Chemotaxonomic Study of *Citrus*, *Poncirus* and *Fortunella* Genotypes Based on Peel Oil Volatile Compounds - Deciphering the Genetic Origin of Mangshanyegan (*Citrus nobilis* Lauriro)

Cuihua Liu¹, Dong Jiang², Yunjiang Cheng¹, Xiuixin Deng¹, Feng Chen³, Liu Fang¹, Zhaocheng Ma¹, Juan Xu¹*

Introduction

Germplasm research provides clues to the origination, development, and even utilization of a biological material and is a prerequisite to collect and protect core collection of plant genetic resources. Information about individual accessions, particularly those found in situ, is often poor, reducing the frequency and efficiency of utilization and the ultimate benefits [1]. Recently, there is growing recognition that the germplasm diversity affects agricultural development, food security, livelihoods, and development aspirations of every country. The collection, preservation, and evaluation of germplasm are of great importance to the world germplasm resources. Information about individual accessions, particularly those found in situ, is often poor, reducing the frequency and efficiency of utilization and the ultimate benefits [1]. Recently, there is growing recognition that the germplasm diversity affects agricultural development, food security, livelihoods, and development aspirations of every country. The collection, preservation, and evaluation of germplasm are of great importance to the world germplasm resources.

Mangshanyegan (*Citrus nobilis* Lauriro), a wild germplasm in the citrus family, was discovered about 30 years ago in the remote mountainous forests of Mangshan, Hunan Province, China [4]. So far two genotypes of round- and sharp-leaf type Mangshanyegan have been found, whereas they have similar fruit types and their fruits can send forth a pleasant and intensive balsamic and floral aroma. Just like in other genotypes of *Citrus* and its relatives, monoterpenoids and sesquiterpenoids play dominant roles in the volatile profile of Mangshanyegan peel oil. Additionally, acids, alcohols, aldehydes, esters, an alkane, an indole, and a diterpene were all identified [5]. Thus, Mangshanyegan is not only a promising and precious resource for the essential oil industry, but also a desirable object for researches regarding mechanisms of aroma production. However, its genetic origin remains uncertain. Therefore, volatile compounds are ideal objects that can provide abundant information for chemotaxonomy study.

In chemotaxonomy study, the chromatographic identification of either volatile or non-volatile natural compounds was a tedious lab work. Recently, progress in chromatographic/spectral techniques and software for automatically analyzing MS data, such as *MzMine* [6], *MathDAMP* [7], *Tagfinder* [8] and *MetAlign* [9], has remarkably facilitated chemotaxonomy and nontargeted functional genomics research [10,11]. The former would play an important role in the study of taxonomy and has been used in chemotaxonomy studies of fungi and plants, and is a promising method for those highly hybrid plants and its closely related species, such as *Citrus* and its related genera [12–14].

As reviewed by Moore [15], *Citrus* and its relatives have some distinguishing characteristics: (1) *Citrus* and its relatives are very ancient, which makes it difficult to trace them back to their origins and diversities; (2) *Citrus* and its relatives are highly heterozygous...
and include many hybrids; (3) polyembryo occurs in most *Citrus* and its relatives, and it’s very possible that nucellar embryos triumph over the single zygotic embryo. The items mentioned above make traditional morphology and geography insufficient to clarify their taxonomy, so various biochemical and molecular markers were used to solve the problem. Since Kesterson et al. [16] and Pieringer et al. [17] analyzed the volatile constituents of different citrus leaf oils in 1964, the chemotaxonomy in *Citrus* and its related genera have been widely reported [18–20].

Luro et al. [21] studied the genetic diversity and chemical diversity among 24 citrus varieties (*C. medica* L.) based on 22 nuclear and 6 cytoplasmic genetic markers along with 43 volatile compounds identified from leaf essential oils. The authors found that the diversity based on leaf oil compositions did not agree with the molecular diversity and was unsuitable for intraspecific phylogenetic studies. However, chemotaxonomy studies on other plants showed that chemical compounds (e.g., Sesquiterpenes dialdehydes, etc.) could be considered as species markers [22]. Furthermore, Hou et al. [23] found that chemotaxonomic classification could be very useful for aquatic assessment in distinguishing phytoplankton communities and extremely advantageous and cost-effective in large ecosystem-scale research. Li et al. [24] found that the evolution and classification of bamboo inferred from leaf n-alkanes were consistent with morphological investigations reported previously. These above studies suggested that chemotaxonomic analysis was a reliable, informative, high-throughput research tool for taxonomy study. Also, it is well known that wild and primitive genotypes, with higher genetic diversity, were significant to the taxonomic classification study [25,26]. Thus, the phylogeny of Mangshanyegan and the chemotaxonomy of *Citrus* and its related genera were assessed in this study based on volatile compounds of their peel oils and volatile profiles from their leaves, which may supply both some new clues to the evolution of citrus and detailed information of the resolved volatile compounds of peel oil for further use, such as in cosmetic industry or citrus breeding programs on sensory flavor quality.

