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Rapid Differentiation of Three *Chamaecyparis* Species (Cupressaceae) Grown in Taiwan Using Solid-Phase Microextraction−Gas Chromatography/Mass Spectrometry, Cluster Analysis, and Principal Component Analysis

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ABSTRACT: Three *Chamaecyparis* species (*C. formosensis*, *C. obtusa* , and *C. obtusa* var. *formosana*) are difficult to distinguish by the naked eye. Therefore, from the chemotaxonomic point of view, it would be valuable to find a simple and rapid method to differentiate these three *Chamaecyparis* species. In this study, the chemical compositions of biogenic volatile organic compounds (BVOCs) from mature leaves were analyzed using solid-phase microextraction−gas chromatography/mass spectrometry (SPME−GC/MS). Then cluster analysis (CA) and principal component analysis (PCA) were conducted for the BVOC constituents to reveal the differences among these three species. Results from SPME−GC/MS showed that the compositions of BVOCs from the three species were distinctly different. Moreover, these species were clearly differentiated according to the results of CA and PCA. In conclusion, the findings of this study suggest that SPME−GC/MS coupled with CA and PCA is a feasible and rapid technique to differentiate *Chamaecyparis* species with similar morphological characteristics.

KEYWORDS: BVOCs, *Chamaecyparis* species, cluster analysis, principal component analysis, SPME−GC/MS

INTRODUCTION

The *Chamaecyparis* genus belonging to the Cupressaceae family is characterized by its horticultural value. This genus includes five species, *C. formosensis*, *C. lawsoniana*, *C. obtusa*, *C. pisifera*, and *C. thyoides*, and one variety (*formosana*) of *C. obtusa*.[1] A number of studies have reported that the *Chamaecyparis* genus has different types of bioactivity, including antimicrobial, anti-insect, and anticancer.[2−4] Apart from the two endemic species, *C. formosensis* and *C. obtusa* var. *formosana*, which are abundant in Taiwan, *C. obtusa*, which is native to Japan, is also found. These three species are often difficult to distinguish by the naked eye, which can lead to problems in application and plant breeding.

There are many methods to differentiate plants, which are generally classified on the basis of their characteristic properties, i.e., taxonomy. In addition, plants can be classified on the basis of differences in their genes or chemical composition.[5,6] To effectively organize and explain the results from genetic or chemical composition, multivariate data analyses (e.g., cluster analysis (CA) and principal component analysis (PCA)) provide more objective interpretations.[7] Researchers have used CA or PCA to study the variability or chemotaxonomy of plants by analyzing the chemical composition of their essential oils.[8,9] Furthermore, Wang et al.[10] characterized five precious woods found in Taiwan using solid-phase microextraction−gas chromatography/mass spectrometry (SPME−GC/MS) to analyze their fragrance compounds, followed by CA and PCA. The SPME technique has been widely utilized in many fields, since it saves time and requires relatively few samples. As mentioned above, SPME−GC/MS is a powerful and rapid technique for chemotaxonomy.

Recently, Chen et al.[11] have studied the phylogenetic relationships of the *Chamaecyparis* genus by analyzing the chemical composition of their leaf essential oils. However, the extraction procedure of essential oil takes a long time and a very large amount of samples for the yield of essential oil to be sufficient for chemical analysis. Hence, it is imperative to find a quick, simple, and solvent-free method for the differentiation of *Chamaecyparis* species. Accordingly, this study analyzes the leaf volatile compounds of three *Chamaecyparis* species in Taiwan using SPME−GC/MS and then differentiate these three species by CA and PCA.

