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Free Volatile Compounds as Chemophenetic Markers—Comparison with ITS2 and ITS1-5.8S-ITS2 Sequence Data for 18 Species of the Genus *Veronica*

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Abstract: (1) Background: The purpose of this study was to compare the free volatile compounds of 18 *Veronica* species (Plantaginaceae), as previously analyzed by gas chromatography coupled with mass spectrometry, with their DNA sequences for internal transcribed spacers ITS2 and ITS1-5.8S-ITS2 of the nuclear ribosomal DNA. (2) Methods: Two sets of DNA sequence data were generated and used for phylogenetic analysis: ITS2 sequences (~360 bp) obtained by next-generation sequencing and ITS1-5.8S-ITS2 sequences (~580 bp) sequenced by the Sanger sequencing method. Clustering from previously analyzed free volatile compounds was performed by Ward's method. (3) Results: Both sets of DNA sequence data showed that the 18 analyzed *Veronica* species were grouped into eight main groups corresponding to the following subgenera: *Pentasepalae*, *Pocilla*, *Chamaedrys*, *Veronica*, *Beccabunga*, *Cochlidiosperma*, *Stenocarpon* and *Pseudolysimachium*. Results of the clustering analysis of free volatile compounds showed better clustering when using microwave-extracted volatiles. Three clusters were detected with the following main compounds: hexahydrofarnesyl acetone, hexadecanoic acid, phytol, caryophyllene oxide and *(E)*-caryophyllene. (4) Conclusion: The phylogenetic analysis of ITS2 data obtained by NGS technology and ITS1-5.8S-ITS2 data obtained by Sanger sequencing resulted in the grouping of 18 *Veronica* species into eight subgenera, which is in accordance with the existing classification. Statistical testing showed that there was no correlation between such clustering of *Veronica* species and clustering that was based on free volatile compounds. The achieved results can be viewed in the light of parallel evolution among some of the species of the *Veronica* genus as well as the fact that volatile compound composition can be influenced by environmental factors or epigenetic modifications.

Keywords: *Veronica*; volatile compounds; chemophenetic markers; molecular phylogeny; ITS1; ITS2; NGS; Sanger

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1. Introduction

The genus *Veronica* of the family Plantaginaceae consists of 450 species, growing mainly in the temperate regions of the Northern Hemisphere, while a smaller number of species grow in the mountains of the tropical and temperate regions of the Southern Hemisphere regions [1,2]. Many species testify to the great ecological adaptability of the genus *Veronica*, in which species grow in wet and dry habitats, by the sea and in the mountains [3].

One of the first phytochemical studies of the genus *Veronica* was related to iridoid glycosides, which proved to be taxonomically important [4,5]. This genus was once placed in the family Scrophulariaceae based mainly on morphological characters, but based on

the results of DNA sequence studies, it was transferred and is currently in the family Plantaginaceae [6]. Albach et al., Jensen et al. and Taskova et al. studied the iridoid glycosides of the genus *Veronica* and concluded that the distribution of these compounds in the different species of the genus is consistent with the molecular phylogeny of the genus, thus showing that the genus chemistry can serve as a good indicator of links between species and within the genus [7–10]. Advances in analytical methods, especially chromatography, followed by electronic detection methods, accelerated chemical studies to the point of metabolic profiling of plant species. In general, research on chemical compounds produced by plants is extremely important because these compounds ultimately affect not only the plant in which they are found but indirectly other plants in the environment as well as the environment as a whole [11]. Iridoid glycosides have been used as chemophenetic markers at different taxonomic levels [12–16]. As a part of research on chemophenetic markers for the genus *Veronica*, Taskova et al. isolated 16 iridoid glycosides and established a link between chemical composition and basic chromosome number [16]. Mehrvarz et al. studied the chemical composition of selected species of the genus *Veronica*, iridoids and flavonoids and investigated their significance for the systematic and phylogenetic assignment of these species. The analysis of four species of the genus *Veronica* (*V. persica*, *V. polita*, *V. francispetae*, *V. siaretensis*) showed a qualitatively constant composition of iridoids of all species, regardless of environmental conditions [17]. Crişan et al. studied 12 species of the genus *Veronica* with LC-MS, analyzing the content of aucubin and catalpol belonging to iridoid glycosides, and also confirmed their importance in chemophenetics [15]. Albach et al. studied the iridoid glycosides aucubin and catalpol in the genus *Veronica* species and the species *Paederota lutea* and, based on the composition of these compounds, concluded that these two genera *Veronica* and *Paederota* are related [18]. Molecular analyses showed that *Paederota* is a sister group next to *Veronica*, and the composition of iridoid glycosides supported this as the same compounds were detected in *Paederota* species [18]. This proves that iridoids are a very good marker for the chemophenetics of plant species, as confirmed by Saracoglu et al. in their studies [19].

The free volatile compounds, as specialized metabolites of the genus *Veronica*, have been much less studied than other metabolites such as glycosides, phenols and flavonoids. Only a few research studies could be found before our research [20–23]. Our recent studies on the composition of volatile compounds in 21 Croatian *Veronica* species indicate the diversity and richness of isolated metabolites, and for most of the *Veronica* species studied, these data were presented for the first time [24].

The molecular phylogeny and taxonomy of the genus *Veronica* are well studied [2,25–29]. Due to the parallel morphological evolution observed in this genus (a taxonomically distinct species develops similar morphological characters) [2], it is not recommended to rely solely on plant morphology for the identification and classification of species in the genus *Veronica*. Therefore, DNA analyses are recommended for reliable and accurate species identification. DNA barcoding regions that have been shown to be useful for the phylogenetics of *Veronica* are ITS1-5.8SrDNA-ITS2 (nuclear genes which are inherited from both parents) and trnL-trnF regions (genes from the chloroplast which are usually inherited from the female parent) [25].

Although the ITS1-5.8SrDNA-ITS2 region is probably the most popular molecular marker in plants for the DNA identification of plant species (DNA barcoding) this sequence can be hypervariable and therefore difficult to analyze when sequenced in a conventional way, using the Sanger method. This is particularly important for plants of hybrid and/or polyploid origin. Since the occurrence of polyploidy has been observed in several species of the genus *Veronica* [25], we hoped that with the new method of next-generation sequencing (NGS, next-generation sequencing), much more accurate identification of species would be possible. With this method, it is possible to sequence many more gene variants (markers) and obtain data of much better quality and higher resolution, revealing genetic diversity such as single nucleotide polymorphisms (SNPs), insertions/deletions (indels), homopolymeric regions and microsatellites, which in aggregate will allow accurate species

identification. The NGS method is already used in many cases: the detection of ITS2 allelic variation in mosquitoes [30], diversity of rDNA unit in *Nicotiana* [31], authenticity of plant foods [32] and identification of medicinal plants [33].

