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Article

Investigation of Diversity by Analyzing the Polymorphism of SSR Markers and the Composition of Leaf and Fruit Essential Oils of 72 Mandarins (*Citrus reticulata* Blanco)

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Abstract: The great genetic and phenotypic diversities characterize the mandarin species (*Citrus reticulata*). The present study aimed to evaluate a part of this diversity with molecular markers and through the composition of leaf (LEO) and fruit peel (PEO) essential oils. Seventy-two cultivars were chosen for this study to represent some wild and cultivated mandarins growing in the same orchards. The essential oils were analyzed via gas chromatography (retention indices) and via gas chromatography coupled with mass spectrometry. The low similarity of 'Tachibana' and 'Korail tachibana' with mandarins and other species suggested that they were pure mandarins but were not involved in the genesis of the cultivated forms. This distinction was also evident at the aromatic level with specific compounds or unusual proportions, such as δ -3-carene in PEO or β -phellandrene at 24.9% in 'Korail tachibana' LEO. 'Kunembo' and 'Ben di gang ju' were genetically and chemically identical, with a high proportion of myrcene (>20%) in their LEO. In general, the genetic diversity of SSR markers was higher than the chemical diversity. From the 72 accessions, 54 genotypes were identified, with only 8 aromatic profiles in PEO and 9 in LEO. This diversity of essential oils of mandarins offers new perspectives for the research and validation of new aromatic properties for food and cosmetic purposes.

Keywords: citrus; phylogeny; genetic diversity; GC-MS; hydrodistillation; cold pressing; yield



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1. Introduction

The term 'mandarin' includes a wide range of citrus fruits commonly called 'easy peelers'. The species *C. reticulata* is an ancestral species, as it has been generated by crossing many cultivated hybrids with other species, such as orange (*C. sinensis*), sour orange (*C. aurantium*), Volkamer lemon and Rangpur lime (*C. limonia*) or Calamondin (*C. madurensis*) [1–3]. Originated from southern China, this species has undergone several phases of speciation in isolated areas such as continental Asia or the Japanese islands and then crosses between these different populations or even with pummelos [4,5]. A study of several mandarin trees carried out on the basis of targeted sequencing around endonuclease sites, a technique known as genotyping by sequencing (GBS), revealed more or less important introgressions of the pummelo genome into the genome of cultivated mandarins such as satsumas (*C. unshiu*) or King (*C. nobilis*) [6]. The genome of the varieties 'Cleopatra' (*C. reshni*), 'Sunki' or 'Sunkat' (*C. sunki*) and 'Shekwasha' (*C. depressa*) is reportedly devoid of it and these are therefore true or pure mandarins [3]. The complexity of the relationships among indigenous and cultivated mandarins across East Asia remains unclear and is a barrier to understanding the origin and domestication of mandarins. Genome sequencing has provided much information available for understanding the evolutionary history of mandarins.

Nanling region in Mangshan (China) was supposed to be the center of mandarin diversification and 'Mangshan' mandarins are a primitive type [4]. Two independent domestication events occurred with interspecific introgression from cultivated pummelo species, resulting in two groups of cultivated mandarins in the North and South Nanling mountains. A new wild species, *C. ryukyuensis*, originating from the Ryukyus Islands (Japan) was identified [7]. There are two subspecies of mainland Asian mandarins, *C. reticulata* (common mandarins) and *C. mangshanyejii*. 'Tachibana' (*C. tachibana*) and 'Shiikuwasha' (*C. depressa*), are supposed to be admixtures of Ryukyuan mandarins with *C. reticulata* and *C. mangshanyejii*, respectively [7]. Apomixis appeared in a mandarin, following an insertion of a MITE (a miniature inverted repeat of transposable element) in the CitRKD1 gene (or CitRWP) [8,9]. This MITE insertion arose in the mangshanyejii population, and the apomixis characteristic spread to other mandarins. Apomixis expressed by the development of somatic embryos has strongly contributed to the maintenance of hybrids that have inherited this characteristic at the expense of hybrids with strictly sexual reproduction [7].

The breeding programs of the 20th century have selected many mandarin × mandarin, mandarin × orange (tangor) or mandarin × grapefruit (tangelo) hybrids that are either spontaneous (Clementine, 'Murcott', 'Ortanique', 'Afourer') or induced ('Orlando', 'Nova', 'Willing', 'Fairchild') [10–12]. Hybridization is not the only way to diversify mandarins, and breeding programs have selected new spontaneous forms belonging to bud mutations in orchards. For example, all clementine cultivars were derived from somatic mutations, as well as cultivars in the *C. deliciosa* group ('Willow leaf', 'Avana', 'Tardivo di Ciaculli', 'de Chios' or 'Augustino') and satumas (*C. unshiu*) [13]. Intervarietal polymorphism in genetic markers such as SSR was generally null between mandarin cultivars that have evolved through mutations [14]. For all these reasons, mandarins in the broadest sense of the term are the most diverse group of citrus species.

The composition of essential oils, a complex mixture of aromatic compounds that characterizes citrus fruits in general and that are found in the skin of fruits, leaves and flowers, can also illustrate this diversity. Generally, each species can be identified by one or more chemical components in the essential oil. Five principal chemical profiles of leaf essential oil (LEO) were observed in mandarin groups characterized by major compounds such as sabinene/linalool, linalool, β -pinene/linalool, γ -terpinene, and methyl N-methyl anthranilate [15–17]. Some singular chemical compositions were also detected, such as that for 'San Jacinto' (*C. reticulata*) or 'Nasranan' (*C. amblycarpa*); however, these citrus species are not pure mandarins because they belong to hybridizations between mandarin and lemon and between mandarin and *C. micrantha*, respectively [14,18]. These five LEO profiles confirmed the high chemical variability of mandarins.

Peel essential oils (PEO) consist almost exclusively of hydrocarbons, with limonene as a major component (up to 96%). However, in some studies, the PEO showed that the percentage of limonene decreased to 65% and that it was associated with γ -terpinene (up to 36%) and terpinolene (up to 1%). α -Pinene (up to 4%), β -pinene (up to 2.5%), myrcene (up to 2%) and sabinene (up to 3%) were present in all samples [16,17,19]. Exceptionally, p-cymene and myrcene can reach 10%. Oxygenated monoterpenes are represented by linalool (up to 4%) and citronellal. Among other compounds, α -sinensal (sesquiterpene aldehyde) is present in almost all samples (up to 0.8%), whereas the aromatic compound methyl N-methyl anthranilate, usually found in LEO as a major component of some cultivars, is rarely identified (up to 1%). The non-terpenoid aldehydes octanal, nonanal and decanal are frequently reported with percentages close to 0.1%. Discriminant analysis confirmed the existence of two clusters with respect to the limonene and γ -terpinene contents [15].

