC system with a split ratio of 30:1. The ion source temperature was set at 200 °C, while the transfer line was 300 °C. The acquisition range was 40–500 amu in electron-impact (EI) positive ionization mode using an ionization voltage of 70 eV [19].

2.5. Identification of the Components of the Volatile Fractions

The identification of the volatile oil components was performed by their retention indices (RI) and their mass spectra and by comparison with a NIST database mass spectral library, as well as with literature data [20,21]. Retention indices were calculated by Elite-5MS capillary columns using an *n*-alkane series (C6–C35) under the same GC conditions as for the samples. The relative amount of each component of the oil was expressed as percent peak area relative to the total peak area from GC/FID analyses of the whole extracts. The quantitative data were obtained from GC/FID analyses by an internal standard method and assuming an equal response factor for all detected compounds.

2.6. Statistical Analysis

A bar plot was drawn to compare the number of compounds belonging to different chemical classes among localities. A Venn diagram and a related table were consequently created to show the number and the identity of the compounds shared by the populations. All the statistical analyses described hereafter were carried out using the percent content value of each compound on the total essential oil, considering the mean value of the three technical replicates for each compound. A principal component analysis (PCA) was carried out. The individuals' plot was separated from the variables' plot to improve the readability of both. The variables' plot was created considering the 43 most contributing variables to the PCA first and second principal components (PCs). The co-variation between climatological/geographical features of populations and VOCs profiles was tested by the Mantel test. To implement the test, distance matrices were built to be later compared based on: the coordinates of the studied populations, the climatological data measured in each study site, and the populations' VOCs profiles. The following climatological data were considered in the analysis: altitude, mean value of the daily minimum temperature (°C), the mean value of the daily maximum temperature (°C), precipitations (mm), solar radiation (MJ/m^2), precipitation-temperature ratio (Table S1) (climatological data were scaled before analysis). All factors were considered on the whole and each one singularly. The co-variation of VOCs content in *H. robertianum* was first evaluated by considering all the compounds together and then divided in chemical classes. Diversity was quantified by Euclidean distance. The correlation method implemented in the Mantel test was the Spearman method (719 permutations). Boxplots were used to represent the distribution of compound content values in the different populations. Analysis of variance (ANOVA) was implemented to assess the significance of the differences in the content of each class of compounds among samples. Whenever data did not comply with the assumptions for implementing parametric ANOVA, the Kruskal–Wallis analysis was performed. If the analysis of variance resulted in a *p*-value less than the significance level of 0.05, the Tukey or the Pairwise Wilcox (Bonferroni corrected for multiple comparisons) post-hoc tests were implemented in case of Parametric or Kruskal–Wallis analysis of variance, respectively. Significant differences emerging by the analysis of variance will be represented above each boxplot by compact letter display so that different letters between two groups (populations) will indicate significant differences. All the statistical analyses were carried out using

R-software, version 4.1.3 (10 March 2022) [22] implemented with the following packages: Venn, factoextra, vegan, ggpubr.

3. Results

The yield of essential oils obtained by steam distillation from inflorescences of *H. robertianum* was different depending on the geographical origin of the samples. More precisely, yields were: JER 0.37%, SIS 0.31%, CAM 0.16%, SUP 0.14%, BAO 0.11%, and DOM 0.10%.

Chemical analysis revealed a total of 79 compounds (Table 2). All compounds were reported as percentages (obtained from GC/FID analyses) of the total essential oil, together with their retention indices (RI) on the Elite-5 MS column, compared to the corresponding values from the literature [20].

Out of the detected compounds, 79 were identified in JER, 71 in SUP, followed by DOM (69), BAO (68), and CAM (66); SIS was the population where *H. robertianum* essential oil was featured by the smallest number of compounds (42). Interestingly, excluding the case of JER, higher yields in essential oil were not related to a higher number of compounds in it. Hexadecanoic acid methyl ester was the main compound among all samples, with percentage of abundance ranging from 12.55% (CAM) to 19.46% (SIS). The other main constituents were heptacosane (6.22%), heneicosane (5.77%), nonadecane (5.70%), tricosane (5.61%), hexacosane (5.24%) and eicosane (5.22%) in SUP; hexacosane (7.59%), pentacosane (6.25%), eicosane (6.14%), tetracosane (5.94%), heneicosane (5.84%) and docosane (5.75%) in JER; 7.9-di-tert-butyl-1-oxaspiro[4.5]deca-6.9-diene-2.8-dione (8.94%), eicosane (8.55%), octadecane (6.75%), heneicosane (6.65%), docosane (5.05%) and *p*-cresol (4.94%) in DOM; *p*-cresol (8.13%), nonadecane (6.94%), eicosane (6.45%), octadecane (5.25%), 7.9-di-tert-butyl-1-oxaspiro[4.5]deca-6.9-diene-2.8-dione (4.93%) and pentacosane (4.84%) in SIS; eicosane (8.22%), heptacosane (7.65%), heneicosane (6.73%), tricosane (6.25%), docosane (5.76%) and pentacosane (5.75%) in BAO; eicosane (9.15%), 7.9-di-tert-butyl-1-oxaspiro[4.5]deca-6.9-diene-2.8-dione (8.45%), heneicosane (7.73%), *p*-cresol (6.65%), nonadecane (6.15) and octadecane (5.85%) in CAM (Table 2).

The identified compounds belong to the following 10 chemical classes: acids, alcohols, aldehydes, esters, ketones, saturated hydrocarbons, unsaturated hydrocarbons, terpenes, oxygenated terpenes, and sesquiterpenes. In the present study the classes of saturated and unsaturated hydrocarbons include compounds that do not have a terpenic nature (and that should be attributed to inflorescences' epicuticular waxes). As regards the adopted classification of terpenic compounds, we chose to separately consider sesquiterpenes, since they resulted to be the most abundant terpenic compounds (ranging from 1.38 to 2.61% in SIS and CAM, respectively); the remaining terpenic compounds were divided based on the presence/absence of oxygen. Moreover, the classification of alcohols, aldehydes, esters and ketones here adopted includes compounds that do not have a terpenic nature, but which have these functional groups. Three compounds were not identified (and consequently grouped in the Unidentified class), and three (o-xylene, anisole, and 2,5-cyclohexadiene-1,4-dione,2,6-bis(1,1-dimethyl ethyl)) were grouped in the Miscellanea class. The relative contribution of the different chemical classes to the total essential oils in the six populations of *H. robertianum* is reported in Table 3.