**Materials and Methods**

**Plant material**

Including the sharp- and round-leaf genotypes of Mangshanye-gan, 30 genotypes of mature fruits belonging to three genera of *Citrus*, *Poncirus*, and *Fortunella* were collected from National Citrus Breeding Centre of China (NCBC), Wuhan in Hubei province, or Citrus Research Institute of Chinese Academy of Agricultural Sciences, Chongqing (CRIC) in 2009 or 2010. Only one sample, Shatian pummelo, was collected from Citrus Experimental Station (CES), Zigui county, Hubei Province. All necessary permits were obtained for this study. Xixian Deng (the authority for NCBC), Dong Jiang (for CRIC), and Wenhua Song (for CES) granted the permission for utilization of samples from corresponding location.

Leaf samples of 29 genotypes collected from NCBC, CRIC, and CES were analyzed. Among them, 25 genotypes were corresponding to that of fruit samples. The mature spring-flush leaves on the third and forth nodes from the biological basal end were collected in July, 2010 and stored at −80°C until analysis.

The detailed information of the leaf and fruit samples was listed in Table 1.

**Standards and reagents**

Internal standards of chlorononane and methyl nonanoate were obtained from Sigma Co. Ltd (St Louis, MO, USA). A standard series of C7–C30 saturated alkanes bought from Supelco (Bellefonte, PA, USA) was used for retention index determination. Methyl tert-butyl ether (MTBE) (high performance liquid chromatography grade) from Tedd (Fairfax, USA) was applied to extracting volatiles. Anhydrous sodium sulfate was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). The sources of volatile standards are listed in Table S1.

**Extraction and volatile analysis of the peel oil**

The sample preparation and the volatile extraction by solvent MTBE were conducted according to Liu et al. [5]. Three independent biological replicates were prepared. Three grams of fruit peel were used for the volatile extraction with 15 mL MTBE. Then 869.7 μg of chlorononane and 400 μg of methyl nonanoate were added as the internal standards. After 1 h of microwave assisted extraction using an FS60 ultrasonic cleaner (Fisher Scientific, Pittsburgh, PA, USA), the organic layer was collected, dried over Na2SO4, and concentrated to a final volume of 1.4 mL under a gentle stream of nitrogen.

The extract was analyzed using a TRACE GC Ultra GC coupled to a DSQ II mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) and equipped with a TRACE TR-5 MS column (30 m × 0.25 mm × 0.25 μm, Thermo Scientific, Bellefonte, PA, USA). The parameters of gas chromatography and mass spectrometer were set according to the method described by Liu et al. [5]. Helium was used as the carrier gas, with a split ratio of 50:1, at a flow rate of 1 mL/min. The temperatures of the injection port, ion source, and MS transfer line were kept at 250, 260, and 280°C, respectively. The oven temperature program adopted the following procedure, which started from 40°C for the initial 3 min, then increased to 160°C at a rate of 5°C/min, kept at 160°C for 1 min, then followed by a ramp of 5°C/min to reach 200°C, held for 1 min, raised to 240°C at a rate of 8°C/min, and finally kept at 240°C for 3 min. The MS was collected in a positive electron ionization mode at 70 eV, obtaining spectra with a scan range from m/z 45 to 400.

The raw data obtained from GC-MS were processed with Xcalibur and AMDIS software. The volatile compounds were identified on the basis of the NIST/EPA/NIH Mass Spectral Library (NIST 2008) and Wiley Registry of Mass Spectral Data 8th edition. Retention indexes were calculated with a homologous series of n-alkanes (C7–C30) [27]. Eighty-one volatile compounds were further positively identified based on the authentic standards (listed in Table S1).

**Solid phase microextraction (SPME) analysis for leaves**

After being ground in liquid nitrogen, 2 g of the finely powdered leaf samples was transferred into a 20 mL Teflon cap vial (Thermo Fisher Scientific) with 5 mL of NaCl aqueous solution (25%, w/v) being added later. Then, the vial was sealed tightly. The extraction procedure described by Liu et al. [28] was applied with minor modifications. The sealed vial was incubated at 40°C for 30 min and the extraction of volatile compounds with a 2 cm, 50/30 μm carbobox divinylbenzene polydimethylsiloxane (CAR/DVB/PDMS) Supelco SPME fiber (Sigma-Aldrich Co. Ltd, St. Louis, MO, USA) was conducted at 40°C for 45 min by agitation at 10 sec intervals. The volatiles were desorbed from the SPME fiber at 230°C for 1 min in the injection port. After each extraction, the fiber was conditioned at 240°C for 3 min. Three replicates for each leaf sample were prepared.