The composition of essential oils can fluctuate depending on many factors such as the environment [20], the stage of fruit maturity [21], the rootstock [22,23], extraction method [24], the health of the tree [25,26], the genotype and the climate changes [27]. Variations in PEO composition can be very significant, as in the case of bergamot PEO, where the proportion of linalyl acetate varied during the ripening period from 17.07% to 40.37% [27]. For trees infected with viroid (CEVd), the proportion of oxygenated com-

pounds in the PEO of ‘Maltaise demi Sanguine’ sweet orange, increased (approximately 50%) with *C. macrophylla* and *C. volkameriana* as rootstocks, while a decrease of 60% to 70% was observed with 4475 citrumelo and *C. reshni* [26]. On healthy trees, the rootstock can also have an influence on the composition of orange PEO: the proportion of limonene varied from 69.51% to 93.34%, respectively, with *C. macrophylla* and *C. aurantium* [26].

However, the questionable identification of the origin of certain varieties in collections can also generate biochemical profiling errors. These varieties are of multiple origins: exchange between germplasm collections, selection of new materials from hybridizations, mutations or plant explorations in regions of natural genetic diversity [28]. Without the use of DNA genetic markers, the assignation of a cultivar name and/or taxonomic classification can be done arbitrarily, leading to possible mistakes or duplication of material. For these reasons, molecular studies are important for the detection of misidentifications and redundancies and for correct assignation [29].

The goal of this work was to investigate the diversity of mandarins at different levels, including genetic and fruit characteristics and essential oil compositions, and to complete the previous works performed on the genetic and chemical characterization of mandarins from INRAE-Cirad Citrus BRC [14–17]. This study was based on 72 mandarin cultivars, and among them, 51 cultivars have not been characterized chemically and 21 genetically.

2. Materials and Methods

2.1. Plant Material

Clonal propagated trees, grafted on Carrizo citrange rootstock, were maintained in the INRAE-Cirad citrus germplasm (BRC ISO9001), located at San Giuliano, Corsica, France (latitude 42°17' N, longitude 9°32' E). These trees were much more than 15 years old and grown in the same pedoclimatic and cultural conditions [30]. Corsican Island was subjected to Mediterranean climate, average rainfall and temperature, 840 mm and 15.2 °C per annum, respectively; the soil was derived from alluvial deposits and classified as fersiallitic, pH range 6.0–6.6. The trees were healthy and without visible insect infestation. The 72 mandarins investigated in this study represent the following 13 species (according to the *Tanaka system*): *C. unshiu*, *C. reshni*, *C. sunki*, *C. tachibana*, *C. nobilis*, *C. reticulata*, *C. deliciosa*, *C. depressa*, *C. tangeina*, *C. succosa*, *C. suhuiensis*, *C. tankan*, *C. erythroa*, mandarin hybrids, tangors and tangelos (Supplementary Materials). This mandarin selection represents a large panel of different phenotypes of this *Citrus* species (Figure 1). Among them, the EO of 19 cultivars were previously analyzed [15–17] and 51 cultivars have not been yet studied. A total of 21 cultivars or accessions were never previously genotyped. The taxonomic positioning of ‘Tachibana’ and ‘Korai tachibana’ varieties in relation to the major species of Asian citrus was carried out by genotyping with the same SSR and InDel markers. Forty-four citrus genotypes were selected to represent the six pure species: *C. maxima*, *C. medica*, *C. ichangensis*, *C. micrantha*, *C. reticulata* and *Poncirus trifoliata* (Supplementary Materials).

2.2. Genotyping

Genomic DNA was extracted from leaf samples using the DNeasy Plant Mini Kit (Qiagen S.A.) according to the manufacturer’s instructions.

The mandarin accessions were genotyped with 44 SSR and InDel markers selected according to their distribution on the 9 genetic linkage groups of the clementine genetic reference map [31] and on the reference sequenced genome [2] (Supplementary Materials). Among these markers, thirty-five were previously used to analyse the mandarin genetic diversity [14]. PCR was performed in a MWG thermocycler as described in [32]. PCR reactions were performed as simplex experiments in a 6 µL volume with 3 µL of PCR master mix (Qiagen kit), 0.35 µM of forward primer with a M13 tail at the 5’-end, 0.35 µM of reverse primer, 0.2 µL of fluorescently labelled M13-tail (6-FAM, NED, VIC or PET (Applied Biosystems, Foster City, CA, USA), 0.12 µL of 5 U/µL Taq DNA Polymerase (Taq’Ozyme OZYA001 from Ozyme, Montigny-le-Bretonneux, France) and 10 ng of DNA template. Amplified DNA samples were separated via capillary electrophoresis, using a

3130XL genetic analyzer (Applied Biosystems) with an internal standard. The data were analyzed with Genemapper™ software v5.0. The ADNid Company/Qualitech Group (Montpellier, France) performed Genotyping.



Figure 1. Photography of the diversity of fruit morphology and color. On the left, fruits of the pure mandarins, and on the right, fruits of cultivars and hybrids.

2.3. Sampling, Peel and Leaf Essential Oils

For each cultivar, approximately 200 g of mature leaves and at least 10 ripe fruits were picked from locations on the same tree, early in the morning and in dry weather, from January to March 2022. The leaves were collected from three trees on the southeast-facing side and located at the tree's periphery. The peel of fresh fully ripe fruits was manually grated with a zester to remove the zest and the essential oil was then separated from the crude extract by centrifugation (10 min at $8000\times g$). Fresh leaves were subjected to water distillation for 2 h 30 using a Clevenger-type apparatus in 2 L flask with a ratio of 200 g of leaves/1 L of distilled water. To avoid any damage, the oil samples were stored at $-20\text{ }^{\circ}\text{C}$ in amber vials until analyzed.

Peel essential oil (PEO) yields were calculated using the essential oil/fresh weight zest ratio. The yields of leaf essential oils (LEO) were not calculated because the volumes of LEO were too low to have a precise measure.

2.4. EO Analysis

The method, protocol, and apparatus for essential oil analysis are identical to the team's previous publication [33]. Each sample was analyzed via dual column gas chromatography and gas chromatography combined with mass spectrometry (GC-MS) in order to determine the chemical composition.

2.4.1. Gas Chromatography (GC) Analysis

GC analyses were performed on a Clarus 500 FID gas chromatograph (PerkinElmer, Courtaboeuf, France) equipped with two fused silica gel capillary columns (length 50 m, internal diameter 0.22 mm and film thickness 0.25 μm), BP-1 (polydimethylsiloxane) and BP-20 (polyethylene glycol). The oven temperature was programmed to increase from 60 to $220\text{ }^{\circ}\text{C}$ at $2\text{ }^{\circ}\text{C}/\text{min}$ and then held in an isothermal state at $220\text{ }^{\circ}\text{C}$ for 20 min, injector temperature: $250\text{ }^{\circ}\text{C}$; detector temperature: $250\text{ }^{\circ}\text{C}$; carrier gas: hydrogen (1.0 mL/min); and

split: 1/60. The relative proportions of the oil constituents were expressed as percentages obtained by peak area normalization without using correcting factors. Retention indices (RIs) were determined relative to the retention times of a series of *n*-alkanes with linear interpolation ('Target Compounds' software of PerkinElmer, Courtaboeuf, France). The essential oil (EO) samples (≈ 30 mg) were diluted in 0.5 mL of chloroform.