MATERIALS AND METHODS

Plant Materials. For this study, we obtained fresh mature leaves of three species of *Chamaecyparis* (*C. formosensis*, *C. obtusa* var. *formosana*, and *C. obtusa*) from different locations in Taiwan in January 2010. Leaves from each location were collected from three healthy trees. *C. formosensis* leaves were collected from the Xitou Tract (tree no. CF01-03), Huisun Experimental Forestry (tree no. CF05-07), Hsin-sheng Nursery (tree no. CF08-10), and Neimaopu Tract (tree no. CF11-13), while *C. obtusa* var. *formosana* leaves were collected from Mt. Chilan (tree no. COF01-03), the Alishan Working Circle (tree no. COF04-06), Taichung (tree no. COF10-12), and Chutung (tree no. COF13-15). *C. obtusa* leaves were collected from the Huisun Experimental Forestry (tree no. CO01-03) and Neimaopu Tract (tree no. CO04-06).

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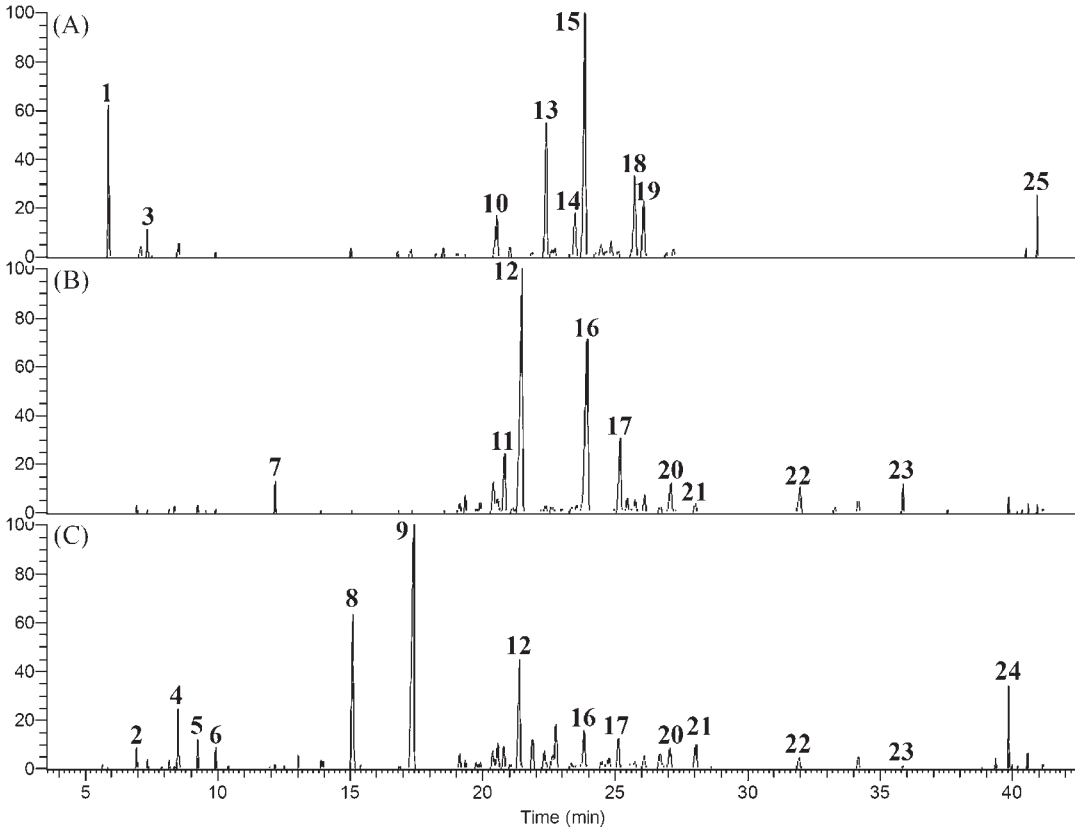

Figure 1. SPME−GC/MS chromatogram of volatile compounds of leaves from *Chamaecyparis* species: (A) *C. formosensis*; (B) *C. obtusa* var. *formosana*; (C) *C. obtusa*. Key: 1, α-pinene; 2, β-pinene; 3, β-myrcene; 4, limonene; 5, γ-terpinene; 6, terpinolene; 7, terpinen-4-ol; 8, L-bornyl acetate; 9, α-terpinyl acetate; 10, β-caryophyllene; 11, β-cedrene; 12, thujopsene; 13, α-humulene; 14, γ-muurolene; 15, germacrene D; 16, compound A; 17, β-himachalene; 18, γ-cadinene; 19, δ-cadinene; 20, γ-cuprenene; 21, β-elemol; 22, cedrol; 23, *cis*-thujopsenal; 24, cembrene; 25, *ent*-16-kaurene.