Table 2. Essential oils composition from inflorescences of six Sardinian populations of *H. robertianum*.

Compound [a]	RI Tab [b]	RI mean [c]	BAO	CAM	DOM	JER	SIS	SUP	Identification [e]
			% [d]	%	%	%	%	%	
2-Methyl-2-pentenal	821	828	0.16 ± 0.03	0.15 ± 0.04	0.35 ± 0.04	0.35 ± 0.04	0.15 ± 0.03	0.28 ± 0.04	NIST. RI
Diacetone alcohol	841	836	0.35 ± 0.04	0.14 ± 0.05	0.12 ± 0.03	0.15 ± 0.04	-	-	NIST. RI
4-Methyloctane	862	861	0.05 ± 0.04	-	0.10 ± 0.04	0.06 ± 0.03	-	-	NIST. RI
o-Xylene	867	869	0.05 ± 0.03	-	0.13 ± 0.05	0.03 ± 0.02	-	0.11 ± 0.03	NIST. RI
Heptanal	901	902	0.43 ± 0.03	0.05 ± 0.03	0.09 ± 0.04	0.05 ± 0.02	0.35 ± 0.04	0.04 ± 0.03	NIST. RI
Anisole	916	916	0.05 ± 0.03	0.06 ± 0.04	0.07 ± 0.04	0.06 ± 0.05	0.03 ± 0.02	0.07 ± 0.02	NIST. RI
6-methyl-5-hepten-2-one	986	985	0.64 ± 0.04	0.24 ± 0.04	0.26 ± 0.03	0.06 ± 0.03	-	-	NIST. RI
Decane	1000	1000	0.04 ± 0.04	-	0.04 ± 0.03	0.03 ± 0.02	-	0.02 ± 0.01	NIST. RI
Octanal	1007	1004	0.05 ± 0.03	0.05 ± 0.02	0.06 ± 0.03	0.04 ± 0.04	0.16 ± 0.05	0.03 ± 0.02	NIST. RI
2-Ethylhexanol	1029	1029	-	-	0.03 ± 0.02	0.03 ± 0.02	-	0.05 ± 0.04	NIST. RI
β-Phorone	1044	1044	1.30 ± 0.11	1.45 ± 0.04	-	0.63 ± 0.04	1.66 ± 0.05	1.13 ± 0.04	NIST. RI
γ-Terpinene	1059	1060	0.04 ± 0.03	0.05 ± 0.03	0.07 ± 0.02	0.04 ± 0.04	-	0.04 ± 0.02	NIST. RI
1-Octanol	1074	1071	0.25 ± 0.05	-	0.05 ± 0.04	0.13 ± 0.03	0.27 ± 0.05	0.28 ± 0.05	NIST. RI
p-Cresol	1076	1077	2.99 ± 0.10	6.65 ± 0.03	4.94 ± 0.04	2.08 ± 0.04	8.13 ± 0.03	3.29 ± 0.02	NIST. RI
Linalool	1097	1099	0.34 ± 0.03	0.06 ± 0.05	0.12 ± 0.03	0.14 ± 0.04	-	0.18 ± 0.04	NIST. RI
2-Phenyl ethanol	1102	1107	0.35 ± 0.04	1.46 ± 0.04	0.93 ± 0.03	0.48 ± 0.03	3.45 ± 0.04	0.33 ± 0.01	NIST. RI
cis-Verbenol	1141	1142	0.06 ± 0.04	0.04 ± 0.04	0.05 ± 0.04	0.05 ± 0.04	-	0.03 ± 0.03	NIST. RI
trans-Verbenol	1145	1148	0.05 ± 0.04	0.05 ± 0.03	-	0.07 ± 0.04	-	0.03 ± 0.02	NIST. RI
Camphor	1150	1154	0.04 ± 0.04	-	0.06 ± 0.03	0.07 ± 0.03	-	0.02 ± 0.01	NIST. RI
β-Phellandren-8-ol	1154	1156	-	-	-	0.06 ± 0.04	-	0.05 ± 0.03	NIST. RI
Pinocarvone	1165	1162	0.15 ± 0.03	0.35 ± 0.04	0.15 ± 0.04	0.34 ± 0.05	-	0.09 ± 0.04	NIST. RI
α-Phellandren-8-ol	1170	1176	0.3 4 ± 0.05	0.05 ± 0.02	-	0.14 ± 0.04	-	0.18 ± 0.07	NIST. RI
Borneol	1173	1179	0.57 ± 0.04	0.03 ± 0.03	0.23 ± 0.04	0.16 ± 0.04	-	-	NIST. RI
Terpinen-4-ol	1182	1186	0.07 ± 0.04	0.06 ± 0.03	-	0.08 ± 0.03	-	0.04 ± 0.01	NIST. RI