2.4.2. Mass Spectrometry

EOs were analyzed with a PerkinElmer TurboMass detector (quadrupole, Perkin Elmer, Courtaboeuf, France), coupled directly to a PerkinElmer Autosystem XL (PerkinElmer), equipped with a fused silica gel capillary column (length, 50 m; internal diameter, 0.22 mm; film thickness, 0.25 μm), and BP-1 (polydimethylsiloxane). Helium was used as carrier gas at 0.8 mL/min, 1/75 split injection and 0.5 μL was injected. The injector temperature was 250 $^{\circ}\text{C}$. The oven temperature was programmed to increase from 60 to 220 $^{\circ}\text{C}$ at 2 $^{\circ}\text{C}/\text{min}$ and then held in an isothermal state for 20 min. The ion source temperature and energy ionization were set at 250 $^{\circ}\text{C}$ and 70 eV, respectively. Electron ionization mass spectra were acquired over a 40–400 Da mass range. Oil samples (≈ 30 mg) were diluted in 0.5 mL of chloroform.

2.4.3. NMR Analysis

^{13}C NMR analyses were performed on an AVANCE 400 Fourier-transform spectrometer (Bruker, Wissembourg, France) operating at 100.623 MHz for ^{13}C , equipped with a 5 mm probe, in CDCl_3 , with tetramethylsilane (TMS) used as internal reference. ^{13}C NMR spectra were recorded with the following parameters: pulse width (PW): 4 μs (flip angle 45 $^{\circ}$); acquisition time: 2.73 s for 128 K data table with a spectral width (SW) of 220,000 Hz (220 ppm); CPD mode decoupling; and digital resolution 0.183 Hz/pt. The number of accumulated scans ranged from 2000 to 3000 per sample (≈ 30 mg of oil sample in 0.5 mL of CDCl_3). Exponential line broadening multiplication (1.0 Hz) of the free induction decay was applied before Fourier-transform.

2.4.4. Identification of Individual Components

The components were identified via three methods. The first one was a comparison of their GC retention indices (RIs) on polar and apolar columns, determined relative to the retention times of a series of *n*-alkanes with linear interpolation ('Target Compounds' software of PerkinElmer), with those of authentic compounds. The second one was based on computer matching against commercial mass spectral libraries and by comparison of spectra with literature data. The last method, used for few samples, was based on a comparison of the signals in the ^{13}C NMR spectra of EOs with those of reference spectra compiled in the laboratory spectral library. In the investigated samples, NMR identified individual components at contents as low as 0.5%.

2.5. Data Analysis

The allelic data obtained with the SSR and Indel markers were used to calculate genetic dissimilarity matrix, using the simple matching dissimilarity index (d_{i-j}) between pairs of accessions (units), with Darwin v6 software [34]. Unweighted neighbour-joining (NJ) analyses were computed to describe the population diversity organization, and the robustness of branches was tested using 1 000 bootstraps [35].

Chemical data were analyzed using R v3.6.3 software (2020) with the g-plots package v3.0.4 to analyze the EO data and determine the relationships between cultivars and components contributing to this diversity. A heatmap clusterization and a HCPC (hierarchical clustering on principal components) were constructed with the function of the package FactoMineR v2.7.

3. Results

3.1. Genetic Diversity in Mandarins

In the total set of 72 mandarins, the 44 markers detected 332 alleles with numbers varying from 1 allele for the IDPSY Indel marker to 15 alleles for the Ci08C05 SSR marker (Supplementary Materials). Fifty-five genetic profiles or genotypes have been detected, and among them, nine genotypes represented several cultivars or accessions (Table 1). While some mandarins are known to be mutant selections (*C. deliciosa* and *C. unshiu*), other genotypic identities are new (('Sweet Small', 'Cleopatra' and 'Xien Khuang'), ('Bombay', 'Antsalaka Diego' and 'Ponkan'), ('Kunembo', 'Ben di gang ju') and ('Bintangor Sarawak', 'Szinkom')). To not burden the tree representation of mandarin genetic diversity, the genetic duplicates of each multiple profile were further removed.

Table 1. List of cultivars sharing the same genotype (each genotype is numbered).

Genotype	Cultivar	Tanaka's System	ICVN
1	Clemendor	<i>C. deliciosa</i>	0100658
1	Avana Apireno	<i>C. deliciosa</i>	0100527
1	Hall	<i>C. reticulata</i>	0100356
1	Thighskin	<i>C. reticulata</i>	0100466
1	Tardivo di Ciaculli	<i>C. deliciosa</i>	0100704
1	Willow Leaf	<i>C. deliciosa</i>	0100131
2	Sweet small	<i>C. tangerina</i>	0100826
2	Cleopatra	<i>C. reshni</i>	0100591
2	Xien Khuang	<i>C. reticulata</i>	0100601
3	Bombay	<i>C. reticulata</i>	0100518
3	Antsalaka Diego	<i>C. reticulata</i>	0100495
3	Ponkan	<i>C. reticulata</i>	0100584
4	Yala	<i>C. reticulata</i>	0100674
4	Pet Yala	<i>C. suhuiensis</i>	0100273
5	Dobashi Beni	<i>C. unshiu</i>	0100342
5	Pusheng	<i>C. unshiu</i>	0100657
5	Saigon	<i>C. unshiu</i>	0100225
5	Sugiyama	<i>C. unshiu</i>	0100446
6	Ellendale	<i>Tangor</i>	0100454
6	Ellendale	<i>Tangor</i>	0100455
7	Scarlett	<i>C. tangerina</i>	0100681
7	Hansen	<i>C. reticulata</i>	0100587
8	Sarawak	<i>Bintangor</i>	0100582
8	Szinkom	<i>C. suhuiensis</i>	0100597
9	Ben di gang Ju	<i>C. unshiu</i>	0100433
9	Kunembo	<i>C. nobilis</i>	0100326

ICVN: International Citrus Varietal Numbering.

The 54 mandarin genotypes were apparently distributed in two clusters (Figure 2). According to the very low bootstrap values, it is impossible to suggest any tangible structure in this genetic diversity, and all the varieties had high genetic distances among them except between 'Geleking' and 'Yellow king'. However, two subgroups could be observed. The first one brought together 'Murcott', 'Rodeking', 'Kinnow', 'Pixie', 'Afourer', 'Palazelli' and 'Fremont' with a bootstrap of 40% for the node. The second subgroup that emerged from this diversity tree associated 'Cleopatra', 'Sunki' (accessions 076 and 705), 'Shekwasha', 'Tachibana', and 'Korai Tachibana', with a high bootstrap of 78%. Nevertheless, this group was characterized by high or even very high genetic distances between the six varieties. The two 'Sunki' mandarins had different genotypes, and accession 705 was genetically closer to 'Shekwasha' than the other accession (076). 'Ben di gang ju' was related to satsumas (86% for the bootstrap of the node); however, the genetic distance between them was nevertheless quite high. 'Yellow king' and 'Geleking' were genetically very close and differed only by two markers.