This study provides an overview of the phytochemical composition of free volatile compounds of selected species of the genus *Veronica* from Croatia, which have been published previously; however, in this work, cluster classification based on free volatile compounds (FVCs) is performed. Moreover, this clustering is compared with molecular classification based on the ITS (internal transcribed spacer) regions of ribosomal genes. One of the main objectives of this study is to investigate whether free volatiles can also be a good chemophenetic marker for classification between species and between genera. Several recent studies indicated the diversity and richness of isolated metabolites from the genus *Veronica*, and some of them proved to be reliable taxonomic markers. The combination of such studies with molecular phylogenetic analyses provides a good basis for exploring the potential relationship between the distribution of free volatile compounds and phylogeny.

2. Materials and Methods

2.1. ITS2 and ITS1-5.8S-ITS2 Sequencing

2.1.1. DNA Extractions

Genomic DNAs of 18 *Veronica* species listed in Table 1 (locations shown in Figure 1) were extracted from young silica-dried leaves using NucleoSpin Plant II, Mini kit for DNA from plants (Macherey-Nagel™; Cat. No. 740770.5, 52355 Düren, Germany) according to the manufacturer's instructions. For each species studied, DNA was separately isolated from three individuals. Voucher specimens were deposited at the Laboratory of Botany herbarium (HPMF-HR), Faculty of Science, University of Split, Croatia.

Table 1. List of 18 *Veronica* species with location collection information used for this research (the same plant material was used for the previously published research [24]).

Taxa	Locality	Latitude	Longitude	Altitude a.s.l. (m)	Voucher no.
V. austriaca L. ssp. *jacquini* i	Brač Island	43°19′07.3″ N	16°36′08.5″ E	564	CROVeS-02-2021
V. cymbalaria	Murter Island	43°48′36.6″ N	15°35′07.4″ E	37	CROVeS-03-2021
V. dalmatica	Dubrovnik	42°39′19.1″ N	18°04′56.9″ E	58	CROVeS-04-2021
V. saturejoides ssp. *saturejoides*	Dinara Mt	44°03′11.3″ N	16°23′29.7″ E	1697	CROVeS-05-2021
V. anagallis-aquatica	Split	43°31′43.5″ N	16°28′45.2″ E	22	CROVeS-06-2021
V. anagalloides	Čikola River	43°49′36.2″ N	16°01′19.4″ E	45	CROVeS-07-2021
V. beccabunga	Baške Oštarije	44°31′32.1″ N	15°10′34.2″ E	908	CROVeS-08-2021
V. catenata	Trakošćan	45°15′30.3″ N	15°56′25.2″ E	240	CROVeS-09-2021
V. longifolia	Oštarije	45°13′36.1″ N	15°16′18.2″ E	311	CROVeS-10-2021
V. acinifolia	Donji Karin	44°07′18.1″ N	15°36′13.7″ E	119	CROVeS-11-2021
V. arvensis	Hvar Island	43°10′42.3″ N	16°36′43.6″ E	38	CROVeS-12-2021
V. chamaedrys	Radoboj	46°09′49.4″ N	15°55′36.1″ E	260	CROVeS-13-2021
V. hederifolia	Zagreb	45°49′40.4″ N	15°58′59.6″ E	192	CROVeS-14-2021
V. montana	Papuk Mt	45°30′38.1″ N	17°39′57.2″ E	761	CROVeS-15-2021
V. officinalis	Kamešnica Mt	43°42′38.7″ N	16°50′47.9″ E	1225	CROVeS-16-2021
V. persica	Samoborsko gorje	45°49′41.6″ N	15°40′32.9″ E	301	CROVeS-18-2021
V. polita	Kaštel Žegarski	44°09′26.1″ N	15°51′56.0″ E	53	CROVeS-19-2021
V. serpyllifolia	Zagreb	45°49′40.3″ N	15°58′59.5″ E	192	CROVeS-20-2021

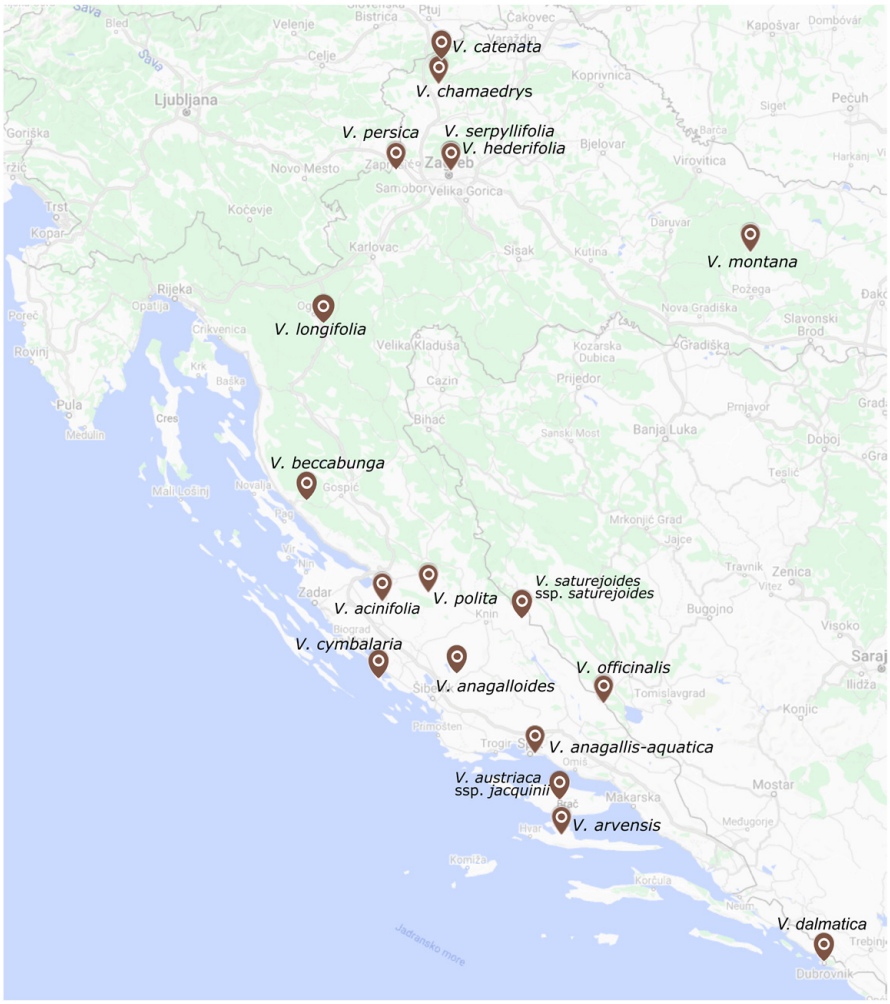

Figure 1. Map of locations of material collection.