Table 2. *Cont.*

Compound [a]	RI Tab [b]	RI mean [c]	BAO	CAM	DOM	JER	SIS	SUP	Identification [e]
Dodecane	1200	1200	0.53 ± 0.04	0.15 ± 0.03	0.16 ± 0.04	0.18 ± 0.04	0.26 ± 0.03	0.32 ± 0.03	NIST. RI
Decanal	1204	1207	0.04 ± 0.04	0.15 ± 0.04	0.05 ± 0.03	0.03 ± 0.02	-	0.07 ± 0.03	NIST. RI
Verbenone	1208	1213	0.06 ± 0.04	0.04 ± 0.04	-	0.07 ± 0.03	-	0.06 ± 0.02	NIST. RI
Geraniol	1253	1252	0.15 ± 0.03	0.35 ± 0.04	0.83 ± 0.02	0.56 ± 0.03	0.45 ± 0.04	0.37 ± 0.04	NIST. RI
2-Decenal. (*E*)	1264	1264	0.05 ± 0.04	0.06 ± 0.03	0.05 ± 0.03	0.06 ± 0.03	-	0.02 ± 0.01	NIST. RI
Nonanoic acid	1271	1270	-	-	-	0.11 ± 0.03	-	0.40 ± 0.54	NIST. RI
Bornyl acetate	1289	1288	0.04 ± 0.03	-	0.03 ± 0.03	0.05 ± 0.03	-	-	NIST. RI
Tridecane	1300	1300	0.55 ± 0.04	0.15 ± 0.02	0.18 ± 0.04	0.15 ± 0.04	0.24 ± 0.02	0.46 ± 0.02	NIST. RI
2.4-Decadienal (*E.Z*)	1302	1308	0.07 ± 0.05	0.05 ± 0.04	0.04 ± 0.03	0.05 ± 0.03	0.09 ± 0.05	0.03 ± 0.03	NIST. RI
2.4-Decadienal (*E.E*)	1319	1321	0.48 ± 0.07	0.16 ± 0.02	0.16 ± 0.03	0.18 ± 0.02	0.18 ± 0.05	0.21 ± 0.03	NIST. RI
Decanoic acid	1372	1366	0.05 ± 0.04	0.15 ± 0.05	0.09 ± 0.04	0.06 ± 0.02	-	0.01 ± 0.01	NIST. RI
1-Tetradecene	1390	1392	-	-	0.06 ± 0.03	0.03 ± 0.02	-	-	MS. RI
Tetradecane	1400	1401	1.62 ± 0.03	1.83 ± 0.03	2.07 ± 0.02	1.14 ± 0.02	-	1.24 ± 0.02	NIST. RI
β-Caryophyllene	1417	1428	0.05 ± 0.04	0.15 ± 0.03	0.07 ± 0.04	0.06 ± 0.03	-	0.01 ± 0.01	NIST. RI
Unidentified	-	1433	0.23 ± 0.04	0.25 ± 0.04	0.74 ± 0.03	0.44 ± 0.04	-	0.18 ± 0.04	-
trans-b-Farnesene	1452	1459	0.15 ± 0.04	0.33 ± 0.03	0.44 ± 0.04	0.33 ± 0.04	-	0.18 ± 0.03	NIST. RI
2,5-Cyclohexadiene-1, 4-dione, 2,6-bis(1.1-dimethylethyl)	1469	1466	0.88 ± 0.12	0.55 ± 0.03	0.83 ± 0.03	0.75 ± 0.03	1.07 ± 0.02	0.83 ± 0.03	NIST. RI
β-Sesquiphellandrene	1537	1530	0.16 ± 0.02	0.12 ± 0.03	0.14 ± 0.03	0.14 ± 0.04	-	0.14 ± 0.05	NIST. RI
Unidentified	-	1533	0.17 ± 0.07	0.28 ± 0.02	0.35 ± 0.03	0.25 ± 0.04	0.34 ± 0.04	0.09 ± 0.04	-
Dodecanoic acid	1566	1565	0.14 ± 0.03	0.44 ± 0.04	0.45 ± 0.04	0.44 ± 0.04	-	0.38 ± 0.04	
1-Hexadecene	1591	1590	0.12 ± 0.01	0.15 ± 0.03	0.15 ± 0.04	0.05 ± 0.04	0.05 ± 0.04	0.14 ± 0.04	MS. RI
2-Hexadecene	1598	1596	0.15 ± 0.04	0.05 ± 0.03	0.16 ± 0.03	0.07 ± 0.04	0.09 ± 0.04	0.07 ± 0.02	MS. RI
Hexadecane	1600	1601	0.38 ± 0.08	0.64 ± 0.05	0.85 ± 0.04	0.57 ± 0.03	-	0.61 ± 0.03	NIST. RI
γ-eudesmol	1630	1634	0.13 ± 0.03	0.06 ± 0.04	0.05 ± 0.04	0.05 ± 0.04	-	0.02 ± 0.01	NIST. RI

Table 2. *Cont.*

Compound [a]	RI Tab [b]	RI mean [c]	BAO	CAM	DOM	JER	SIS	SUP	Identification [e]
Pentadecane. 2.6.10-trinethyl	1647	1647	0.32 ± 0.02	0.34 ± 0.05	0.35 ± 0.04	0.26 ± 0.03	-	-	NIST. RI
7-heptadecene	1673	1674	0.86 ± 0.03	0.13 ± 0.03	0.24 ± 0.05	0.15 ± 0.02	0.08 ± 0.05	0.62 ± 0.03	MS. RI
1-heptadecene	1680	1679	-	0.15 ± 0.04	0.15 ± 0.04	0.03 ± 0.02	0.07 ± 0.04	0.03 ± 0.01	MS. RI
(*E*)-3-Heptadecene	1686	1683	0.33 ± 0.03	0.24 ± 0.05	0.24 ± 0.04	0.12 ± 0.03	0.11 ± 0.02	0.08 ± 0.01	MS. RI
(*Z*)-3-Heptadecene	1692	1693	-	0.25 ± 0.04	0.24 ± 0.03	0.17 ± 0.03	0.13 ± 0.04	0.14 ± 0.02	MS. RI
Heptadecane	1700	1703	2.05 ± 0.03	2.59 ± 0.02	3.35 ± 0.04	2.04 ± 0.03	2.25 ± 0.04	2.15 ± 0.04	NIST. RI
(*E*)-2-Heptadecene	1702	1709	0.24 ± 0.04	0.48 ± 0.04	0.44 ± 0.04	0.25 ± 0.03	0.12 ± 0.04	0.21 ± 0.02	MS. RI
(*Z*)-2-Heptadecene	1716	1713	0.55 ± 0.04	0.35 ± 0.03	-	0.25 ± 0.03	-	-	MS. RI
Farnesol	1718	1720	1.64 ± 0.09	1.95 ± 0.04	1.84 ± 0.03	0.86 ± 0.04	1.38 ± 0.08	1.23 ± 0.03	NIST. RI
Methyl tetradecanoate	1722	1725	0.85 ± 0.04	0.56 ± 0.02	0.94 ± 0.14	0.54 ± 0.04	0.70 ± 0.04	0.40 ± 0.01	NIST. RI
1-Octadecene	1791	1793	-	-	0.16 ± 0.04	0.44 ± 0.15	-	0.40 ± 0.54	MS. RI
Octadecane	1800	1806	2.79 ± 0.12	5.85 ± 0.04	6.75 ± 0.04	4.26 ± 0.04	5.25 ± 0.04	4.75 ± 0.43	STD. RI
Phytane	1811	1810	0.93 ± 0.04	1.36 ± 0.03	1.56 ± 0.03	0.95 ± 0.04	0.44 ± 0.04	1.42 ± 0.02	NIST. RI
3-Methyloctadecane	1874	1874	0.75 ± 0.03	1.36 ± 0.03	1.74 ± 0.04	0.74 ± 0.03	-	1.36 ± 0.02	NIST. RI
Nonadecane	1900	1903	-	6.15 ± 0.04	-	5.74 ± 0.05	6.94 ± 0.02	5.70 ± 0.03	STD. RI
7.9-di-tert-butyl-1-oxaspiro[4.5]deca-6.9-diene-2.8-dione	1916	1910	-	8.45 ± 0.04	8.94 ± 0.04	0.93 ± 0.03	4.93 ± 0.03	1.39 ± 0.04	NIST. RI
Hexadecanoic acid methyl ester	1926	1934	15.50 ± 0.45	12.55 ± 0.04	13.47 ± 0.14	16.81 ± 0.05	19.46 ± 0.15	18.08 ± 0.05	NIST. RI
Hexadecanoic acid	1942	1939	-	0.64 ± 0.03	0.38 ± 0.05	0.66 ± 0.04	0.45 ± 0.04	0.45 ± 0.04	NIST. RI
Eicosane	2000	2006	8.22 ± 0.07	9.15 ± 0.04	8.55 ± 0.04	6.14 ± 0.19	6.45 ± 0.04	5.22 ± 0.03	STD. RI
Heneicosane	2100	2105	6.73 ± 0.04	7.73 ± 0.05	6.65 ± 0.06	5.84 ± 0.04	4.81 ± 0.06	5.77 ± 0.15	STD. RI
Methyl octadecanoate	2127	2124	0.68 ± 0.06	2.37 ± 0.08	2.85 ± 0.05	2.78 ± 0.03	0.74 ± 0.03	2.35 ± 0.03	NIST. RI
Docosane	2200	2203	5.76 ± 0.02	5.76 ± 0.17	5.05 ± 0.04	5.75 ± 0.04	4.48 ± 0.05	4.02 ± 0.03	STD. RI