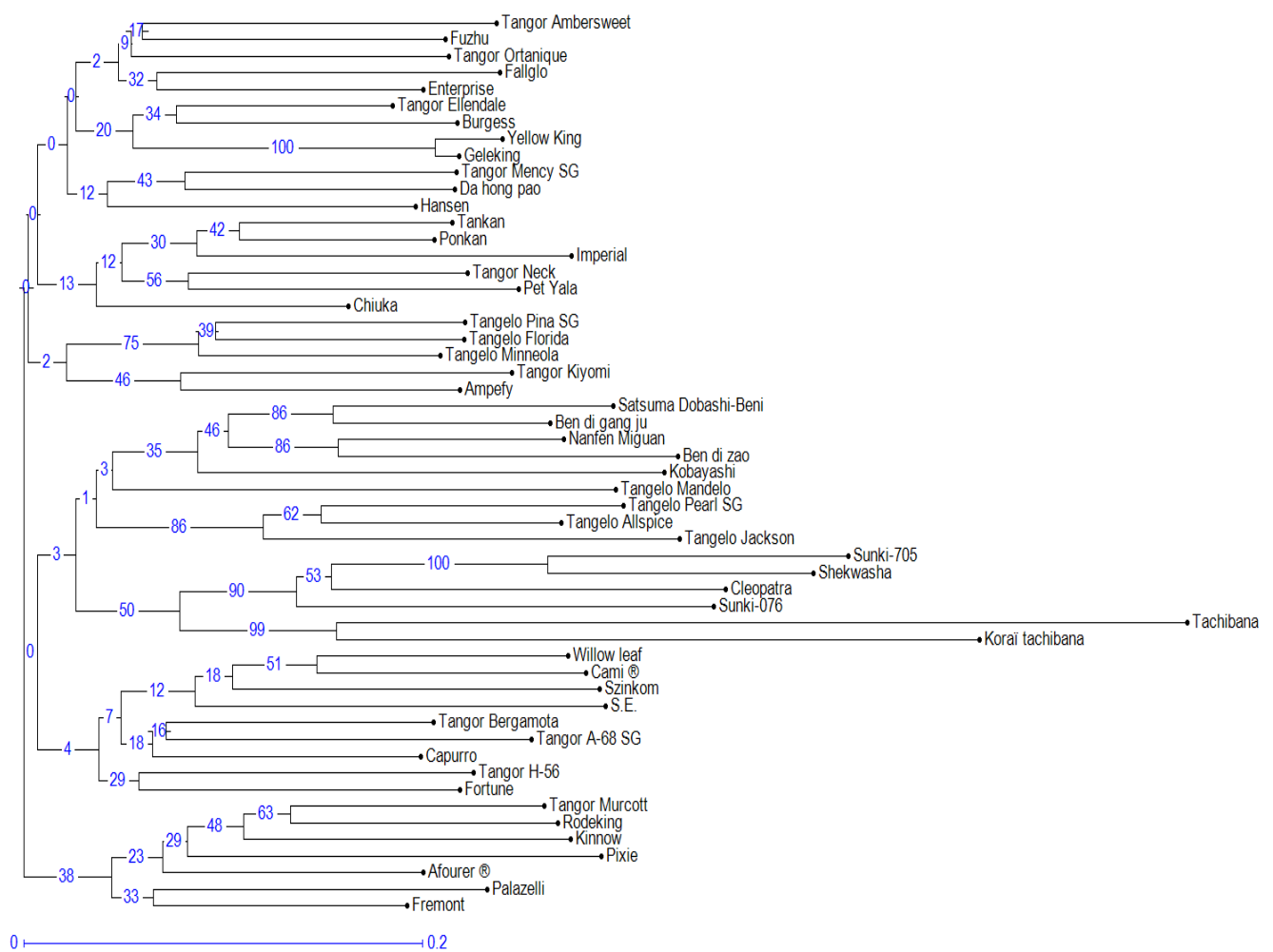


Figure 2. NJ tree of the genetic relationships between 54 mandarin genotypes based on the 44 SSR and InDel markers.

‘Tachibana’ and ‘Korai tachibana’ were the two mandarins with the largest genetic distances from other mandarins. Seventeen and twenty markers out of forty-four, with at least one specific allele, distinguished the genotype of ‘Tachibana’ and ‘Korai tachibana’ from other mandarins, respectively. For example, there were four specific alleles for ‘Cleopatra’ and only three for ‘Sunki-705’. This discrepancy demonstrates the uniqueness of the ‘Tachibana’ and ‘Korai tachibana’ genotypes and their probable nonparticipation in the genesis of the other mandarins of this study.

Because of the high genetic distances between ‘Tachibana’, ‘Korai tachibana’ and other mandarins, their placement in the mandarin group could be questioned. To test the hypothesis of a possible interspecific hybrid status, their genotypes were compared with those of other ancestral Asian citrus species (Figure 3).

Five clusters corresponding to the five true species were distinguished in this diversity tree. Low intraspecific genetic diversities characterized the citron and poncirus species (short branch length linking each varieties in the genetic tree) and high intravarietal distances in mandarin or pummelo species. Despite high genetic distances from other mandarin varieties, ‘Korai tachibana’ and ‘Tachibana’ linked to the mandarin branch with a high percentage of association (bootstrap of 99%). Therefore, these two varieties can be considered pure mandarins but with distant genetic relationships with the main mandarins of the present study.