2.1.2. PCR Amplification and Sequencing by Sanger

The ITS1-5.8S-ITS2 region of 35S rDNA was amplified by PCR using the primers ITS-1 (5′GTTTCCGTAGGTGAACCTGC3′) described by Bezić et al. [34] and ITS-4 (5′TCCTCCGCTTATTGATATGC3′) described by White et al. [35]. The amplified products were visualized and confirmed by 1% agarose gel electrophoresis, extracted from the gel and isolated using a Plasmid Mini Kit (Qiagen, Hilden, Germany). Purified DNA was sent to Macrogen (Amsterdam, The Netherlands) for sequencing. The ITS1-5.8S-ITS2 sequences were deposited in GenBank under the following accession numbers: OQ564378-OQ564387 (Table S1).

2.1.3. Sequence Analysis

The DNA sequences were assembled and aligned in ClustalW implemented in MEGA11 [36], and the alignment was manually refined. Alignments in fasta format are available as Datas S1 and S2. To infer phylogenetic relationships from the newly obtained *Veronica* ITS sequences and other closely related *Veronica* species, the sequences were subjected to similarity search against the non-redundant nucleotide sequence database using the NCBI (National Centre for Biotechnology Information) BLASTN network service. Four species from the Plantaginaceae family were taken into analysis as outgroup species, and details of their GenBank accession numbers and publications are shown in Table S2 [8,10,25,28,37–40]. The final dataset of ITS2 sequences contained 57 sequences, and there were a total of 327 positions. The evolutionary history was inferred by using the Maximum Parsimony method implemented in MEGA 11 under default parameters. Tree

#1 out of 5 most parsimonious trees (length = 323) is shown in Figure 2. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data and ambiguous bases were allowed at any position (partial deletion option).

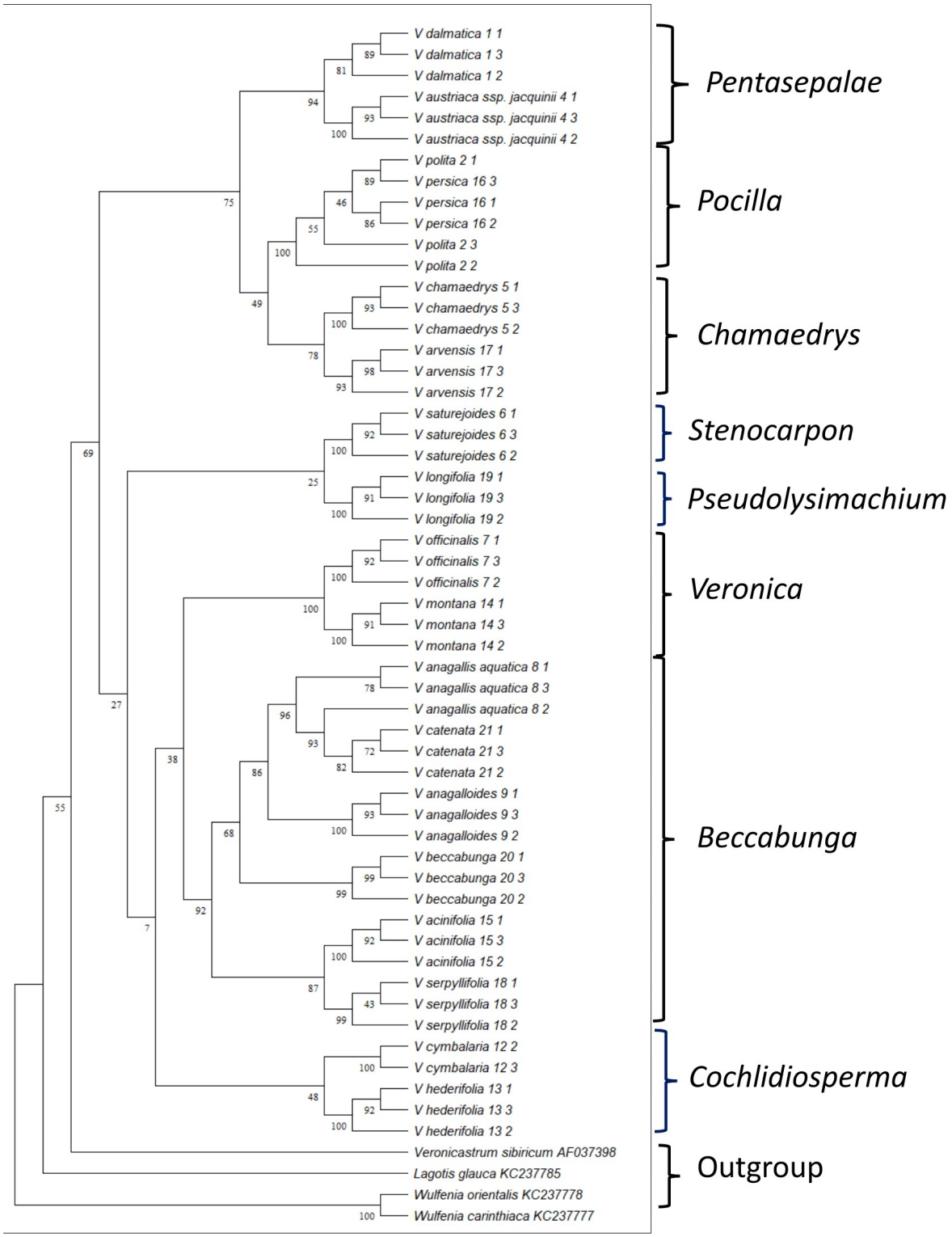

Figure 2. Evolutionary relationships of the studied *Veronica* species inferred from ITS2 sequence analysis by the Maximum Parsimony method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Subgeneric affiliation is shown.

The final dataset of ITS1-5.8S-ITS2 sequences contained 28 sequences, and there were a total of 528 positions in the final dataset. The evolutionary history was inferred by using the maximum likelihood method and general time-reversible model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.4567)). All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data and ambiguous bases were allowed at any position (partial deletion option). The bootstrap consensus tree was inferred from 500 replicates. Evolutionary analyses were conducted in MEGA11 [36].