Table 2. *Cont.*

Compound [a]	RI Tab [b]	RI mean [c]	BAO	CAM	DOM	JER	SIS	SUP	Identification [e]
9-Tricosene	2279	2279	1.45 ± 0.04	1.20 ± 0.02	1.08 ± 0.05	1.25 ± 0.03	0.95 ± 0.04	1.03 ± 0.04	MS. RI
Tricosane	2300	2303	6.25 ± 0.04	4.13 ± 0.04	3.84 ± 0.04	2.73 ± 0.03	3.74 ± 0.03	5.61 ± 0.36	STD. RI
Tetracosane	2400	2402	4.94 ± 0.04	2.80 ± 0.04	3.44 ± 0.12	5.94 ± 0.03	3.84 ± 0.04	3.93 ± 0.05	STD. RI
Pentacosane	2500	2503	5.75 ± 0.03	2.96 ± 0.04	3.15 ± 0.05	6.25 ± 0.04	4.84 ± 0.04	4.04 ± 0.03	STD. RI
Unidentified	-	2590	-	-	0.18 ± 0.04	0.34 ± 0.04	-	0.13 ± 0.06	-
Hexacosane	2600	2602	4.64 ± 0.03	1.33 ± 0.04	3.24 ± 0.03	7.59 ± 0.08	4.06 ± 0.04	5.24 ± 0.04	STD. RI
Heptacosane	2700	2702	7.65 ± 0.05	1.76 ± 0.03	3.94 ± 0.04	5.63 ± 0.04	4.55 ± 0.04	6.22 ± 0.02	STD. RI
Octacosane	2800	2801	5.30 ± 0.03	0.35 ± 0.04	1.44 ± 0.05	1.54 ± 0.04	2.26 ± 0.08	4.62 ± 0.04	STD. RI
Nonacosane	2900	2900	1.24 ± 0.05	-	-	1.84 ± 0.05	-	1.27 ± 0.07	STD. RI

[a] Compounds are listed in order of their elution on an Elite-5 column. [b] Retention indices according to Adams [20] unless stated otherwise. [c] Retention indices determined on an Elite-5 column using a homologous series of *n*-hydrocarbons (mean of three replicates). [d] (mean ± SD of three replicates). BAO = Bau Onu, CAM = Capo Mannu, DOM = Domusnovas, JER = Jerzu, SIS = Sant'Isidoro, SUP = Su Planu. [e] Method of identification: STD = pure compound; MS = mass spectrum; NIST = comparison with library [21]; RI = retention indices in agreement with literature values.

Table 3. Relative abundance (%) of the different chemical classes in the essential oils obtained from the six Sardinian populations of *H. robertianum*.

Class	BAO	CAM	DOM	JER	SUP	SIS
Acids	0.18	1.23	0.91	1.28	1.23	0.45
Alcohols	3.59	8.11	5.96	2.72	3.95	11.85
Aldeydes	1.27	0.67	0.79	0.75	0.68	0.93
Esters	17.07	15.47	17.30	20.18	20.83	20.91
Ketones	2.48	10.63	9.52	2.19	2.63	6.59
Saturated hydrocarbons	65.58	55.02	54.91	64.41	62.55	53.96
Unsaturated hydrocarbons	3.69	3.00	2.91	2.83	2.72	1.61
Terpenes	0.98	1.42	1.62	0.99	1.46	0.44
Oxygenated terpenes	1.64	0.68	1.23	1.33	0.94	0.45
Sesquiterpenes	2.13	2.61	2.54	1.44	1.59	1.38
Miscellanea	0.99	0.61	1.03	0.85	1.01	1.10
Unidentified	0.39	0.54	1.26	1.02	0.41	0.34

BAO = Bau Onu, CAM = Capo Mannu, DOM = Domusnovas, JER = Jerzu, SIS = Sant'Isidoro, SUP = Su Planu.

Saturated hydrocarbons are the most abundant class (ranging from 53,96% in SIS to 65,58% in BAO), followed by esters (from 15.47% in CAM to 20.91% in SIS), alcohols (from 2.72% in JER to 11.85% in SIS), and ketones (from 2.19% in JER to 10.63% in CAM). Number of VOCs in the studied populations varied too (Figure 3).