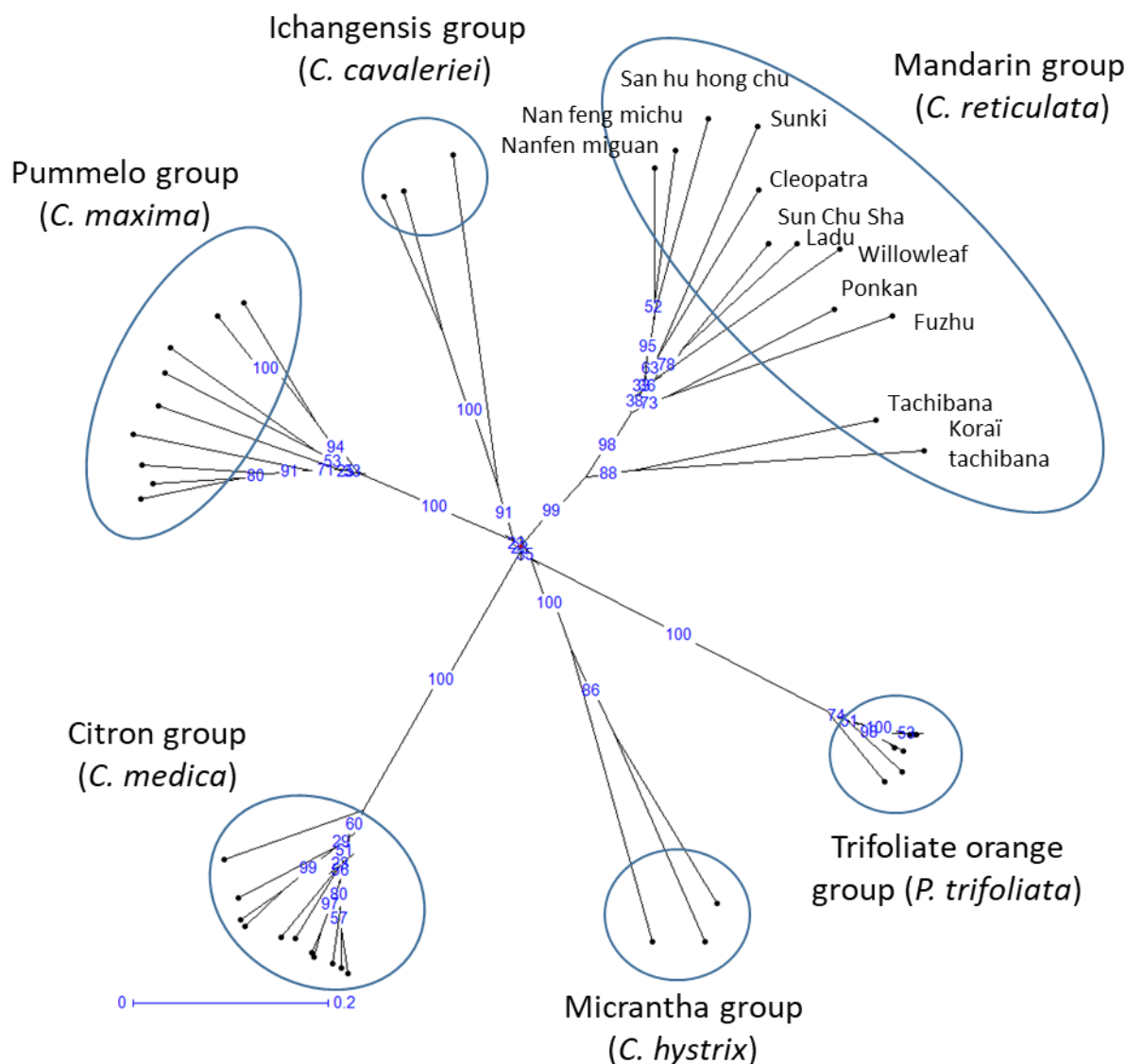


Figure 3. NJ tree representing the genetic diversity relationships of ‘Tachibana’ and ‘Korai tachibana’ with the true citrus species named according to [5].

‘Murcott’ is considered a tangor; however, seven markers (loci) rejected this hypothesis because it carried no common alleles with the orange genotype. Tangor ‘A-68’ did not seem to be a true tangor either, since four markers (loci) did not carry alleles in common with orange. In tangelos, ‘Allspice’ and ‘Pearl’ would not be first-generation hybrids because six and four loci, respectively, had no alleles in common with grapefruit. The same conclusion was made for the ‘Mandelo’ tangelo, where ten markers had no common alleles with grapefruit.

3.2. Variation in PEO Yield

The essential oil yield was variable among mandarins, ranging from 0.04 g to 8.84 g per 100 g of fruit zest for ‘Sugiyama’ satsuma and ‘Neck’ tangelo, respectively (Supplementary Materials). The number of varieties according to oil yield class shows that this trait is quantitative and therefore has multigenic control (Figure 4). The satsuma-type mandarins that differed from each other with little or no genetic polymorphism were the group with the lowest PEO yield (on average of 0.5%), while the eight tangors and eight tangelos had much higher yields (on average 5.2% and 4.1%, respectively) but with high variation (SD greater than 2.8). Varieties of the *C. deliciosa* group, with the same SSR genotype profile,

also showed variable yields from 0.9% for ‘Tightskin’ to 5.3% for ‘Tardivo di Ciaculli’. A high variation was also observed in the PEO yield between ‘Yala’ and ‘Pet Yala’, although they carried the same genotype (2.2/7.2%). On the other hand, there were also cases where the production of PEO was in accordance with the proximity or the genetic identity. For example, the PEO yields of the three ‘Cleopatra’-type accessions (‘Sweet Small’, ‘Cleopatra’ and ‘Xien Khuang’) were close (1.5% to 1.9%), as well as for the ‘Ponkan’-type mandarins (3.9 to 4.6%), ‘Hansen’ and ‘Scarlet’ (2.4 and 2.5%) or ‘Szinkom’ and ‘Bintangor Sarrawak’ (4.7 and 5.1%). The distribution was slightly skewed, with a greater representation of yields below the average value of the batch of the studied mandarins (Supplementary Materials).

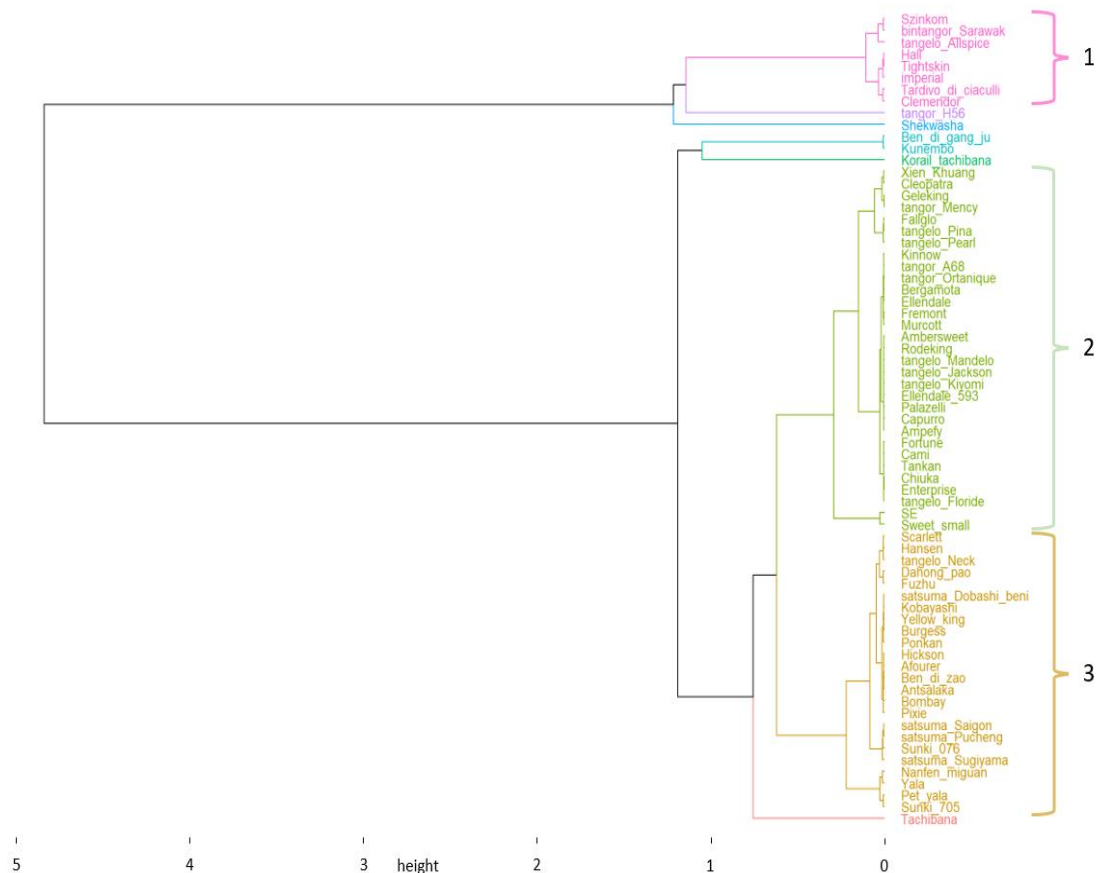


Figure 4. Dendrogram obtained via hierarchical clustering on principal components representing the cultivar diversity supported by the 15 major aromatic compounds of PEO.