**Data analysis**

For volatile compounds of the peel oils, the peak areas in the total ion current chromatogram (TIC) were processed by the software of Xcalibur. The corrected peak areas (CPAs) of target
compound were calculated based on internal standards. Chlorononane was used to calculate the CPA of \( \beta \)-myrcene and \( \alpha \)-limonene, and methyl nonanoate was used for all the other volatile compounds. At first, when each peak area of the internal standards (chlorononane and methyl nonanoate) in Kaime satsuma mandarin was set as 1 for calculating CPAs of different volatile compounds, the chromatographic peak area of each corresponding internal standard in every sample was normalized respectively. Then, every peak area of targeted volatile compounds was divided by a corresponding CPA of the internal standard in every sample. The result was named as the corrected peak area of target compound (CPA-TC), which was used for Hierarchical Cluster Analysis (HCA) and Principal Component Analysis (PCA).

For HCA, the CPA-TCs were transformed via \( \log_2 \) with the MultiExperiment Viewer (MeV) version 4.7.4 software (http://www.tm4.org, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA). The average linkage clustering was performed based on the Pearson correlation [10]. The complete dataset including all replicates was employed for HCA, whereas only the mean values of volatile compounds in each sample were used for PCA. After autoscaling pretreatment with CPA-TCs was done as van den Berg et al. [29], the functions of Precomp and Plot in R version 2.14.2 software (http://www.R-project.org, R

Table 1. Citrus genotypes used in this study.

| Abbreviation* | Common name | Scientific Name | Sampling location and time |
|---------------|-------------|----------------|---------------------------|
| Early         | early-flowering type trifoliate | Poncirus trifoliata L. Raf. | NCBC *, 2009 |
| Trifoliolate  | Trifoliolate orange | Poncirus trifoliata L. Raf. | NCBC, 2009 |
| Kumquat-HZAU  | Hongkong kumquat | Fortunella hindsi Swingle | NCBC, 2009 |
| Kumquat-CRIC  | Hongkong kumquat | Fortunella hindsi Swingle | CRIC *, 2010 |
| Ningbo        | Ningbo Meiwa Kumquat | Fortunella crossifolia Swingle | NCBC, 2010 |
| Calamondin    | Calamondin | C. madurensis Lour. | NCBC, 2009 |
| Sanshan Xiangyuan | Sansshan | C. wilsonii Tanaka L. | CRIC, 2010 |
| Yuzu          | Yuzu | C. junos Sieb. | CRIC, 2010 |
| FC            | Finger citron | C. medica L. | NCBC, 2010 |
| Eureka        | Eureka lemon | C. limon (L.) Burm. f. | NCBC, 2009 |
| Limonera      | Limonera lemon | C. limon (L.) Burm. f. | CRIC, 2010 |
| Rough         | Rough lemon | C. jambhiri (L.) Lush | NCBC, 2009 |
| Lime          | Lime | C. aurantifolia Swingle. | NCBC, 2009 |
| Ichang        | Ichang papeda | C. ichangensis Swing. | NCBC, 2010 |
| HP            | Honghe papeda | C. honghensis, Y. L. D. L | CRIC, 2010 |
| Liang         | Liangping pummelo | C. grandis Osbeck | CRIC, 2010 |
| Kaopan        | Kaopan pummelo | C. grandis Osbeck | NCBC, 2010 |
| Shatian       | Shatian pummelo | C. grandis Osbeck | CES*, 2009 |
| Tachibana     | Tachibana orange | C. tachibana Makino | CRIC, 2010 |
| Mang-HZAU-09  | Mangshanyegyan (sharp leaf) | C. nobilis Lauriuro | NCBC, 2009 |
| Mang-HZAU     | Mangshanyegyan (sharp leaf) | C. nobilis Lauriuro | NCBC, 2010 |
| Mang-SL-CRIC  | Mangshanyegyan (sharp leaf) | C. nobilis Lauriuro | CRIC, 2010 |
| Mang-RL-CRIC  | Mangshanyegyan (round leaf) | C. nobilis Lauriuro | CRIC, 2010 |
| Kaimé         | Kaimé satsuma mandarin | C. unshiu Marcow | NCBC, 2010 |
| Dao           | Daoxian wild mandarin | C. reticulata Blanco | NCBC, 2010 |
| Jiang         | Jiangyong wild mandarin | C. reticulata Blanco | NCBC, 2010 |
| Cha           | Chazhi gan mandarin | C. reticulata Blanco | CRIC, 2010 |
| Ponkan        | Ponkan mandarin | C. reticulata Blanco | NCBC, 2010 |
| Huangyan      | Huangyanbendizao tangerine | C. reticulata Blanco | NCBC, 2009 |
| Hua           | Huanongbendizao tangerine | C. reticulata Blanco | NCBC, 2009 |
| Nanfeng       | Nanfungmiu mandarin | C. reticulata Blanco | NCBC, 2009 |
| Clementine    | Clementine tangerine | C. reticulata Blanco | NCBC, 2009 |
| Mang-T        | Mangshan wild tangerine | C. reticulata Blanco | NCBC, 2010 |
| Seike         | Seike navel orange | C. sinensis Osbeck | NCBC, 2010 |