2.1.4. NGS (Illumina Sequencing) of ITS2 Region and Data Analyses

Preparation of Illumina libraries, PCR and sequencing was outsourced to Novogene (Cambridge, UK). Target ITS2 region was amplified from genomic DNA extractions using ITS3 (5′ GCATCGATGAAGAACGCAGC 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) universal primers described by White et al. [35]. The same amount of PCR products from each sample was pooled, end-repaired, A-tailed and ligated with Illumina adapters. NovaSeq PE250 Sequencing System was used to sequence the libraries. Paired-end reads were assigned to samples based on their unique barcode, after which barcode and primer sequences were removed. Quality filtering of the raw reads was performed with Novogene proprietary pipeline and included discarding reads shorter than 60 bp, reads with >10% of uncertain nucleotides (N) and reads with >50% of low-quality nucleotides (Qscore $\leq$ 5). The quality of paired-end reads at every step was inspected with FastQC v0.11.8 [41]. Clean reads were merged using FLASH v1.2.7 [42], and chimeras were removed by comparing them to the Gold database using UCHIME algorithm v.7.0.1001 [43]. The resulting effective clean tags were further screened for contamination by comparison with the UNITE database [44] of the eukaryotic nuclear ribosomal ITS regions using SortMeRNA v2.1 [45]. Tags that did not match any of the *Veronica* plant species in the database were discarded from further analyses. Contigs (ribotypes) of the ITS2 region for each sample were assembled de novo as fragments using MIRA v5rc1 [46] in draft mode. Assembly in fragment mode is recommended for single gene projects or small plasmids. Since the tag number was high (mean 90,774, min. 32,731–max. 116,647), lossless digital normalization was enabled, and other assembly parameters were kept as default. Contigs that contained more than 20% of reads and displayed highest coverage were selected as representative ribotypes, and the longest of them were selected for phylogenetic analyses (Table S4). Purification of effective tags, quality control and assembly of contigs were performed using the resources of the Isabella computing cluster hosted by the University Computing Centre, University of Zagreb (SRCE), Croatia.

2.2. Review Protocol

In order to obtain data for the cluster analyses of volatile compounds, the data for the free volatile compounds (FVCs) for 18 selected *Veronica* species used for this paper were obtained from one previously published paper by our team [24]. The volatile compounds were isolated by two methods: hydrodistillation in a Clevenger-type apparatus (Šurlan, Medulin, Croatia) and microwave-assisted extraction (Milestone 'ETHOS X' microwave laboratory oven, 1900 W maximum, Sorisole, Italy) for 2.5 h using 30–50 g of dried plant material. The distillate consists of two layers: a lipophilic layer collected in a side tube using a pentane/diethyl ether trap and a water layer (hydrosol). For this study, we only used volatile compounds from lipophilic layer, because it is standard procedure in most papers when obtaining data for cluster analyses of volatile compounds [47–51]. For this paper, we presented percentages of FVCs from both extractions in Table 2. Percentages for FVCs were obtained with gas chromatography–mass spectrometry. Method for FVC analyses is presented in detail in paper by Dunkić et al. [24].

Table 2. Free volatile compounds most abundant in all investigated *Veronica* species—Clevenger hydrodistillation (HD) and microwave extraction (MW) (relative percentage, %).

Subgenus/Species	Hexahydrofarnesyl Acetone		Hexadecanoic Acid		Caryophyllene Oxide		*(E)*-Caryophyllene		Phytol		Pentacosane		Germacrene D	
	HD	**MW**	**HD**	**MW**	**HD**	**MW**	**HD**	**MW**	**HD**	**MW**	**HD**	**MW**	**HD**	**MW**
Pentasepalae														
V. austriaca ssp. *jacquinii*	17.12	8.61	32.17	22.17	13.98	6.64	8.01	1.79	4.58	6.58	1.03	5.91	-	0.68
V. dalmatica	7.72	3.44	1.13	2.65	0.52	7.52	3.48	39.53	41.22	2.75	0.25	0.35	3.87	1.89
Pseudolysimachium														
V. longifolia	9.08	9.28	9.74	6.14	5.58	1.53	4.13	1.43	13.63	37.18	6.81	4.71	3.87	-
Beccabunga														
V. acinifolia	15.37	16.17	3.35	4.52	7.71	5.52	4.46	6.51	15.63	39.88	-	5.75	0.43	1.83
V. anagallis-aquatica	27.17	25.97	4.65	4.77	4.36	2.55	5.49	3.29	9.42	14.56	-	-	1.28	0.88
V. beccabunga	6.13	9.56	2.72	4.74	4.22	1.62	2.75	2.95	27.31	34.54	0.51	1.13	0.42	0.42
V. catenata	17.75	17.22	10.02	5.81	1.55	6.52	4.11	2.48	29.92	42.26	0.28	-	-	-
V. serpyllifolia	7.92	6.54	12.28	7.71	4.19	14.74	2.11	6.83	39.79	18.72	0.98	0.18	0.67	3.24
V. anagalloides	14.33	19.12	13.67	9.17	4.91	8.58	4.07	4.01	9.58	14.88	2.01	5.43	2.22	3.07
Veronica														
V. montana	6.86	9.17	9.24	5.81	7.28	2.61	0.13	0.44	18.53	37.03	10.47	14.90	-	-
V. officinalis	3.25	6.82	13.21	12.40	4.65	4.15	3.12	3.12	32.61	16.89	11.89	0.15	-	-
Stenocarpon														
V. saturejoides	6.88	17.72	6.14	6.64	34.53	8.43	9.43	8.49	-	22.47	-	0.48	2.61	5.11
Chamaedrys														
V. arvensis	6.35	17.55	3.17	17.42	14.11	7.11	6.21	3.25	7.54	22.57	0.71	-	1.25	2.45
V. chamaedrys	10.82	16.69	5.73	15.83	6.25	1.22	2.43	1.05	31.66	18.88	0.56	8.36	1.02	0.12
Pocilla														
V. persica	10.31	18.47	7.35	5.31	10.11	3.14	9.29	2.62	20.21	23.71	-	5.27	0.75	0.35
V. polita	10.28	10.82	6.75	5.69	7.55	1.48	6.57	4.17	31.18	19.88	0.36	1.76	1.06	0.07
Cochlidiosperma														
V. cymbalaria	36.33	13.35	0.75	15.72	10.92	32.72	3.95	6.13	-	3.71	0.71	0.49	1.42	2.34
V. hederifolia	28.85	59.15	7.25	1.57	4.59	0.51	4.11	1.10	18.53	14.58	0.21	-	1.47	-

2.3. Statistical Analyses

Cluster Analyses Based on Free Volatile Compounds

Cluster analysis (CA) was based on chemical constituents obtained by classical extraction—hydrodistillation with Clevenger apparatus and microwave-assisted water extractions in an amount of at least 2%. Dendrograms of Euclidean distances were prepared according to Ward's method to verify the affinities determined in the molecular analysis. CA was performed using the Statistica 7 software (StatSoft Inc., Tulsa, OK, USA).

The relationship between matrices of Euclidean distances describing chemical components of species and genetic distances obtained by Sanger and NGS methods was assessed using Mantel test as implemented in vegan package for R v4.2.2 [52]. Spearman rank correlation was chosen as a measure of association, and significance was calculated through 9999 permutations. Genetic distances for the Mantel test were calculated in MEGA11 [34] using Kimura 2-parameter model with 1000 bootstrap iterations [53].

3. Results

3.1. ITS2 and ITS1_5.8S-ITS2 Data Analyses

With the aim of evaluating the usefulness of next-generation sequencing (NGS) for the authentication of species from the genus *Veronica*, we performed NGS sequencing of the ITS2 region of 18 *Veronica* species (data available as BioProject PRJNA944611). Each species was represented by three plants. On average, 99086 paired-end reads were produced per sample (Table S3). After quality control, filtering and chimera removal, an average of 99.4% of reads remained and were used to generate effective clean tags. The proportion of contaminant non-*Veronica* species tags was generally low (4.8% on average), except for sample E (*V. chamaedrys*) where 30.9% of tags were removed (Table S3).