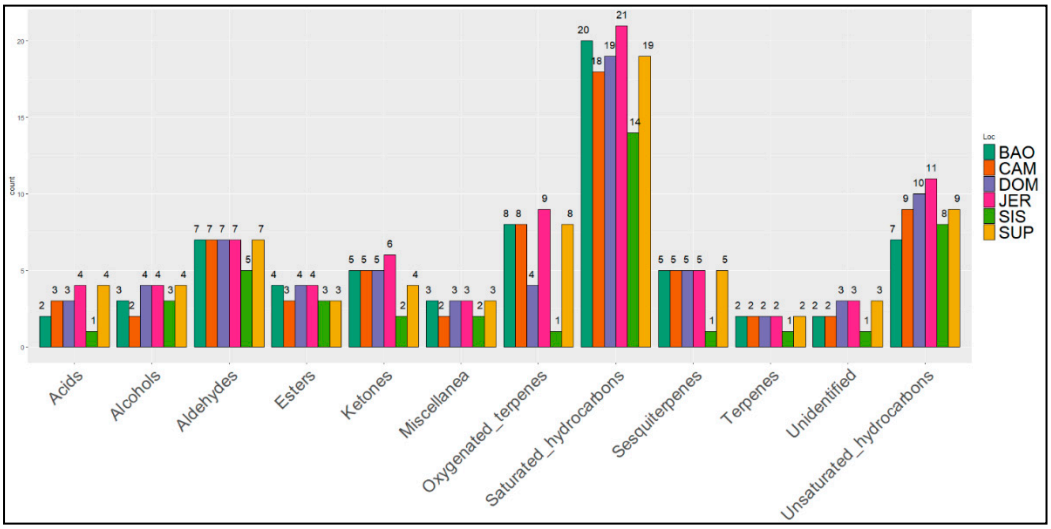

Figure 3. Bar plot reporting the number of VOCs in each chemical class in the different populations, represented by different colors, as described in the legend (BAO = Bau Onu, CAM = Capo Mannu, DOM = Domusnovas, JER = Jerzu, SIS = Sant'Isidoro, SUP = Su Planu). The number above each bar indicates the number of compounds in that population.

The classes of compounds that were featured by the presence of the highest number of compounds in each population are saturated hydrocarbons, followed by unsaturated hydrocarbons, oxygenated terpenes, and aldehydes. On the other hand, the class hosting the fewer compounds is terpenes. The essential oil obtained from JER showed the highest number of compounds in all chemical classes. The lower number of VOCs in SIS are due to the lack of several compounds mainly belonging to oxygenated terpenes (geraniol was the only oxygenated terpene detected in this sample) and saturated hydrocarbons (compounds, such as 4-methyl octane, tetradecane, hexadecane, pentadecane, 2,6,10-trinethyl,3-methyloctadecane and nonadecane, have not been identified in this population).

A Venn diagram (Figure 4) was realized to illustrate qualitative differences and similarities in volatile profiles among the six Sardinian populations of *H. robertianum*.

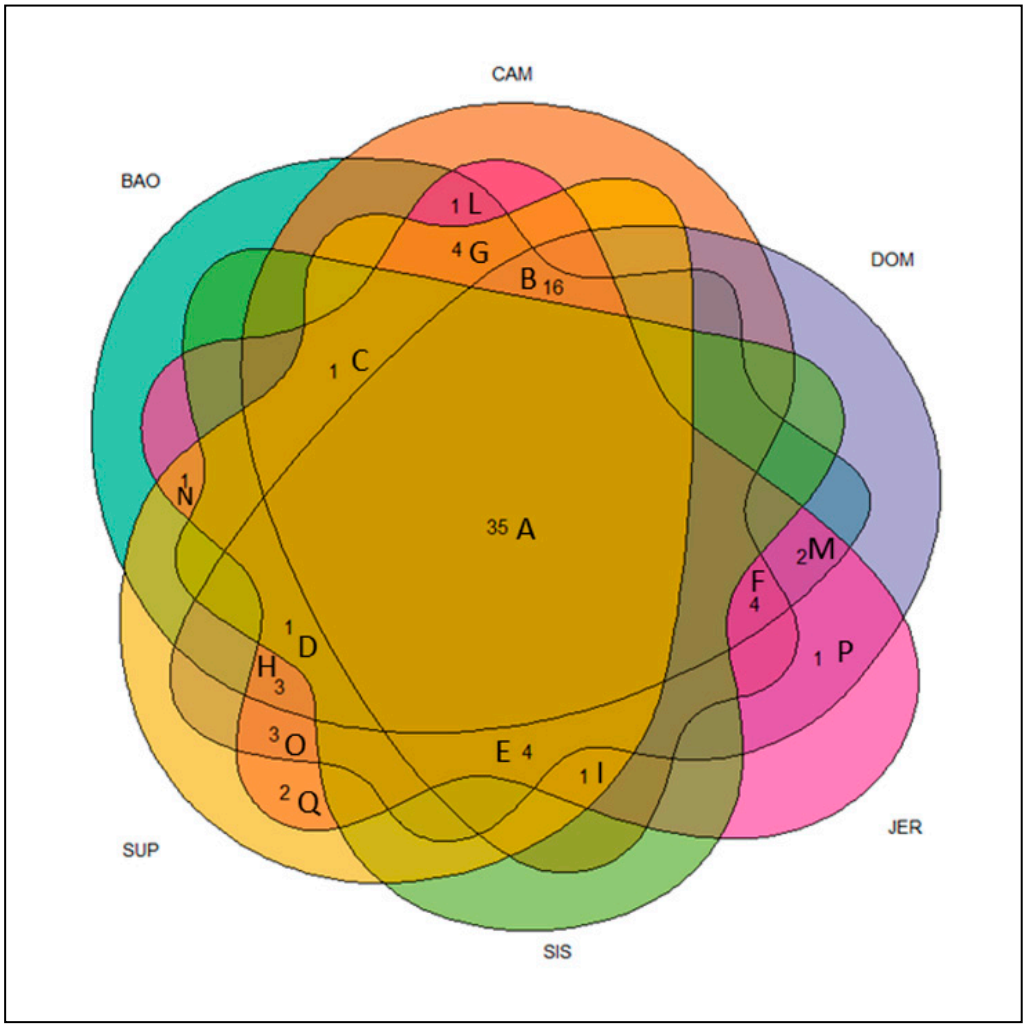

Figure 4. Venn diagram with the number of compounds shared among Sardinian populations of *H. robertianum*. The letters refer to the supplementary table (Table S2) reporting the identity of the compounds shared by populations (BAO = Bau Onu, CAM = Capo Mannu, DOM = Domusnovas, JER = Jerzu, SIS = Sant'Isidoro, SUP = Su Planu).

Figure 4 and the related table (Table S2) highlight the number of compounds shared among the different populations. In our study, a core of 35 compounds (44.3% of the total number of compounds detected) were shared among all samples. The other 16 compounds (20.25%) were shared by CAM, DOM, JER, BAO, and SUP populations. Besides these two large groups of compounds, accounting for 64.55% of the compounds detected, the remaining 35.45% was diversely shared in the different populations. It is worthy of note that there was no compound exclusive of a single population; two compounds (β-phellandren-8-ol and nonanoic acid) were shared only by JER and SUP, and only one (1-tetradecene) by DOM and JER.