All sampled trees were over 30 years old and were identified by Yen-Ray Hsui (Chungpu Research Center, Taiwan Forestry Research Institute). Voucher specimens were deposited at the Laboratory of Wood Chemistry (School of Forestry and Resource Conservation, National Taiwan University), and fresh leaves were kept frozen at −80 °C until analysis.

Extraction of Biogenic Volatile Organic Compounds from Leaves. The manual SPME device and fiber were purchased from Supelco Co. (Bellefonte, PA). Then 65 μm poly(dimethylsiloxane)−divinylbenzene (PDMS/DVB) fiber was used to extract the volatile compounds of leaves from *Chamaecyparis* species. The PDMS/DVB fiber was conditioned as recommended by the manufacturer prior to the extraction.

The SPME extraction procedure was based on that of Chen et al.,[12] with slight modifications. Fresh leaves were clipped into small pieces, and 150 mg samples were placed in 20 mL vials closed by poly(tetrafluoroethylene) (PTFE)/silicone septa and then heated for 5 min in a 50 °C water bath. The adsorption time of each extraction was 15 min at 50 °C, and each sample was desorbed at a GC inlet for 5 min at 250 °C.

Qualitative Analysis of Biogenic Volatile Organic Compounds. Qualitative analyses used both GC with flame ionization detection (GC-FID) and GC/MS. GC-FID analysis was carried out using a Trace GC Ultra (Thermo Scientific, Waltham, MA) with an FID (Thermo Scientific) equipped with a 30 m × 0.25 mm × 0.25 μm DB-5ms column (J&W Scientific, Folsom, CA). The temperatures of the injection port and the detector were 250 and 270 °C, respectively. Samples were desorbed in the split mode (split ratio 10:1). The oven temperature program was from 60 to 80 °C at 3 °C/min then heating at 8 °C/min to 120 °C, subsequent heating at 1 °C/min to 140 °C, and finally heating at 10 °C/min to 220 °C. Helium was used as the carrier gas at a flow rate of 1 mL/min. GC/MS analysis also used a Trace GC Ultra with a PoLaris Q MSD detector (Thermo Scientific). The parameters of the column, temperatures of the injection port and the detector, split ratio, oven temperature program, and carrier gas and its flow rate of GC/MS were identical to those of GC-FID. The temperatures of the transfer line and the ion source were 250 and 230 °C, respectively. Electron impact mass spectra were acquired over the mass range of 50−400 amu at an ionization energy of 70 eV. The Kovats indices (KIs)[13] were calculated for all volatile compounds using a homologous series of *n*-alkanes (C_9−C_{21}) on the DB-5ms column. Identification of individual components was done using the Wiley/NBS Registry of Mass Spectral Database (version 7) and NIST MS Search (version 2), published literature, and several authentic reference compounds.

Statistical Analysis. The method of Chen et al.[11] was employed to evaluate the similarity of volatile compounds extracted from fresh leaves. CA and PCA were performed with the MVSP software (Multivariate Statistical Package for Windows, version 3.1, Kovach Computing Services, Anglesey, U.K.).