3.3. Diversity of Mandarin PEO Composition

A total of 59 compounds were detected in the PEO of 69 mandarin cultivars, where limonene was the major compound, varying from 53.8 to 97.3% (Supplementary Materials). Among the 69 cultivars, four unique PEO profiles were distinguished from the others by atypical compositions corresponding to a single cultivar. ‘Tachibana’ showed a limonene/ γ -terpinene profile but with a specific compound, δ -3-carene, and one α -phellandrene also present in ‘Korai tachibana’. The profile of ‘Korai tachibana’ differed due to the absence of γ -terpinene but especially due to the presence of a high proportion of β -phellandrene (5.2%), while it was only present between 0.1 and 0.3% in other mandarin PEOs. The cultivars ‘Ben di gang ju’ and ‘Kunembo’ had a very high proportion of myrcene (>30%), while this compound did not exceed 2.1% in the other cultivars. The profile of ‘Shekwasha’ was composed of 53.8% limonene (the lowest level), 23.4% γ -terpinene, 8.9% p-cymene (the highest proportion) and the presence of two specific compounds (carvone and γ -cadinene). The rest of the mandarin cultivars represented three profiles characterized by the major compounds as follows: limonene/ γ -terpinene/p-cymene, limonene/ γ -terpinene

and limonene (>92%). It was observed that methyl N-methyl anthranilate was detected (0.3–0.8%) in ‘Tardivo di Ciaculli’, ‘Clemendor’, ‘Imperial’, ‘S.E.’ and tangor ‘A68’, as was nootkatone, a pummelo-specific compound, in tangor ‘H56’, ‘Kobayashi’ and tangor ‘Kiyomi’ (0.1–0.2%).

Only 15 compounds exceeded the threshold of 1% for at least one cultivar. The variation in several compounds was correlated (Supplementary File). For example, limonene was negatively correlated (>0.8) with α -thujene, α -pinene, γ -terpinene and terpinolene. A positive correlation (>0.8) existed with α -thujene, α -pinene, γ -terpinene and terpinolene. The 15 aromatic compounds were used to construct the dendrogram revealing the PEO mandarin diversity. In this dendrogram, apart from the 5 peculiar profiles detailed above, 3 clusters group the other cultivars (Figure 4).

The cultivars of Group 1 were characterized by a relatively low limonene content (<80.0%), an average of p-cymene (3.2%), α -pinene (2.2%) and γ -terpinene (16.8%). Group 2 was distinguished by the average values of α -thujene (0.20%), β -pinene (0.74%), α -terpinene (0.11%), terpinolene (0.15%) and limonene (84.8 to 94.3%). Group 3 gathered the PEO profiles characterized by a very high limonene content ranging from 93.1 to 97.3%.

3.4. Diversity of Mandarin LEO Composition

Seventy-three compounds were detected in the LEO of 64 varieties (Supplementary Materials). Some mandarin varieties or hybrids of mandarins showed atypical or specific chemical profiles (Figure 5). The variety ‘Koraï’ (or ‘Koraï tachibana’) was distinguished by two major compounds: β -phellandrene (24.9%), present in the other varieties but generally in proportions lower than 1% and β -pinene (20%). The profiles of ‘Ben di gang ju’ and ‘Kunembo’ were similar but peculiar compared to the other mandarins: γ -terpinene (24%), myrcene (22%) and geranyl acetate (1.3 and 7.0%). Proportions of β -pinene (25.5%), γ -terpinene (24.8%) and linalool (15.1%) distinguished the ‘Afourer’ LEO profile from other mandarins. ‘Sunki076’ PEO was characterized by a high proportion of thymyl oxide (19.7%), which was the second most abundant compound after γ -terpinene (34.7%). The chemotype of ‘Sunki971’ differed from that of ‘Sunki076’ by a higher proportion of sabinene (47.7%) and linalool (18.7%), a lower amount of γ -terpinene (3.4%) and the absence of thymyl methyl oxide. The LEO composition of ‘Da hong pao’, ‘Fuzhu’ and ‘SE’ was similarly characterized by linalool (49.5–55.8%), thymyl methyl oxide (6.8–8.4%), γ -terpinene (6.9–7.6%), (E)- β -ocimene (4.4%–7.8%) and thymol (3.6–6.3%). Such high proportions of thymol are unusual in mandarin except for ‘Jackson’ tangelo (3.3%). The LEO of ‘Tardivo di Ciaculli’, ‘Imperial’, ‘Tightskin’, ‘Hall’, ‘Clemendor’, and ‘H56’ tangor was characterized by a very high proportion of methyl N-methyl anthranilate (49.7–68.7%) and γ -terpinene (14.2–27.3%). The PEO of ‘A68’ tangor contained the highest amount of methyl N-methyl anthranilate (78.8%) but with only 0.1% γ -terpinene.

The LEO of ‘Fallglo’ and ‘Enterprise’ were similar but also peculiar compared to other mandarins with δ -3-carene (7.6 and 6.9%) and nerol (4.5 and 3.5%). The singularity of the ‘Ambersweet’ LEO amount was based on 45.3% of (E)- β -ocimene, which did not exceed 13.3% in other mandarins. The high proportion of β -pinene (41.1%) was a specificity of ‘Yala’ LEO. Very similar or even identical chemical profiles were also observed for [‘Ben di zao’ and ‘Neck’ tangor], [‘Scarlett’ and ‘Hansen’], [‘Bintangor Sarrawak’ and ‘Szinkom’], the different satsuma cultivars [‘Saigon’, ‘Pucheng’, ‘Sugiyama’ and ‘Dobashi Beni’] and for [‘Geleking’ and ‘Yellow king’].

A total of 23 compounds exceeded the threshold of 3%, and 42 exceeded the threshold of 1%. Among the 23 major compounds, only terpinen-4-ol and sabinene showed a high positive correlation (>0.8) (Supplementary Materials). A study of the mandarin diversity structure was performed using the 23 major compounds (Figure 6).