Out of 18 *Veronica* species, the identity of 11 species was successfully confirmed by blasting randomly chosen 5000 sequences for the ITS2 region, as these data were consistent with the results of anatomical-morphological identification. For the remaining seven species, identification based on the ITS2 region was doubtful and did not agree with their morphological identification. For these samples, an additional analysis of the complete ITS1-5.8S-ITS2 region was performed by the PCR amplification of this region and its classical Sanger sequencing (Table S1).

The number of reads/tags after the clean and merge procedures is shown in Table S2. Clean effective tags were assembled into anywhere between 21 to 237 contigs per sample, with one largest contig/ribotype recreated with the highest coverage and a length of approximately 360 nucleotides for most samples. Information on the assembled contigs supported by at least 20% of the reads for each sample is listed in Table S3, and their nucleotide sequences are given in fasta format in Data S1. These contigs/ribotypes that had the highest coverage were selected as representative ITS2 ribotypes, deposited in GenBank under unique GenBank accession numbers (Table S1), and the longest of those were chosen for phylogenetic analyses (Figure 2).

Both sets of DNA sequence data (ITS2 and ITS1-5.8S-ITS2) showed that the 18 analyzed *Veronica* species were divided into eight major groups corresponding to the following subgenera: *Pentasepalae*, *Pocilla*, *Chamaedrys*, *Veronica*, *Beccabunga*, *Cochlidiosperma*, *Stenocarpon* and *Pseudolysimachium* (Figures 2 and 3). The most numerous subgenus was *Beccabunga*, which contained six species: *V. beccabunga*, *V. catenata*, *V. acinifolia*, *V. serpyllifolia*, *V. anagaloides* and *V. anagalis-aquatica*. This subgenus is much better supported in the phylogenetic tree with ITS1-5.8S-ITS2 sequences (bootstrap support = 99) (Figure 3) than in the tree containing only ITS 2 sequences (bootstrap support = 92) (Figure 2). Monophyletic group *Cochlidiosperma*, with two species *V. cymbalaria* and *V. hederifolia*, was the least supported group in both the ITS2 and the ITS1-5.8S-ITS2 phylogenetic trees (Figures 2 and 3). The subgenera *Stenocarpon* and *Pseudolysimachium* are represented by only a single species *V. saturejoides* and *V. longifolia*, respectively.

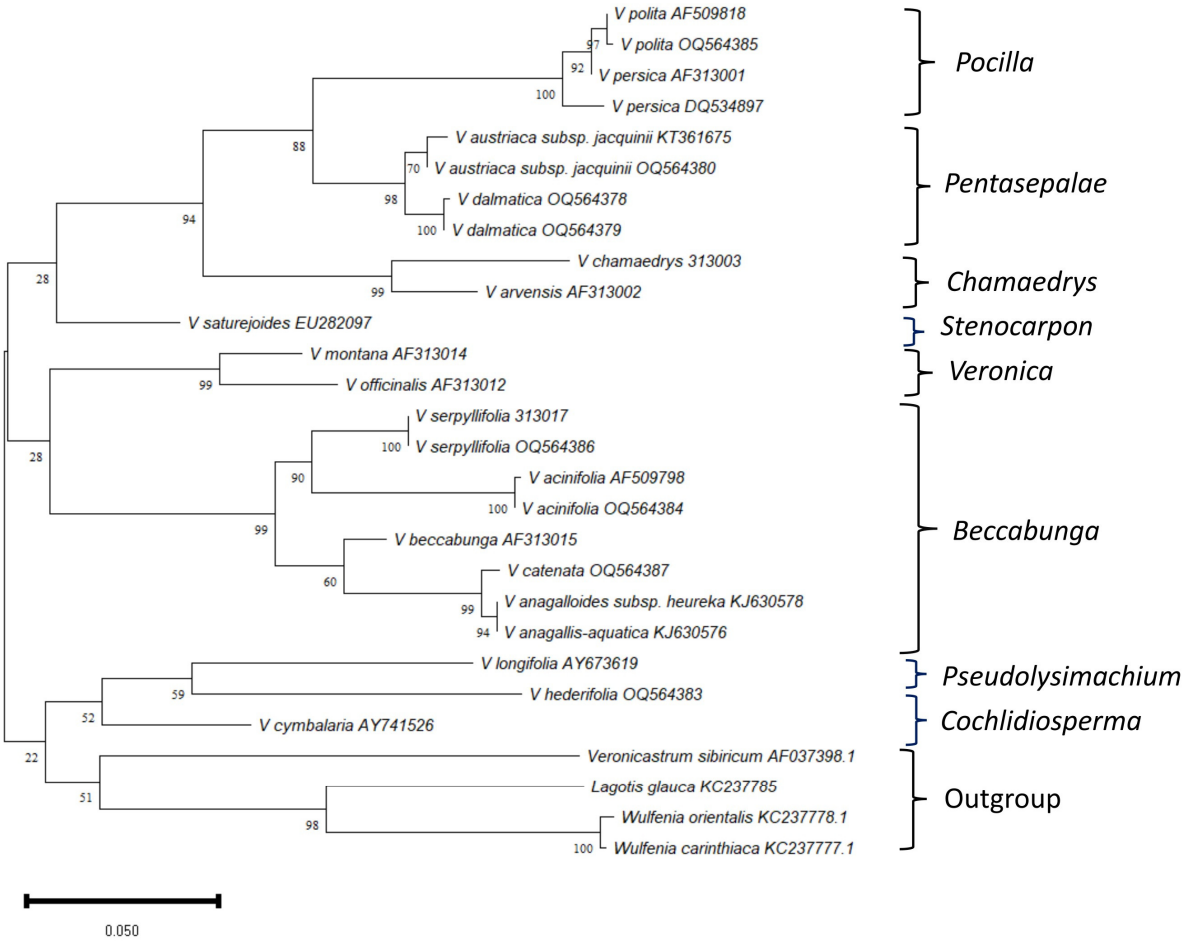

Figure 3. Evolutionary relationships of the studied *Veronica* species inferred from ITS1-5.8S-ITS2 sequence analysis by the maximum likelihood method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Subgeneric affiliation is shown.

A comparison of the two phylogenetic analyses and their results—the two phylogenetic trees—suggests that the analysis of the longer ITS1-5.8S-ITS2 sequences yielded better subgenus classification than the analysis of the ITS2 sequences. This is likely due to the fact that longer Sanger-generated sequences (~580 bp) produce more variable and informative sites (positions) than shorter NGS sequences (~360 bp). Thus, our results indicate that ITS2 data are of rather limited value for *Veronica* species identification and phylogenetic analysis.