PCA carried out on *H. robertianum* VOCs content in the different populations explained in its first two dimensions the 56.1% of total data variability. In the individuals' plot (Figure 5, panel A) different localities are reported as colored dots. The different populations clustered apart in the PCA space. The variables' plot (Figure 5, panel B) shows the 43 most contributing variables to the PCA's first two dimensions.

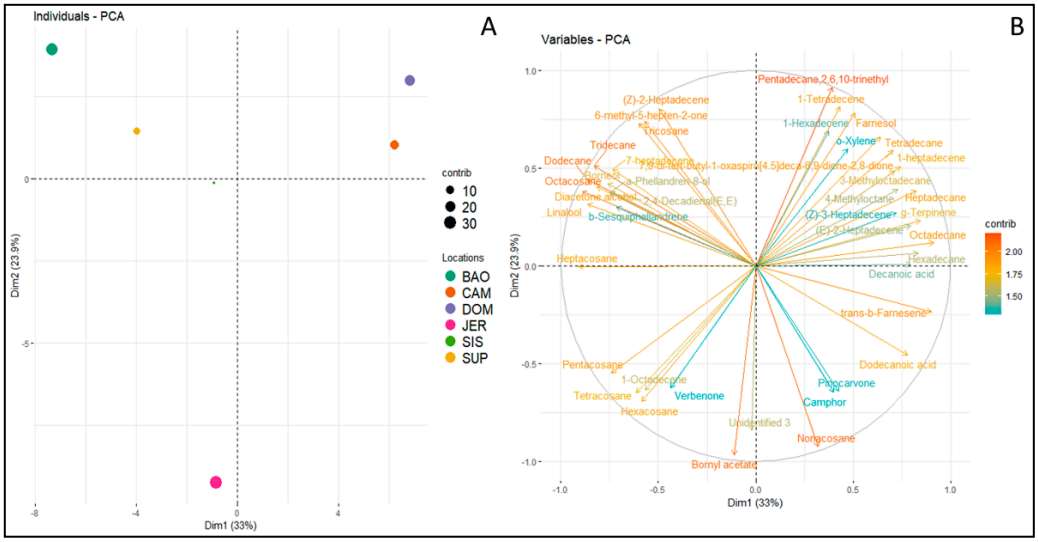

Figure 5. PCA plots of individuals (**A**) and variables (**B**). In panel A, measurements are represented by colored dots, different colors indicating different geographical origin of the samples, as reported in the legend (BAO = Bau Onu, CAM = Capo Mannu, DOM = Domusnovas, JER = Jerzu, SIS = Sant'Isidoro, SUP = Su Planu). Dot size is proportional to the contribution of the population to the analysis. Panel B reports the 43 most contributing variables to the PCA. Variables are represented as arrows originating from the intersection between the two principal axes. Arrows length and color (from hot to cold color) are proportional to the contribution of each variable to the PCA. Variables pointing in the same direction should be considered directly proportional, while variables pointing in opposite directions should be considered inversely proportional. Variables pointing towards clusters of individuals should be considered highly representative of the features of that cluster.

CAM and DOM clusters are determined by a high content in the compounds occupying the top-right quadrant of variables' plot. JER is featured by a high content of compounds pointing to the negative portion of the principal component 2 axis. Compounds occupying the top-left quadrant characterize BAO and partly SUP. SIS is plotted near the axis origin so we should consider the contribution of this population to the analysis very low.

To delve into the differences between populations emerging from the PCA, the Mantel test was implemented to assess whether the physical distance between populations and/or the climatological factors considered could explain the diversity in the VOCs profiles. The Mantel test confirmed a relationship between the physical distance between populations and VOCs profiles diversity (Mantels statistic r = 0.50, significance $p = 0.048$), meaning that the more the populations were distant, the more different were their VOCs profiles. Out of the chemical classes of compounds identified in the essential oils, only saturated hydrocarbons exhibited a significant relation with the physical distance between populations (Mantels statistic r = 0.54, significance $p = 0.024$). On the contrary, no relation between VOCs profile diversity and climatological factors was assessed, neither considering the climatological factors on the whole or singularly.

The distribution of content values of the compounds belonging to the different classes is represented by boxplots (Figure 6).

The classes of compounds could be divided into two main categories based on the abundance of their compounds in *H. robertianum* samples. Compounds belonging to the classes of esters, saturated hydrocarbons, alcohols, terpenes, and ketones ranged between the 5.45% (esters) and 1.47% (ketones) mean content on the total essential oil (Figure 6, panel A). Compounds belonging to sesquiterpenes, unsaturated hydrocarbons, acids, oxygenated terpenes, and aldehydes ranged between the 0.57% (sesquiterpenes) and 0.13% (aldehydes) mean content on the total essential oil (Figure 6, panel B). Compounds assigned to the miscellanea class and unidentified compounds were present at 0.36% and 0.29%, respectively (Figure 6, panel B). Black dots in the graph represent outliers, each one of them

a single compound detected in very high or very low concentration with respect to the other compounds of the same class in that population. Once checked for the correctness of the values, outliers were maintained in the graph and in statistical analysis because of their significance in the VOCs profiles. ANOVA found no significant differences between populations as regards the compounds' content in the different classes.