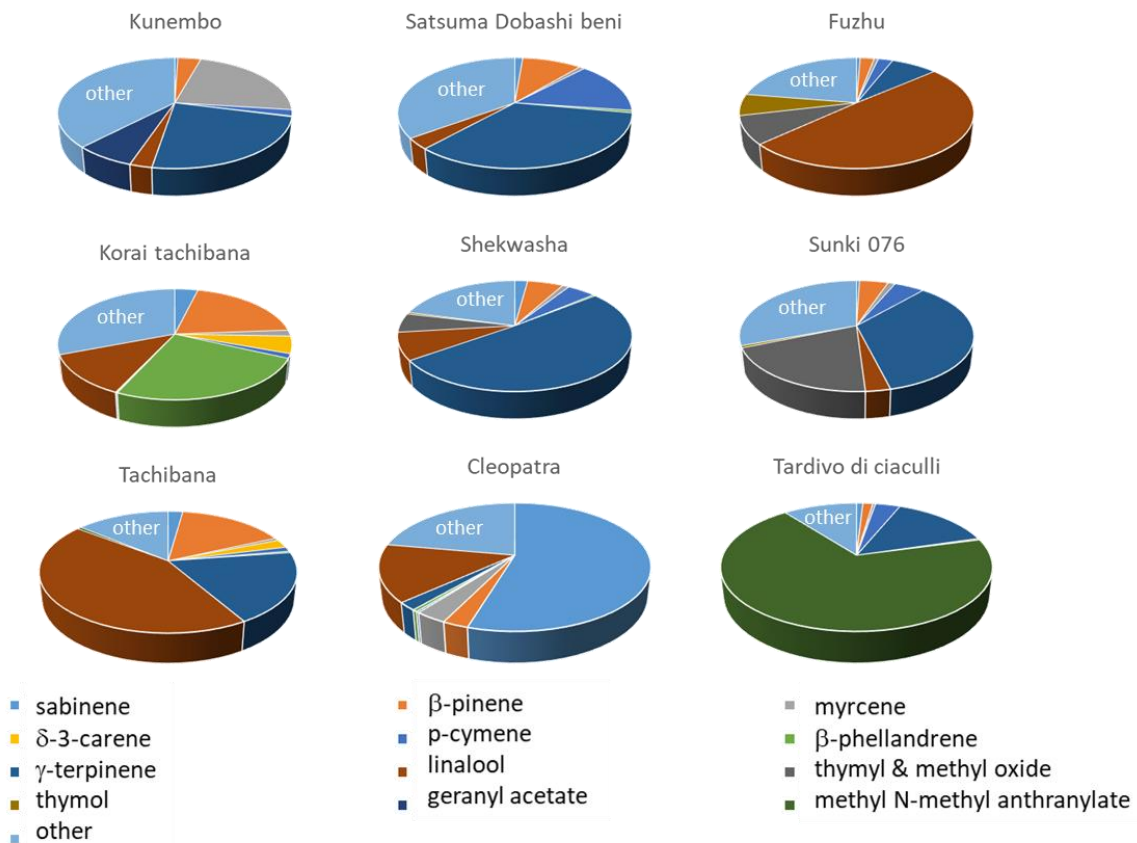


Figure 5. Representation of 9 LEO profiles considering the proportion of 12 major compounds observed for the pure mandarins.

Group 1 included seven mandarins characterized by the profile β -pinene (13.4–45.4%)/linalool (17.4–29.6%). The mandarins of Group 2 clustered based on high proportions of linalool (36.5 to 64.9%). Among them, ‘Tachibana’ stands out for its linalool (43.8%), γ -terpinene (18.9) and β -pinene (16%) contents. Subgroup b was characterized by a low content of α -pinene (0.1%). The mandarins of Group 3 were distinguished from others by γ -terpinene (20 to 50.9%) as the major compound. This group can be subdivided into three according to the α -pinene (3.2–5.4%) for subgroup a and high proportions of myrcene (21.8%) and geranyl acetate (4.1%) for the two mandarins of subgroup b. ‘Hansen’, ‘Scarlett’ and satsuma cultivars, characterized by p-cymene (13.9–15.7%), β -elemene (6.2–6.9%) and β -caryophyllene (mean amount of 3.1%), composed subgroup c. The major LEO compound of Group 4 was methyl N-methyl-anthranilate (49.7–78.8%). This compound characterized mandarins of the *C. deliciosa* group and some of their hybrids, such as ‘H56’ and ‘A68’ tangors. These tangor differed by a very low proportion of γ -terpinene (0.1%), while this compound represented 14.2–27.3% of the LEO for the other mandarins of this Group 4. Group 5 assembled the largest number of varieties (27), characterized by a profile with a majority of sabinene (28.5 to 54.7%). This group was divided into two subgroups according to the content of linalool, from 12.7 to 23.9% for 19 cultivars and from 1.9 to 5.7% for 8 cultivars.

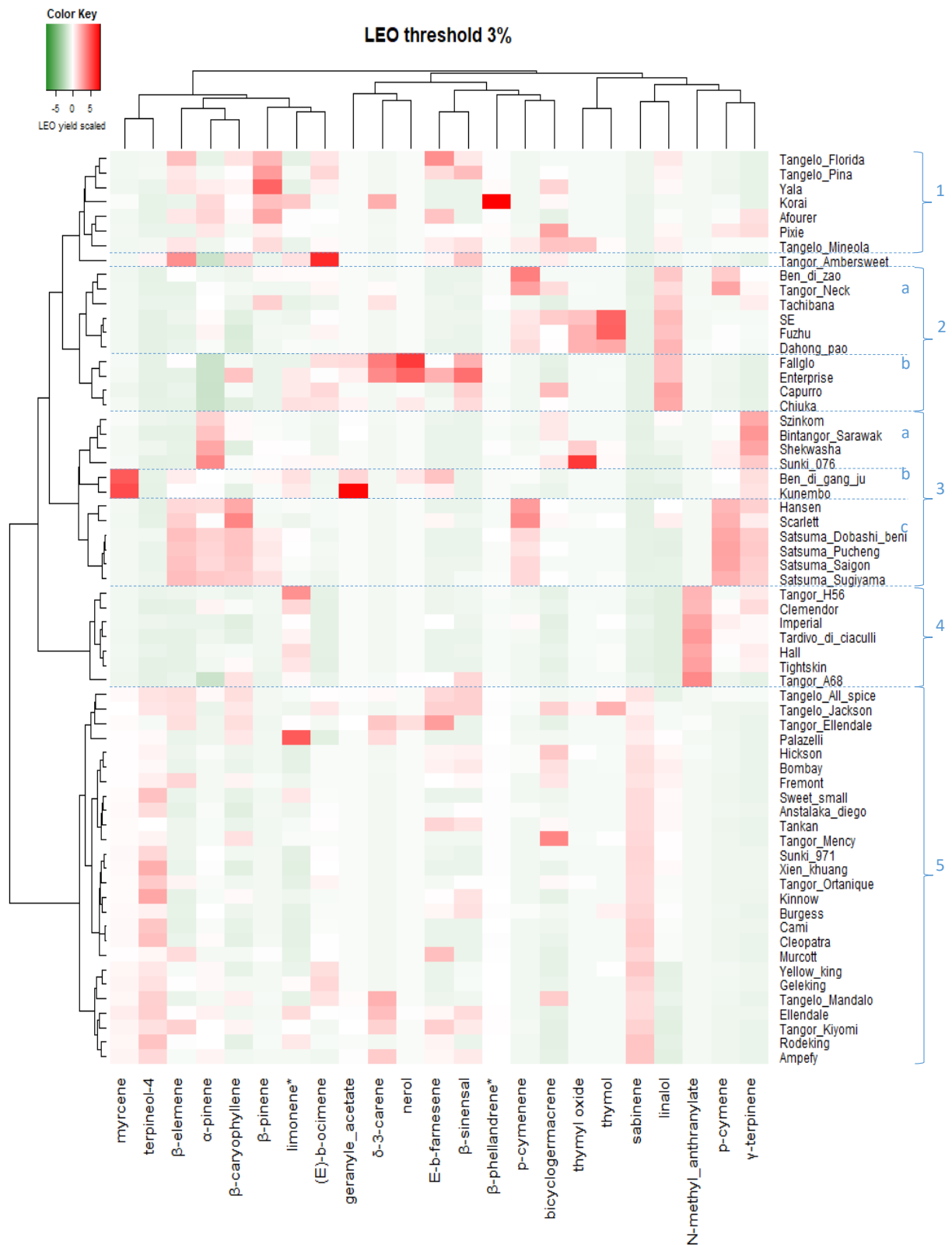


Figure 6. Hierarchical clustering and heat-map analysis of mandarin varieties and aromatic compounds of LEO exceeding 3% in at least one cultivar. Red represents high levels, and green represents low levels. Data were reduced and centered. The different clusters are numbered, and subgroups are identified by an alphabetical letter.