*Collected from National Citrus Breeding Centre of China (NCBC).
*Collected from Citrus Research Institute of Chinese Academy of Agricultural Sciences (CRIC).
*Collected from Citrus Experimental Station (CES), Zigi county, Hubei Province, China.
*The samples in bold were collected only for peel oils analysis, while the samples in italic were collected only for leaf volatile analysis, and the samples in normal font were for both analyses. The abbreviations of fruit samples belonging to Sinocitrus were marked with underline.

doi:10.1371/journal.pone.0058411.t001
Development Core Team) were employed for PCA. The raw dataset of leaf volatile profiles was preprocessed according to non-targeted method with Metalign software Package (version 200410, http://www.metalign.nl, Plant Research International, Wageningen, The Netherlands) referring to Lommen [9] and Tikunov et al. [10], and then the preprocessed result (Table S2) was subjected to HCA using MeV based on Cosine correlation and single linkage method.

**Results**

**Volatile compounds detected in peel oils**

In this study, a total amount of 160 volatile compounds were detected in the peel oils (Table S1), among which 81 were definitely identified, 64 tentatively identified, and 15 unidentified. The above 145 identified compounds were grouped into the following 19 classes: acids (3 compounds), alcohols (9), aldehydes (11), alkane (1), diterpene (1), esters (6), furans (2), monoterpenes (16), monoterpenic alcohols (15), monoterpenic aldehydes (4), monoterpenic esters (3), monoterpenic ketones (4), monoterpenic oxides (4), sesquiterpenes (36), sesquiterpenic alcohols (11), sesquiterpenic aldehydes (2), sesquiterpenic ketone (1), sesquiterpenic oxide (1), benzene compounds (10).

In this study, five novel compounds, (E)-3-carene-2-ol, x-copaene-11-ol, (Z,E)-9,9-dimethyl-9,12-cyclodeca-1,5-diene were tentatively identified in citrus fruit for the first time. For Mangshanyegan, x-copaene-11-ol was found in peel oil of both genotypes, while 8-isopropenyl-1,5-dimethyl-cycloelca-1,5-diene was detected only in the peel oil of sharp-leaf genotype (Table S1).

**HCA results of peel oils**

In this study, the mass spectral data of volatile profiles supplied abundant information to the chemotaxonomy study of Mangshanyegan. HCA was conducted throughout 90 data sets of peel oils from all 30 samples, including the sharp-leaf and round-leaf Mangshanyegan collected from CRIC in 2010 and the sharp-leaf collected from NCBC in 2009 and 2010.

The HCA results indicated that these 30 genotypes could be clustered into 6 groups: Group 1: one Citrus nobilis and two Fortunella hindsii; Group 2: two Poncirus trifoliata; Group 3: four Citrus reticulata; Group 4: two Citrus limon, one C. jambhiri, one C. medica, and one C. aurantiifolia; Group 5: three C. grandis and one C. tachibana; Group 6: one C. madurensis, one C. junos, one C. unshiu, one C. sinensis, and eight C. reticulata (Figure 1A).

The genotypes in Group 3, 4, 5, and 6 all belonged to Citrus genus, and the samples of the other two genera in this study were found in Group 1 and 2, respectively. Group 4, 5, and 6 represented Citrophorum (citron), Cephalocitrus (pummelo), and Sinocitrus (mandarin), respectively. This result confirmed the hypothesis that C. grandis (pummelo), C. medica (citron), and C. reticulata (mandarin) were the three basic species of the cultivated Citrus [30]. Each group could be recognized as a citrus true species together with its hybrids or descendants except C. tachibana Makino, a wild species from Japan, which was located in the group of pummelo instead of mandarin, and postulated as an individual species in Swingle classification system [31].

Three genera used in this study were almost separated, which was in accordance with previous studies [30,32], except for Group 1 which included two Fortunella hindsii genotypes and Ichang papeda that belonged to Citrus genus. Also, it is worthy to note that the taxonomy of Ichang papeda was still controversial (this will be discussed later). This cluster dendrogram showed that Citrus and its related genera fell into different groups and the group of Fortunella hindsii Swingle located the most distantly. Mangshanyegan fell into a different group from the other three recognized groups of true basic species of the cultivated Citrus, and these four groups were sharply distinguished from each other (Figure 1A).

In the group of Mangshanyegan, the sharp-leaf ones collected from NCBC and CRIC in 2010 were grouped together, which neighbored with the sharp-leaf one from NCBC in 2009, whereas the round-leaf Mangshanyegan harvested from CRIC in 2010 was distant from the three sharp-leaf ones (Group 3 in Figure 1A). The results obviously indicated that the geographical and temporal influences on HCA were less than that of the genetic factor, which agreed with Merle et al. [33] and suggested the experimental reliability of the HCA result.