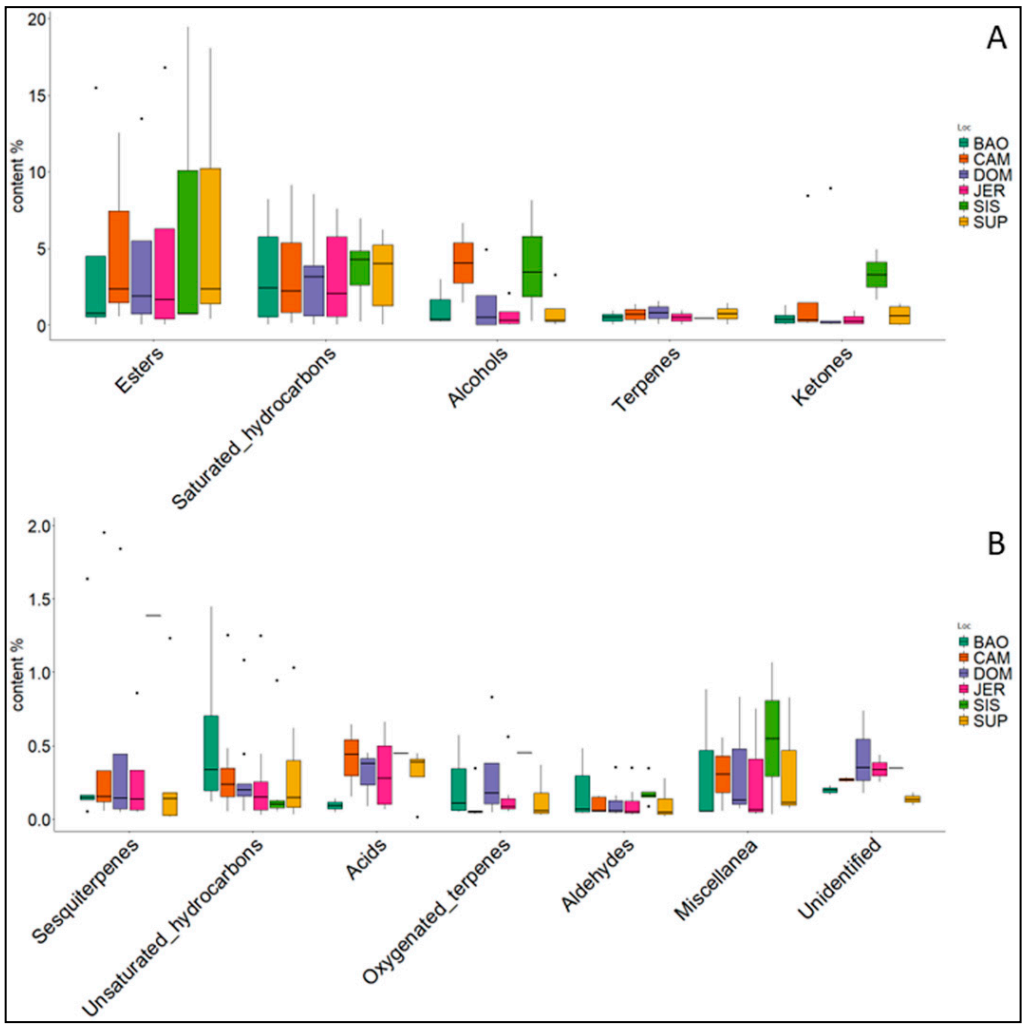

Figure 6. Boxplot representing the distribution of values in each class of compounds in the six populations (BAO = Bau Onu, CAM = Capo Mannu, DOM = Domusnovas, JER = Jerzu, SIS = Sant'Isidoro, SUP = Su Planu). On the *x*-axis the classes of compounds, and on the *y*-axis the % content on the total essential oil are reported, respectively. Different populations are represented by different colors as reported in the legend on the right. (**A**) values distribution of VOCs present at higher percentages in the essential oil; (**B**) values distribution of VOCs present at lower percentages in the essential oil. Each boxplot reports 50% of the measured values (inside the box), comprised between the first quartile value (lower side of the box) and the third quartile value (upper side of the box); the median is indicated by the black line inside the box, while whiskers join the first and third quartiles with lower and higher measured value, respectively (outliers are reported by black dots).

4. Discussion

The objective of this study was to give a contribution to the knowledge of VOCs produced by *H. robertianum*, a food-deceptive orchid largely distributed in the Mediterranean basin. Moreover, we evaluated the influence of environmental features on the composition of essential oils obtained from inflorescences sampled in different Sardinian populations. Sardinia Island (Italy) is one of the Mediterranean hotspots of biodiversity, due to the high number of endemic species, amounting to 15% of the total native flora [23]. A long and peculiar paleogeographic history, prolonged geographical isolation, and high geomorphological diversity have significantly contributed to this richness [24], since isolation is one of the main causes of speciation. Consequently, Sardinian plant species are often found featured by singular chemical profiles [25–27], different from those observed in the other region of the Mediterranean area.

Sardinian orchids have been largely studied, as documented by the wide available literature [14,28–31]; but, to the best of our knowledge, this is the first study focusing on the characterization of essential oils obtained from orchids in Sardinia.

VOCs are produced by plants to address a variety of ecological and physiological tasks. To evaluate whether the environmental features affected the essential oil composition of Sardinian *H. robertianum* we selected six populations growing in diverse ecological conditions. In this study, we identified a higher number of compounds in comparison to those previously reported for this species [19,32–34], the number of VOCs varying considerably in the studied populations. While there was no compound exclusive of a single population on the other hand, Sardinian samples shared the 44% of the identified compounds, and hexadecanoic acid methyl ester was the main VOC in all the populations. Moreover, considering the chemical classes of compounds individually, no statistically significant difference emerged between the analyzed samples. Nevertheless, besides similarities, quantitative differences in the essential oil yield and in the abundance of some compounds among the six populations were observed and multivariate statistics separated *H. robertianum* populations based on their chemical profiles.

We found a positive correlation between diversity in chemical profiles and geographical distances between populations, meaning that distant populations are more diverse in their VOCs. The latter result diverges from the one reported in Romano et al. [33], which did not find a correlation between chemical profiles diversity and geographical distances between populations.

Since VOCs have a role in abiotic stress resistance [1,6], we characterized the studied populations in their daily (minimum and maximum) temperature, precipitation, solar radiation and in the precipitation-to-temperature-ratio since these factors may easily lead to a stress condition in plants if at sub- or supra-optimal levels. *H. robertianum* samples were collected in cooler and wetter populations (BAO and JER), intermediate populations (DOM and CAM) and warmer and dryer populations (SIS and SUP). Though Mantel test failed to find a covariation between climatological and chemical diversity, experimental and PCA results reflected the populations' climates. In fact, warmer and drier environments (SIS and SUP) resulted in poorer and similar VOCs profiles and intermediate environments (DOM and CAM) resulted in similar chemical profiles, while *H. robertianum* growing in BAO and JER, cooler and wetter study sites, showed typical and very well-characterized chemical profiles.

Populations settling on identical lithology or vegetation in the present study presented very different VOCs profile (see for example the cases of SIS and SUP or JER and DOM), for this reason and in accordance with Romano et al. [33], we concluded that these two factors scarcely influence VOCs variation in *H. robertianum*.