3.2. Statistical Analyses

3.2.1. Cluster Analyses Based on Free Volatile Compounds

The complete table of volatile compounds of these *Veronica* species was previously published in a paper by Dunkić et al. [24]. Free volatile compounds were extracted with two methods of hydrodistillation, with the Clevenger apparatus and microwave hydrodistillation. Table 2 lists the compounds with the highest relative percentages for all species selected for genetic research. Cluster analyses were performed for this study, and the results are shown in Figures 4 and 5. A total of 40 volatiles were used for this analysis. Figure 4 shows the clustering for the volatiles extracted with the Clevenger apparatus. Two main groups can be observed, which we described with five major compounds: phytol, hexahydrofarnesyl acetone, caryophyllene oxide, *(E)*-caryophyllene and hexadecanoic acid. The first group includes *V. persica*, *V. acinifolia*, *V. chamaedrys*, *V. arvensis*, *V. cymbalaria*, *V. anagalloides*, *V. longifolia*, *V. austriaca* ssp. *jacquinii*, *V. polita*, *V. montana*

and *V. anagallis-aquatica*. This group cannot be described with only one chemotype, but we can observe three subgroups. The subgroup *V. persica*/*V. acinifolia*/*V. chamaedrys* can be described with compounds in the following order: phytol > hexahydrofarnesyl acetone > caryophyllene oxide > (*E*)-caryophyllene > hexadecanoic acid. The subgroup *V. arvesis*/V. cymbalaria/V. anagalloides/V. longifolia/V. austriaca ssp. jacquinii is defined with compounds in the following order: hexahydrofarnesyl acetone ≈ hexadecanoic acid ≈ caryophyllene oxide > (*E*)-caryophyllene ≈ phytol. The third subgroup *V. polita*/*V. montana*/*V. anagallis-aquatica* can be described with compounds in the following order: phytol ≈ hexahydrofarnesyl acetone > hexadecanoic acid ≈ caryophyllene oxide > (*E*)-caryophyllene. The second cluster consists of *V. serpyllifolia*, *V. hederifolia*, *V. dalmatica*, *V. saturejoides*, *V. catenata*, *V. officinalis* and *V. beccabunga*. This cluster is specified by the main compounds in the following order: phytol ≈ caryophyllene oxide ≈ hexahydrofarnesyl acetone > hexadecanoic acid > (*E*)-caryophyllene.

In Figure 5, which shows the clusters of volatiles extracted with a microwave apparatus, three clusters can be observed. The first cluster includes *V. persica*, *V. polita*, *V. serpyllifolia*, *V. arvensis*, *V. chamaedrys*, *V. saturejoides*, *V. anagalloides* and *V. anagallis-aquatica*. This cluster is specified by the major compounds in the following order: hexahydrofarnesyl acetone > phytol > hexadecanoic acid ≈ caryophyllene oxide > *(E)*-caryophyllene. The second cluster consists of *V. officinalis*, *V. austriaca* ssp. *jacquinii*, *V. cymbalaria*, *V. dalmatica* and *V. hederifolia*. This cluster is specified by major compounds in the following order: phytol ≈ caryophyllene oxide ≈ hexahydrofarnesyl acetone > hexadecanoic acid > (*E*)-caryophyllene. It can be seen that this type of percentage ratio of volatile compounds is observed in the second cluster for Clevenger isolation (species compared in both clusters are *V. officinalis*, *V. dalmatica* and *V. hederifolia*). The third cluster includes *V. acinifolia*, *V. montana*, *V. longifolia*, *V. catenata* and *V. beccabunga*. For all these species, the prevailing compound is phytol, and other compounds are evenly distributed in relative percentage.

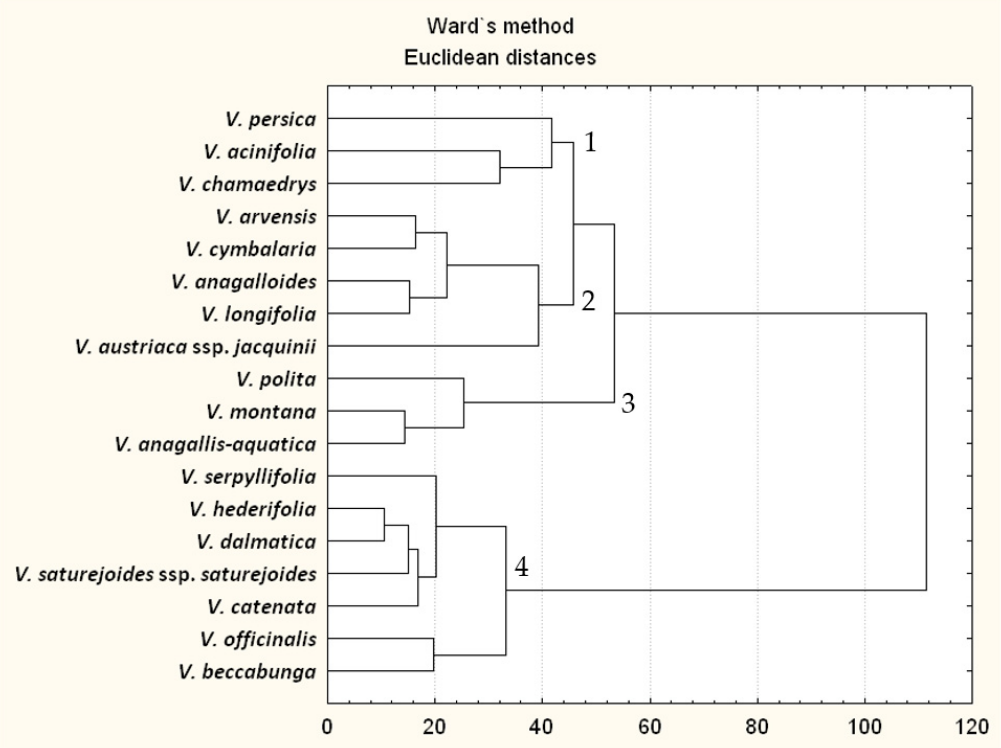

Figure 4. Dendrograms of Euclidean distances using Ward's method based on chemical constituents obtained by classical extraction—hydrodistillation with Clevenger apparatus in an amount equal to or greater than 2%. 1-phytol; 2-hexahydrofarnesyl acetone; 3- phytol/hexahydrofarnesyl acetone; 4-phytol/caryophyllene oxide/hexahydrofarnesyl acetone.