In the analysis of HCA, four samples of Mangshanyegan were used (see Table 1), and two accessions of Hongkong kunequats (one from NCBC and the other from CRIC) were investigated. However, only one accession of other citrus and its relatives was utilized. Accordingly, the differences of the sample numbers may cause a weighted bias among different genotypes, and the independent group of Mangshanyegan might result from this bias in sampling, which blurred its genetic divergence among samples.

To minimize the bias due to samples size and maximize the sample size, the sharp-leaf and round-leaf Mangshanyegan collected from CRIC in 2010 were used for HCA. Thus, although only five groups were obtained, the cluster result was almost identical to that with only either one of them included, and was different from that with four (Figure 1A) or three Mangshanyegan samples (data not shown). It was shown that Mangshanyegan was merged with the pummelos, forming the new Group 3 in Figure 1B, in which Mangshanyegan neighbored with Tachibana. Notably, the other four groups were hardly changed between Figure 1A and Figure 1B.

**PCA results of peel oils**

PCA was applied to test the HCA results. The samples in each group in Figure 1B were colored individually (Figure 2A and Figure 2B). The first component explained 13.47% of the variance, and Citrus and Poncirus were clearly separated on the PC1 axis; the second component explained 13.84% of the variance, and all three genera used in the study were separated very clearly on the PC2 axis. Although the first two principal components explained only about 29% of the variance, three genera used in the study were distinguished from each other. Dots representing genotypes of Poncirus and Citrus were compact, whereas the two samples of Fortunella were scattered (Figure 2B). In general, the results of PCA were almost consistent with the results of HCA (Figure 2B).

**HCA results of leaf volatile profiles**

To verify the above results of HCA and PCA, the volatile compounds of leaf samples collected in 2010 were extracted by SPME and further analyzed with the assistance of MetaAlign software. The aligned data obtained from MetaAlign (Table S2) was subjected to MeV for HCA. The HCA results of leaf volatile profiles were almost in line with the results of HCA and PCA based on volatile compounds of peel oils. C. tachibana was clustered into the branch of mandarin, and Mangshanyegan was grouped with three pummelos (Figure 3).

Notably, Honghe papeda was clustered into the group of Citrus genus (Figure 3). Whereas, HCA of volatile compounds in peel oil demonstrated that the Ichang papeda fell into the group of Fortunella genus (Figure 1A and 1B).
Discussion

The origin of Mangshanyegan

Previous study speculated that Mangshanyegan (Citrus nobilis Laurio) belongs to the King mandarin which might be a natural tangor (C. reticulata × C. sinensis) [32], whereas C. sinensis originated from the introgression of C. reticulata genotype with C. grandis [15,34]. In the present study, Figure 1B and Figure 3 showed that Mangshanyegan belonged to the group of pummelo instead of mandarin and neighbored with Tachibana (Figure 1B) because the volatile profile of Mangshanyegan peel oil was similar to those of Tachibana (wild mandarin) and pummelo, indicating that Mangshanyegan is ancient and not pure mandarin genetic background. This is further supported by previous study as well. Li et al. [25] suggested that among 19 wild mandarin accessions and 33 loose-skin mandarin landraces, Mangshanyegan formed an individual group in the dendrograms constructed using nuclear simple sequence repeat (nSSR) and chloroplast simple sequence repeat (cpSSR) markers systems. In the nSSR tree, the Mangshanyegan group was the most distant one and very close to a mandarin landraces group (including Kuigan, Choupigan etc.), which was possible hybrids of mandarin with pummelo or with sweet orange [35]. And similar results were obtained except that the Kuigan group was the most distant group instead of Mangshanyegan group which was the second most distant group at the cpSSR loci [25].

However, it was reported that in the natural habitat of Mangshanyegan, there was neither any orange nor any pummelo found [36,37]. Furthermore, it was reported that Mangshanyegan was a more primitive species than Mangshanyeju (C. reticulata blanco), and even might be the ancestor of Tachibana [36,38]. Li and Liu [39] suggested that Mangshanyegan might be the intermediate type between the Ichang papeda and loose skinned mandarin. However, the above mentioned previous studies on Mangshanyegan were mainly based on morphology analysis and zymological analysis. The contradictions mentioned above could not be completely resolved in this study. An accurate conclusion will likely require genome resequencing.

In Figure 1A, Tachibana was clustered into the group of pummelo (Cluster 5) rather than the group of mandarin. In the Swingle’s classification system, all mandarins and tangerines belonged to C. reticulata Blanco except Tachibana mandarin (C. tachibana Makino) and Indian wild orange (C. indica Tanakain). Based on the analyses of isozyme, chromosome, chloroplast DNA, and mitochondrial DNA, it was suggested that Tachibana was different from the mandarins originating from China and India, and it was separated from other mandarins at an early date [34,40,41]. According to our study, it could be concluded that C. tachibana Makino was distinct from other species of Citrus genera.
However, previous reports never demonstrated that Tachibana was clustered with pummelo based on molecular markers [34,42–44]. Thus, it was deduced that the possible reason for Tachibana falling into the cluster of pummelo in Figure 1A might be the uneven bias caused by the overwhelming sample number of Mangshanyegan, which emphasized the importance of population balance as the premise of chemotaxonomy. Possibly, owing to the much more similar volatile profile of peel oils between Tachibana and Mangshanyegan than that between Tachibana and other mandarins, Tachibana neighbored with Mangshanyegan in Figure 1B.