In order to further investigate VOCs variation in *H. robertianum*, we compared our results with those obtained in our previous study [19] in which the essential oil of *H. robertianum* collected in Piedmont (Italy) was obtained and analyzed through the same extraction and analytical procedures as in this work. A comparison between Sardinian and Piedmonts' populations highlighted huge differences in their VOCs profile (Figure S1). In

fact, *H. robertianum* sampled in Piedmont was characterized by the presence of 11 compounds not detected in Sardinian populations (Figure S2 and Table S3), while only 6 compounds were shared with all Sardinian populations, thus evidencing strong differences in both the number and the identity of VOCs between insular and peninsular *H. robertianum* populations. Differently from Sardinian populations, the most abundant compound in Piedmont's essential oil was pentacosane (40.17%), followed by *p*-cresol (15.28%) and nonanal (4.41%). Moreover, the total amount of aldehydes, alcohols, and esters strongly differed from those observed in this study.

It is interesting to note the considerable differences between our results and those available in the literature. These differences can be attributed to the different methodological approaches performed. We analyzed the essential oil produced and stored in the inflorescence's tissues while generally studies on orchids VOCs focus on scents emitted in the environment by the plants, mainly to interact with pollinators.

Romano et al. [33] who have recently described the floral scent emitted by *H. robertianum* sampled in Basilicata (Italy), reported volatile profiles characterized by ethyl dodecanoate, hexadecane, β-bisabolene, σ-selinene and β-sesquiphellandrene as the most frequent compounds, with very high variability in VOCs composition and abundance between all the analyzed samples. These compounds have not been detected in our study, or they were present in some populations and in very low amounts. Gallego et al. [32], who analyzed Spanish populations of *H. robertianum,* found a very high percentage of terpenes, mainly α-pinene, β-pinene, and limonene, compounds not found in Sardinian samples. Among VOCs, terpenes are those predominantly involved in abiotic stress resistance especially towards heat, drought and high irradiation in land plants (bryophytes, herbaceous and woody plants) [35–39]. Terpenes were present in very low quantities in our populations and this could be explained by the extraction procedures we implemented and by the fact that we sampled *H. robertianum* in its ecological optimum (natural populations) so that our individuals should be considered not or only marginally stressed. However, among the studied populations BAO and JER are featured by very low temperatures and this stress factor could be the reason why they resulted in very typical chemical profiles by the multivariate statistical approach. Similarly SIS and SUP, featured by a milder, less-stressful climate, resulted less complex in their volatile profiles, indicating a marginal investment in the synthesis of defensive secondary metabolites.

The results obtained in our study confirmed the high variability in the volatile profile of this food-deceptive orchid. Considering that Romano et al. [33] documented a huge variation in VOCs emitted by the same plants in two different years, we can hypothesize that there is a limited genetic influence in shaping VOCs profiles in *H. robertianum*. However, despite the issues that may derive from making phylogeographic inferences based on VOCs production and emission of wild occurring species [5], the influence of genetics in shaping volatile profiles in *H. robertianum* is corroborated by the increased similarity of Sardinian populations if compared to the Piedmont population. In fact, more similar VOCs profiles in Sardinian samples may derive from the founder effect that must have affected our study species in the colonization of the island. In the present study, distant populations appeared significantly more diverse in their VOCs profiles than populations close to each other. Moreover the climate in the study sites seems to influence VOCs profiles in *H. robertianum*, since cooler and wetter environments resulted in very different VOCs profiles in comparison to those produced in warmer and dryer and intermediate environments. Considering what has been said so far, the hypothesis that environmental factors do play a role in shaping VOCs profiles in *H. robertianum* should be maintained. Further studies providing to cover a wider spectrum of ecological variables would guarantee new insights on the ecological factors responsible for the differences in VOCs here described.

It is interesting to note the presence in all Sardinian samples of some saturated hydrocarbons, such as tricosane, pentacosane, and heptacosane. These compounds are usually emitted in large amounts by sex-deceptive orchids [40] since they are also found in the cuticle of virgin females of some pollinators. However, in our study, we cannot correlate

these secondary metabolites to the reproductive strategies of *H. robertianum*, since it is a food-deceptive orchid.

Food-deceptive orchids are pollinated by generalist pollinators, just emerged from the soil and without experience. Increasing the variability of orchids scents is a strategy to avoid insects' negative learning that associates a certain floral scent with the lack of food reward. This strategy could partly explain the great variability in the VOCs content we measured in Sardinian populations of *H. robertianum*.

5. Conclusions

Within a project aiming to analyze the volatile profile of Sardinian orchids, we characterized the essential oils obtained from *H. robertianum* inflorescences. Moreover, we evaluated through a multivariate statistical approach whether the environmental conditions of the different growing sites affected the volatile profiles. We found that Sardinian samples were characterized by different volatile profiles with respect to those previously reported in the literature. Saturated hydrocarbons represented the most abundant class in all populations, both for their total abundance and the number of detected compounds. All essential oils shared a great number of VOCs (approximatively 44%), and there was no compound exclusive of a single population. The differences in the composition of the *H. robertianum* essential oils here described were positively correlated with geographical distance separating populations and reflected the climatological features of the sampling sites. On the contrary lithology and vegetation did not affect VOCs production in the species. Our results confirmed the high variability of the volatile profile in *H. robertianum*. Further studies are required to investigate whether this variability depends on genetic features, or it is mainly influenced by environmental parameters not considered in this study.

Moreover, the analyses of the essential oils obtained from the same populations in different years could give us useful information to better understand the influence of genetics on VOCs production.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14121125/s1, Table S1: Detailed environmental data on collection sites; Table S2: Integration table to Figure 4; Table S3: Integration table to Figure S2; Figure S1: PCA plots of individuals and variables comprehensive of Ponzone population; Figure S2: Venn's diagram with the number of compounds shared among Sardinian and Ponzone populations.

Author Contributions: Conceptualization, F.S.R.d.C., P.C. and C.S. (Cinzia Sanna); methodology, F.S.R.d.C., A.D.A., C.S. (Cinzia Sanna) and C.S. (Cristina Sottani); investigation, P.C., A.D.A., C.S. (Cinzia Sanna), F.S.R.d.C. and F.S.; data curation, A.D.A.; writing—original draft preparation, A.D.A. and C.S. (Cinzia Sanna); writing—review and editing, A.D.A., C.S. (Cinzia Sanna), F.S.R.d.C., P.C. and A.C.; supervision, C.S. (Cinzia Sanna), P.C. and F.S.R.d.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors are grateful to Vincenzo Rodi who provided the orchid's pictures in Figure 1.

Conflicts of Interest: The authors declare no conflict of interest.

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