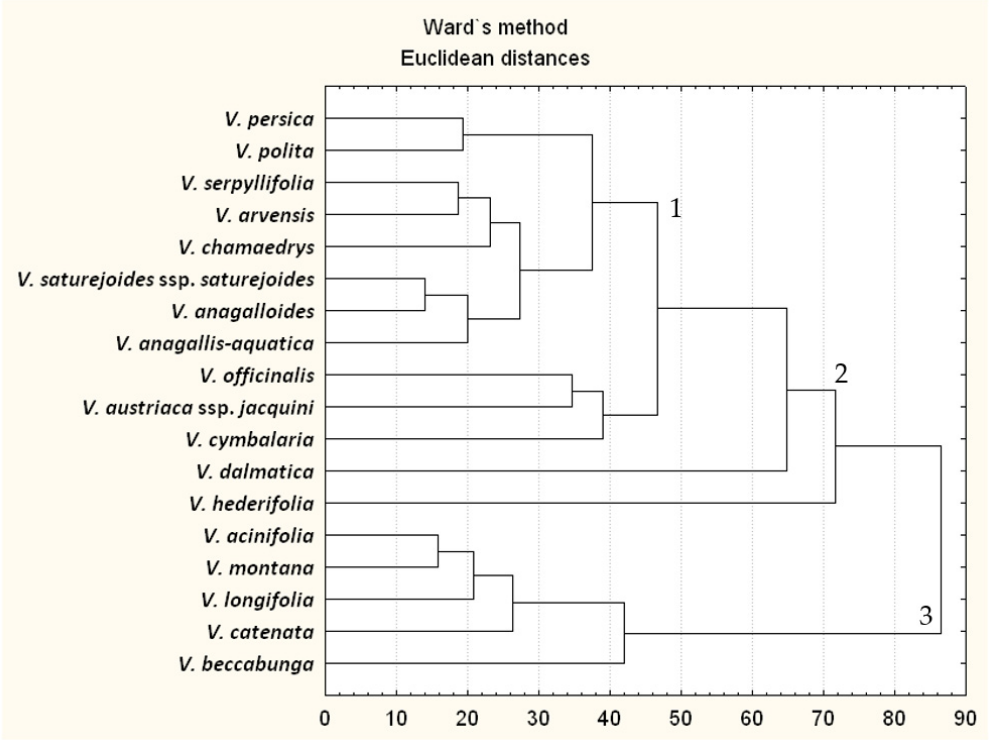

Figure 5. Dendrograms of Euclidean distances using Ward's method based on chemical constituents obtained by microwave-assisted water extractions in an amount equal to or greater than 2%**.** 1-hexahydrofarnesyl acetone; 2-phytol/caryophyllene oxide/hexahydrofarnesyl aceton; 3-phytol.

3.2.2. Mantel Test

In order to compare the grouping of the 18 studied *Veronica* species based on the ITS sequences with the grouping based on volatile components, a Mantel test was performed to compare the Euclidean distances of volatile components and Kimura genetic distances from DNA sequence data (Data S3). However, the result showed that there was no correlation between these two sets of data, and it was not statistically significant.

4. Discussion

Although it was possible to identify most of the analyzed *Veronica* species studied from their ITS2 sequence data generated by NGS sequencing, more accurate identification was achieved by using the longer ITS1-5.8S-ITS2 sequences obtained by classical Sanger sequencing. In addition, better results of the phylogenetic analysis and clustering of *Veronica* species to eight subgenera were obtained based on ITS1-5.8S-ITS2 sequences than with data based on ITS2 sequences. This is likely due to the fact that longer sequences generated by the Sanger sequencing method (~580 bp) produced more variable and informative positions than shorter NGS sequences (~360 bp). However, the abundance of ITS2 sequences obtained with NGS technology, along with ITS1 sequences also sequenced with NGS for 18 *Veronica* species, which are not shown in this paper because they are less informative than the ITS2 sequences, will be used for the detailed analysis of the diversity of contigs/ribotypes within each species and their molecular evolution and genome structure, and such analyses are underway.

The results of the two phylogenetic analyses (based on ITS2 and ITS1-5.8S-ITS2) are largely consistent with previous publications. According to our and previously published data on molecular-phylogenetic analyses of the *Veronica* species [1,2,6,8,10,28,54,55], the species from Croatia selected for this research belong to eight subgenera: *Pseudolysimachium* (*V. longifolia*), *Beccabunga* (*V. acinifolia*, *V. anagallis-aquatica*, *V. beccabunga*, *V. catenata*, *V. serpyllifolia*, *V. anagalloides*), *Veronica* (*V. montana*, *V. officinalis*), *Chamaedrys*

(*V. arvensis*, *V. chamaedrys*), *Pentasepalae* (*V. austriaca*, *V. dalmatica*), *Stenocarpon* (*V. saturejoides*), *Pocilla* (*V. persica*, *V. polita*) and *Cochlidiosperma* (*V. cymbalaria*, *V. hederifolia*).

Comparing the two clusters created based on the free volatile compounds (Figures 4 and 5) and based on the molecular data for ITS regions (Figure 2), it can be said that clustering based on the volatile compounds from microwave extraction resulted in clusters that better fit the molecular clusters. The first cluster (Figure 5) consists of species belonging to *Chamaedrys*, *Pocilla*, *Stenocarpon* and *Beccabunga* subgenera. *Chamaedrys* and *Pocilla* are closely related in the molecular ITS clusters. The second cluster includes species from *Cochlidiosperma*, *Veronica* and *Pentasepalae* subgenera. The third cluster consists of species from *Veronica* and *Beccabunga* subgenera. In Figure 2, these two subgenera, *Veronica* and *Beccabunga*, are closely related. Compounds in these species that were present in all species regardless of the habitat and conditions under which they grew include hexahydrofarnesyl acetone, hexadecanoic acid, caryophyllene oxide and *(E)*-caryophyllene. Other compounds also present in almost all species samples are phytol, germacrene D and pentacosane. The different relative percentages and their mutual ratio are probably the result of the ecological conditions in which they live. Since no correlation was found between the ITS cluster and the volatile cluster, it can be concluded that these compounds are not good interspecies (belonging to the same genus) chemophenetic markers, but some of them can be defined as chemophenetic markers for the whole genus *Veronica*. To back up this result, we reviewed volatile compounds studied in other genera belonging to the family Plantaginaceae and found that compositions of volatiles differed in major compounds but had some of the same compounds as *Veronica* species. Hammami et al. studied essential oil of *Plantago afra* and found that the major constituents were thymol (14.3%), 3-[4-(t-Butyl) phenyl] furan-2,5-dione (12.7%), hexadecanoic acid (8.9%) and eudesmane [56]. Al-Mazroa et al. studied essential oils of *Plantago amplexicaulis* and *Plantago boissieri*, and the results showed the main composition for *P. amplexicaulis* was hexadecanoic acid and 3 -methyl undecane. *P. boissieri* major compounds were found to be bicyclo-2,2, 1-heptane,2-(2-propenyl and l-dodecane-3-ol [57]. Essential oil major constituents of *Conobea scoparioides* were found to be thymol methyl ether (62%), thymol (16%) and α-phellandrene (14%) in a study by de Lima et al. [58]. In another study by Brandao et al., *Dizygostemon riparius* essential oil major constituents were found to be endo-fenchyl acetate and endo-fenchol, followed by *(E)*-caryophyllene and caryophyllene oxide in smaller relative percentages [59]. Bajer et al. studied the essential oil composition of *Plantago lanceolata* leaves and found the following major compounds: hexadecanoic acid, linalool and pentyl vinyl ketone. They also identified hexahydrofarnesyl acetone but in small relative percentages (1.81–2.99%) [60]. In another study on the essential oils of *Plantago lanceolata* and *Plantago major*, the major compounds identified were different from those in Bajer et al.'s research: metaraminol, bifemelane, metossamina and pterin-6-carboxylic acid in *P. lanceolata* and 2-dodecen-1-yl (-) succinic anhydride, benzenemethanol, α-(1-aminoethyl)-2,5- dimethoxy, dl-phenylephrine and nortriptyline in *P. major* [61]. Fons et al. also studied *Plantago lanceolata* essential oil and found the major compounds of leaf essential oil to be oct-1-en-3-ol and *(E)*-4(3-oxo-2.6.6-trimethylcyclohex-2-en-1-yl)-3-buten-2-ol [62]. They found hexahydrofarnesyl acetone as a major compound but only in the fruits of this plant [62]. One more study on the Plantaginaceae family reported hexahydrofarnesyl acetone as a detected compound in the FVC composition. Roudbaraki et al. studied *Digitalis nervosa* leaf volatile compounds and found that the major compounds were *trans*-pinocamphone and hexadecanoic acid, followed by caryophyllene oxide and phytol [63]. Comparing all these results to the identified volatiles of *Veronica* species and their relative percentage, it can be concluded that the identified volatile compounds of the genus *Veronica* were not found outside the genus in this combination, at least not until this moment. Some major compounds mentioned earlier in these identified percentage ratios can be defined as chemophenetic markers for the genus *Veronica*. Hexahydrofarnesyl acetone is particularly noteworthy, which appears in high percentages in all *Veronica* species studied and appears in other Plantaginaceae genera in much smaller relative percentages than in *Veronica* species. Other reported identi-