4. Discussion

Our study includes mostly natural mandarin hybrids or hybrids provided from breeding programs (tangors, tangelos or between mandarins) and only a few pure varieties, such as 'Shekwasha', 'Cleopatra', Sunki or 'Ponkan'. 'Tachibana' and 'Korai tachibana'. The genetic distances between pure mandarins and cultivars were high. Their genetic peculiarity was especially reflected for 'Korai tachibana' with a chemical profile never observed before with an original major compound (not detected in such proportion in any other citrus species). This mandarin does not seem to have been studied before or elsewhere under another name. 'Tachibana' is supposed to be a hybrid between mandarins from two populations that evolved in different Asian regions, *C. rukuyensis* and mainland *C. reticulata* [7]. 'Korai' (sometimes designated 'Korai tachibana') could be an interspecific hybrid between 'Yuzu' (*C. junos*) and an unknown citrus [36]. Our results do not support this hypothesis. 'Yuzu' is a mandarin/*ichangensis* hybrid [37]. Since our study did not reveal any particular genetic relationship with *C. ichangensis*, the linkage of 'Korai tachibana' with 'Yuzu' could then arise from alleles of the mandarin ancestor of 'Yuzu' that would also be present in 'Korai tachibana'. 'Ben di gang ju', which is genetically and chemically similar to 'Kunembo', is closely related to the satsuma mandarins; therefore, it could be involved in their genesis, as suggested by [36]. 'Kunembo' was considered as an admixture of *common* mandarin, pummelo and a small amount of *mangshanyaju* mandarin and one of the parents of the *yukunibu* mandarin group [7]. 'Shekwasha' and 'Tachibana' genotypes are thought to be half from the genome of *C. ryukyuensis* and half from different admixtures of *mangshanyaju* and *common* mandarin populations. Their particular genetic origins could explain their chemical particularities discovered during our study.

Identical genotypes were detected: 'Kunembo' and 'Ben di gang ju' satsuma, 'Scarlett' and 'Hansen', 'Sarawak' Bintangor and 'Szinkom', 'Yellow king' and 'Geleking'. These genetic identities were confirmed by biochemical profiles of similar or close EO compositions between cultivars of the same genotype. Homonymy was also revealed: the two accessions of 'Sunki' are genetically and chemically very different. It is likely that this homonymy is related to an erroneous phenotypic characterization of Sunki's morphotype or that several genotypes correspond to the same phenotype. The classification of 'Ben di gang ju' in the satsuma group is at odds with our results, both chemical and genetic. Its similarity with 'Kunembo' could suggest that this mandarin originated from Japan and that it could be part of the mandarins that have generated different cultivated forms, such as satsuma [36].

In some situations, there is a concordance between the results of genetic diversity and chemical diversity of essential oil compositions, especially in the proximity of chemical profiles for cultivars with close or identical genotypes. This is the case for 'Kunembo' and 'Ben di gang ju', 'Scarlett' and 'Hansen' and for mandarins of the *C. deliciosa* group. However, there are also large differences between the two studies, particularly for the cultivars sharing a close LEO profile, which is contrasted with the high genetic diversity between these same cultivar. Such discrepancies and concordances have already been reported in similar citrus studies [38]. 'A68' and 'H56' tangors have LEO chemical profiles very similar to those of *C. deliciosa*, in agreement with their parental origin. However, they are genetically very different; therefore, they cannot be considered members of the *C. deliciosa* group.

In our list of mandarins, the composition of essential oils of 20 cultivars had already been studied 20 years ago [15–17]. The chemical profiles were globally unchanged except for variations in the proportion of some compounds. For example, in 'Kunembo', the proportion of myrcene was lower in the former study: 24% vs. 31% in PEO and 15% vs. 23% in LEO. In 'Tardivo di Ciacculi', γ -terpinene in 2001 vs. 2022 reached 17.3% vs. 19.6% of PEO and 24.1% vs. 14.2% of LEO, as well as 54.45% vs. 68.7% methyl N-methyl anthranilate in LEO. These variations over time may be the consequence of different sampling dates in the development or maturity of the fruits or of a different climate between the two years of study (annual effect or climatic change in duration). Several publications have already mentioned these variations over time [27,39,40].

5. Conclusions

The different areas of diversification during evolution and natural interspecific crosses as well as crosses from breeding programs have contributed to the great diversity of the mandarin group. Genetic diversity revealed very different genotypes, such as ‘Korai’ and ‘Tachibana’, from those of modern cultivars, likely representing ancient subpopulations originating from different centers of diversification from the ancestors of cultivated mandarins. The study of the composition of essential oils revealed a great diversity of profiles, especially in pure mandarins and more particularly in the leaves, where limonene did not dominate as in PEO. Synonymous genotypes were detected using DNA markers and confirmed by EO analyses. Conversely, the ‘Sunki’ accessions diverged both genetically and chemically. This diversity of essential oils of mandarins offers new perspectives for the research and validation of new aromatic properties for food and cosmetic purposes. Originality is a marketing asset sought by the cosmetics and food industry to develop and market new products. The aromatic diversity of mandarin trees revealed in this work could thus be exploited directly for the creation of new aromas and fragrances, and can suggest to breeders crosses between genotypes to combine parental aromatic compounds and create new profiles.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae9050577/s1>, and all are grouped in the Excel table as follows: Sheet “mandarin varieties”: List of the 72 mandarin accessions, identification number and origin. Sheet “markers”: the name, type, genome position, primer sequences, annealing temperature of PCR and reference for the 44 genetic markers. Sheet “mandarin genotypes”: the size of alleles of the 44 markers for 70 mandarin accessions. Sheet “Allele-marker”: the number and size of alleles for each marker detected among the 70 mandarins. Sheet “citrus genotypes”: the alleles (in nucleotides) detected by the 44 markers in the additional 36 citrus accessions representing the different ancestral and true species, a grapefruit and a sweet orange. Sheet “Structure”: Results of structure software analysis (with $k = 2$) representing the proportion of mandarin (red) and pummelo (blue) in the 54 mandarin genotypes. Sheet “PEO yield”: The PEO yield in g/100 g of zest for 71 mandarin accessions and graphical representations of the distribution. Sheet “LEO composition”: Percentage of each aromatic compound of leaf essential oils from 64 mandarin accessions. Sheet “PEO composition”: Percentage of each aromatic compound of peel essential oils from 69 mandarin accessions. Sheet “PEO correlation”: figure of the matrix of Pearson’s correlation coefficient between the 15 major compounds of PEO (>1% threshold). Sheet “LEO correlation”: figure of the matrix of Pearson’s correlation coefficient between the 23 major compounds of LEO (>3% threshold). References [41–47] are cited in the supplementary materials.

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