Phylogenetic classification of other citrus

In Figure 1A and Figure 1B, the cluster dendrogram trees clearly showed that the cluster of *Fortunella hindsii* Swingle located most distantly. Previous studies suggested that *Fortunella* was the most primitive whereas *Citrus* was the most advanced genus among the “True Citrus Fruit Trees” [45,46]. Based on the 11000 unigenes from a Clementine EST library, it was found that *Poncirus trifoliata* located in a cluster of citron-limes-lemon, whereas kumquat (*Fortunella japonica*) remained genetically distant to other citrus [47]. However, according to Barkley et al. [48], the group of *Poncirus* accessions was very distant from the four other citrus groups of mandarins, pummelos, citrons, and papedas, while kumquat was closer to the four citrus groups on the basis of genomic SSR. In this study, it should be noted that the volatile profiles of Kumquat was dominated by $\beta$-myrcene instead of $d$-limonene. The mean CPA-TC ratio between $\beta$-myrcene and $d$-limonene in Hongkong kumquat from NCBC and CRIC was about 192 and 206, respectively. Thus, the ratio was inferred as the predominant differences that caused the largest distance among cluster of Kumquat and other clusters in the HCA, as shown in Figure 1.

The taxonomy of *C. ichangensis* is controversial. With its flower resembling that of *Citrus* and its leaf resembling that of *Papeda*, *C. ichangensis* was grouped into the Papeda subgenus in Swingle’s system [32]. However, in the Tanaka’s system, it was classified into the subgenus Metacitrus [49]. The result of this study was in accordance with that of Handa et al. [50] and Nicolosi et al. [34]: *C. ichangensis*, which was distinct from the other samples of *Citrus* genus, located in the cluster of *Fortunella*.

By combining the previous morphological and biochemical criteria with molecular marker (RAPD, RFLP, and SCARs) analyses, Biswas et al. [51] supposed that Papeda was different from other *Citrus* species. Furthermore, Biswas et al. [52] found that Ichang papeda fell into the kumquat sub-cluster with 25 randomly selected SSR primer pairs among 40 species of *Citrus* and its related genera. In this study, Honghe papeda was close to *Citrus* genus while Ichang papeda was close to *Fortunella* genus. However, only the fruit peel of *C. ichangensis* and leaf of Honghe papeda were sampled, respectively. Therefore it needs more study in the same sample set.

In Figure 1A and Figure 1B, Chazhigan mandarin fell into the cluster of mandarin, while in Figure 3, Chazhigan mandarin was more distant from the other clusters of *Citrus* genus than the cluster of *Poncirus*. Volatile profile of Chazhigan mandarin revealed that the most abundant and predominant compound of its leaf was dimethyl anthranilate rather than $d$-limonene (Figure S1), which might be one of the reasons for its divergence from other genotypes in the *Citrus* genus in Figure 3.
Validity of chemotaxonomic analysis for interspecies phylogenetic studies

The cluster results in this study agreed with the Swingle classification system very well. Luo et al. [47] also obtained a good agreement of diversity relationships with the established taxonomy and phylogeny among the species of citrus and its related genera based on EST-SSR markers. In addition, with 24 citron varieties employed in their study, Luo et al. [21] suggested that the diversity estimated by leaf oil composition was unsuitable for intraspecific phylogenetic studies. Here, ripe fruits of 30 accessions and 29 leaf samples belonging to three genera of *Citrus*, *Poncirus*, and *Fortunella* and 18 species in total were collected in this study, and it could be deduced that chemotaxonomic analysis based on volatile compounds in both fruit peel and leaf is suitable for interspecies phylogenetic studies.

The loss of flavor traits in citrus cultivars compared with wild genotypes

Liu et al. [5] found that β-myrcene and (Z)- and (E)-linalool oxides were the characteristic aroma compounds of Mangshanye- gan and (Z)- and (E)-linalool oxides had a flower, woody, green, linalool-like note [5]. In this study, it was very interesting that among 30 investigated fruit samples, (Z)- and (E)-linalool oxides were only identified in Daoxian wild mandarin, Jiangyong wild mandarin, Mangshyengan (sharp-leaf & round-leaf), Clementine tangerine and Liangping pummelo. Also, (Z)- and (E)-linalool oxides were at trace levels in both Clementine tangerine and Liangping pummelo. Among the 14 fruit samples of Sinocitrus samples (with underline in Table 1), Daoxian wild mandarin, Jiangyong wild mandarin, Mangshyengan (sharp-leaf & round-leaf) were wild genotypes and the others were all commercial cultivars. It was demonstrated that (Z)- and (E)-linalool oxides were not detected in the cultivated loose-skin mandarins except Clementine (trace level). The loss of flavor traits in cultivars might be attributed to breeding and selection that favors yield, disease resistance, and pleasant fruit appearance and in which flavor and aroma have been ignored for a long period of time. As a result, some superior flavor traits of wild genotypes were gradually lost.