fications of this compound are studied in seeds or fruits, so they are not comparable to our study [62,64]. A similar situation is found with caryophyllene oxide, *(E)*-caryophyllene, phytol, germacrene D and pentacosane. After reviewing all these Plantaginaceae volatiles, it can be concluded that hexadecanoic acid is not a good chemophenetic marker as it appears in many other species outside the *Veronica* genus. Our study is not the first that found compounds that are chemophenetic markers for the genus but not for the subgenus level. Mehrvarz et al. found similar results in their study, in which they detected constant and characteristic iridoid and flavonoid profiles in selected *Veronica* species, which is useful in analyzing taxonomic problems at a specific level (intergenus level) [17] but not for distinguishing species belonging to the same genus. Reviewing the glycosides that were detected in the species of genus *Veronica*, Taskova et al. found that aucubin and catalpol were found in all the species they investigated (many of them were part of this study by our team: *V. polita*, *V. persica*, *V. chamaedrys*, *V. cymbalaria*, *V. montana*, *V. officinalis*, *V. longifolia*, *V. anagallis-aquatica*, *V. peregrina*, *V. beccabunga*, *V. serpyllifolia*, *V. acinifolia*) [10]. In their research, they also found no correlation between clade membership and phytochemical components due to intraclade variability. This is also the case with free volatile compounds from our research. Taskova et al. also concluded in their research of the New Zealand snow hebe that chemical profiles can provide valuable data for taxonomic problems at the subsection rank [65]. Further research on the free volatile compounds as chemophenetic markers could include some sections of the genus *Veronica* that grow in the Southern Hemisphere, such as *Hebe*, *Parahebe*, *Heliohebe*, *Detzneria* and *Derwentia*, as they were once considered to be different genera.

The Mantel test for the comparison between the Euclidean distances of volatile components and Kimura genetic distances from DNA sequence data showed that there was no correlation between the chemical and genetic data groups. Genetic distances are typically based on molecular markers that evolve slowly over time and are subject to random mutations. Moreover, the observed cases of parallel evolution and in the genus *Veronica* [2,37] can additionally complicate the correlation of genetic data with the chemical composition of volatile components. Volatile components as well as other plant metabolites may be influenced by more rapid changes such as changes in gene expression, environmental factors or epigenetic modifications. Therefore, it is important to consider multiple lines of evidence when studying the evolutionary relationships between organisms, including genetic, morphological and biochemical data. Further studies will shed more light on this interesting question.

5. Conclusions

This is the first report of the comparison between the free volatile compounds and DNA sequence data in Croatian *Veronica* species and a useful contribution to the better understanding of interspecies relationships in this genus. We did not find any correlations between the *Veronica* subgenera membership and the composition of free volatile compounds. This could be explained by the fact that volatile components as well as other plant metabolites may be influenced by more rapid changes such as changes in gene expression, environmental factors or epigenetic modifications. Major components identified by the classical (hydrodistillation) and green (microwave) methods of extraction regardless of the habitat isolated in all 18 species selected for this study were hexahydrofarnesyl acetone, hexadecanoic acid, phytol, *(E)*-caryophyllene and caryophyllene oxide. Since these compounds were not identified as major compounds in these relative percentage ratios in any other genera from the Plantaginaceae family, they could be considered as chemophenetic markers for the genus.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae9050524/s1eo. Table S1: List of taxa, GenBank accession numbers for the ITS2 and ITS1-5.8S-ITS2 sequences of *Veronica* species obtained in this study, Table S2: List of taxa, GenBank accession numbers for the ITS1-5.8S-ITS2 sequences and references for the previously published sequences used in this study, Table S3: Number of raw reads, effective tags and contigs/ribotypes obtained after cleaning, merging, chimera removal and assembly of ITS2 region amplicons of *Veronica* plants with NovaSeq Illumina platform, Table S4: Statistics of assembled contigs/ribotypes of ITS2 region of *Veronica* plants that incorporated > 20% of clean tags, Data S1: Sequences of assembled contigs/ribotypes of ITS2 region of *Veronica* plants that incorporated > 20% of clean tags, Data S2: Sequences of ITS1-5.8S-ITS2 region of *Veronica* species obtained in this study and combined with earlier published sequences from GenBank, Data S3: Results of Mantel test.

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Data Availability Statement: Raw sequence reads obtained with the NovaSeq Illumina platform for the ITS2 region were submitted to the NCBI Sequence Read Archive (SRA) and are available under BioProject PRJNA944611, biosample accessions SAMN33750235—SAMN33750287. The most representative ITS2 sequences from NGS datasets (dominant ribotype) were deposited in GenBank under accession numbers OQ594828- OQ594880, and the ITS1-5.8S-ITS2 sequences that were sequenced by the Sanger sequencing method were deposited in GenBank under accession numbers OQ564378-OQ564387.

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