Supporting Information

**Figure S1** Total ion current chromatograms (TIC) (**A, B, C** and **D, E**) and mass spectra (**D, E**). (**A**) The global TIC of Chazhigan mandarin. (**B**) The TIC of dimethyl anthranilate (a part of A). (**C**) The TIC of the authentic standard of dimethyl anthranilate. (**D**) Mass spectrum of dimethyl anthranilate in B. (**E**) Mass spectrum of dimethyl anthranilate in C. time, retention time; m/z, mass-to-charge ratio. (TIF)

**Table S1** The corrected peak areas of target volatile compounds detected in fruit peels of whole sample set. (XLS)

**Table S2** The aligned data obtained from Met-align. (XLS)

Acknowledgments

We thank Dr. Shaping Zhu (Citrus Research Institute of Chinese Academy of Agricultural Sciences, Chongqing) for providing fruit and leaf samples for courtesy.

**Author Contributions**

Conceived and designed the experiments: JX XD FC. Performed the experiments: CL LF. Analyzed the data: CL YC. Contributed reagents/materials/analysis tools: DJ ZM. Wrote the paper: CL.

References

1. Fowler C, Hodgkin T (2004) Plant genetic resources for food and agriculture: assessing global availability. Anna Rev Environ Resour 29: 143–179.

2. Deng XX, Hu CG, Hsu H, Gou WW, Yi H (2000) A preliminary study of citrus germplasm conservation and its evaluation by RAPD analysis. Acta Hortic (ISHS) 535: 99–106. Available: http://www.actahort.org/books/535/535_10.htm. Accessed 2013 Feb 19.

3. Schmidt MR, Wei W (2006) Loss of agrobiodiversity, uncertainty, and perceived control: a comparative risk perception study in Austria and China. Risk Anal 26: 455–470.

4. Li RT (2008) Investigation on botanical characters of wild mandarins from Human Province. Human Agric Sci 5: 30–51. (in Chinese)

5. Liu C, Cheng Y, Zhang H, Deng X, Chen F, et al. (2012) Volatile constituents of wild citrus Mangshyengan (*Citrus nobilis* Lourio) peel oil. J Agric Food Chem 60: 2617–2628.

6. Katajamaa M, Oresic M (2005) Processing methods for differential analysis of LC/MS profile data. BMC Bioinf 6: 179.

7. Baran R, Kochi H, Saito N, Suematsu M, Soga T, et al. (2006) MathDAMP: a package for differential analysis of metabolic profiles. BMC Bioinf 7: 530.

8. Luedemann A, Strassburg K, Erban A, Kopka J (2008) TagFinder for the LC/MS profile data. BMC Bioinf 6: 179.

9. Lommen A (2009) MetAlign: interface-driven, versatile metabolomics tool for hyphenated full-scan mass chromatometry data preprocessing. Anal Chem 81: 3079–3086.

10. Tims T, Lommen A, de Vos CH, Veeken HA, Bissel RJ, et al. (2005) A novel approach for non-targeted data analysis for metabolomics. Large-scale profiling of tomato fruit volatiles. Plant Physiol 139: 1125–1137.

11. Skogerson K, Wohlgemuth G, Barupal DK, Fieth N (2011) The volatile compound BinBase mass spectral database. BMC Bioinf 12: 321.

12. Aliferis KA, Cubeta MA, Jabaji S (2011) Chemotaxonomy of fungi in the *Rhizoctonia solani* species complex performing GC/MS metabolite profiling. Metabolomics. Available: http://dx.doi.org/10.1007/s11306-011-0380-1. Accessed 2013 Feb 19.

13. Fraga BM (2012) Phytochemistry and chemotaxonomy of *Sideritis* species from the Mediterranean region. Phytochemistry 76: 7–24.

14. Radulovic N, Dekic M, Jokovic M, Vukicevic R (2012) Chemotaxonomy of Serbian *Tavulium* species inferred from essential oil chemical composition: the case of *Tavulium aureus* L. sp. *aurantioides*. Chem Biodivers 9: 106–122.

15. Moore GA (2001) Oranges and lemons: clues to the taxonomy of *Citrus* from molecular markers. Trends Genet 17: 536–540.

16. Kesterson JW, Pieringer AP, Edwards GJ, Hendrickson R (1964) Application of gas-liquid chromatography to the citrus leaf oils for the identification of kinds of citrus. Proc Am Soc Hortic Sci 84: 199–203.

17. Pieringer AP, Edwards GJ, Woford RW (1964) The identification of citrus species and varieties by instrumental analysis of citrus leaf oils. Proc Am Soc Hortic Sci 84: 204–212.

18. Merle H, Moron M, Blazquez MA, Boira H (2004) Taxonomical contribution of essential oils in mandarins cultivars. Biochem Syst Ecol 32: 491–497.

19. Lin Z, Hua Y (1992) Systematic evolution relation of chemical components of the essential oils from 11 taxa of citrus leaves. Acta Bot Sin 34: 135–139.

20. Gonzalez-Mas MC, Rambla JL, Alamar MC, Gutierrez A, Granell A (2011) Comparative analysis of the volatile fraction of fruit juice from different citrus species. PLoS One 6: e22016.

21. Luo F, Venturini N, Costantini G, Paolini J, Ollitrault P, et al. (2012) Genetic and chemical diversity of citrus (*Citrus medica* L.) based on nuclear and cytological markers and leaf essential oil composition. Phytochemistry 77: 186–196.

22. Wayman KA, de Lange PJ, Larsen L, Sansom CE, Perry NB (2010) Chemotaxonomy of Persicoverna: sesquiterpene dialdehyde variants are species markers. Phytochemistry 71: 766–772.

23. Hou Y, Liang W, Zhang L, Cheng S, He F, et al. (2011) Freshwater algae chemotaxonomy by high-performance liquid chromatographic (HPLC) analysis. Front Environ Sci Eng China 5: 84–91.

24. Li R, Luo G, Meyers PA, Gu Y, Wang H, et al. (2012) Leaf wax n-alkane chemospecies of bamboo from a tropical rain forest in Southwest China. Plant Syst Evol 298: 731–738.

25. Li YZ, Cheng YJ, Yi HL, Deng XX (2006) Genetic diversity in mandarin landraces and wild mandarins from China based on nuclear and chloroplast species markers. Phytochemistry 71: 766–772.

26. Motamayor JC, Lachenaud P, da Silva EMJW, Loor R, Kuhn DN, et al. (2008) Geographical and genetic population differentiation of the Amazonian chocolate tree (*Theobroma cacao* L.). PLoS One 3: e5311.
27. Vandendoel H, Kratz PD (1963) A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. J Chromatogr A 11: 463–471.
28. Liu C, Zhang H, Dai Z, Liu X, Liu Y, et al. (2012) Volatile chemical and carotenoid profiles in watermelons [Citrullus vulgaris (Thunb.) Schrad. (Cucurbitaceae)] with different flesh colors. Food Sci Biotechnol 21: 531–541.
29. van den Berg RA, Hoefsloot HC, Westerhuis JA, Smilde AK, van der Werf MJ (2006) Centering, scaling, and transformations: improving the biological information content of metabolomics data. BMC Genomics 7: 142.
30. Scora RW (1975) On the history and original of citrus. Bull Torrey Bot Club 102: 369–375.
31. Tanaka T (1954) Species problem in Citrus: A critical study of wild and cultivated unites of citrus, based upon field studies in their native homes (Revisio Aurantiacearum IX). Tokyo: Japanese Society for the Promotion of Science. pp. 1–141.
32. Swingle WT (1943) The botany of citrus and its wild relatives of the orange subfamily (family Rutaceae, subfamily Aurantioideae). In: Webber HJ, Batchelor LD, editors. The Citrus Industry. Berkeley: University of California Press. pp. 128–474.
33. Merle H, Moron M, Blazquez MA, Boira H (2004) Taxonomical contribution of essential oils in mandarins cultivars. Biochem Syst Ecol 32: 493–497.
34. Nicolosi E, Deng ZN, Gentile A, Malfa SL, Tribulato GC (2000) Citrus phylogeny and genetic origin of important species as investigated by molecular markers. Theor Appl Genet 100: 1155–1166.
35. Cheng YJ, de Carmen VM, Meng HJ, Guo WW, Tao NG, et al. (2005) A set of primers to analyze the chloroplast DNA diversity in Citrus and its relatives. Tree Physiol 25: 661–672.
36. Li RT, Zhang YN, Chen ML, Jiang KJ (2009) Taxononomy analysis on wild mandarins originating from Mangshan Mountain. Guangdong Agric Sci 8: 11–13. (in Chinese)
37. Chen ML, Wei WN (1987) Studies on genetic relationship among wild loose skin mandarins originated from Hunan Province. Proceedings of the national conference of fruit crop resources. (Held in Chongqing, China, 1987, in Chinese)
38. Li WB, Liu GF, He SW, Zhang YN (1993) Studies on the origin and evolutionary of Chinese loose skin mandarins based on isozyme analysis. Hunan Agric Sci 3: 5–6. (in Chinese)