RESULTS AND DISSCUSION

Biogenic Volatile Organic Compounds Analysis. To investigate the biogenic volatile organic compounds (BVOCs) emitted from three *Chamaecyparis* species (*C. formosensis*, *C. obtusa* var. *formosana*, and *C. obtusa*), we used HS-SPME to extract the volatile compounds emitted from fresh leaves and then analyzed the composition of BVOCs using GC-FID and GC/MS. Results from SPME−GC/MS chromatograms (Figure 1) showed that

Table 1. Constituents and Compositions of BVOCs of Leaves from Three *Chamaecyparis* Species[a]

KI[b]	rKI[c]	compound	CF 01-03	CF 05-07	CF 08-10	CF 11-13	COF 01-03	COF 04-06	COF 10-12	COF 13-15	CO 01-03	CO 04-06
936	939	α-pinene	13.33	8.87	8.07	8.89	–[d]	–	–	–	–	–
977	979	β-pinene	–	–	–	–	3.85	3.08	–	1.17	4.98	1.18
981	978	(3*Z*)-octen-2-ol	1.46	–	–	–	–	–	–	–	–	nd
991	991	β-myrcene	1.73	1.14	1.32	1.48	1.36	–	–	–	1.58	–
1013	1011	δ-3-carene	nd[e]	1.46	–	1.29	nd	nd	–	nd	nd	nd
1022	1017	limonene	–	–	–	–	–	–	–	–	4.78	3.34
1064	1060	γ-terpinene	nd	nd	nd	nd	–	–	–	–	2.68	1.27
1088	1089	terpinolene	–	–	–	–	–	–	–	–	1.39	–
1284	1288	L-bornyl acetate	–	–	–	–	–	–	–	–	13.28	11.31
1345	1351	α-cubebene	–	–	–	1.07	–	–	–	–	nd	nd
1349	1348	α-terpinyl acetate	nd	nd	nd	nd	nd	nd	nd	nd	21.70	25.79
1388	1388	β-cubebene	nd	nd	nd	nd	–	–	1.25	1.76	nd	nd
1392	1390	isolongifolene	nd	nd	nd	nd	1.37	1.92	1.06	–	–	–
1414	1411	α-cedrene	nd	nd	nd	nd	4.96	5.74	2.98	2.81	nd	nd
1417	1419	(*E*)-β-caryophyllene	3.19	2.38	2.74	2.69	1.75	1.74	1.87	2.31	nd	1.57
1422	1420	β-cedrene	nd	nd	nd	nd	5.65	7.20	4.07	3.37	1.41	1.61
1427	1432	β-copaene	–	–	–	–	–	–	–	1.01	nd	nd
1433	1431	thujopsene	nd	nd	nd	nd	23.49	17.14	22.37	21.38	6.00	9.25
1445	1441	aromadendrene	nd	nd	nd	nd	nd	nd	nd	nd	–	2.24
1450	1454	α-humulene	8.66	4.38	5.35	5.91	–	–	–	–	nd	nd
1459	1463	*cis*-cadina-1,4-diene	nd	nd	nd	nd	nd	nd	nd	nd	1.18	1.09
1470	1479	γ-muurolene	5.82	7.94	9.09	6.85	–	1.07	–	–	–	–
1474	1485	germacrene D	40.06	41.20	35.10	37.81	nd	nd	nd	nd	nd	nd
1476		compound A	nd	nd	nd	nd	9.71	16.47	21.88	19.02	2.64	3.52
1486	1495	γ-amorphene	1.30	1.87	2.13	2.01	nd	nd	nd	nd	nd	nd
1493	1500	α-muurolene	1.34	1.47	2.25	2.20	nd	nd	nd	nd	nd	nd
1497	1500	β-himachalene	nd	nd	nd	nd	6.19	7.14	6.38	6.12	1.83	2.60
1502	1504	cuparene	nd	nd	nd	nd	1.04	1.09	–	–	nd	nd
1507	1513	γ-cadinene	2.28	4.11	5.26	4.77	1.49	1.61	1.77	2.08	nd	nd
1512	1523	δ-cadinene	6.30	7.76	10.34	8.14	1.89	2.23	2.17	2.35	nd	nd
1528	1533	γ-cuprenene	nd	nd	nd	nd	3.30	3.40	3.16	3.35	1.31	1.93
1528	1538	α-cadinene	–	–	1.13	1.07	nd	nd	nd	nd	nd	nd
1543	1549	β-elemol	nd	nd	nd	nd	–	–	–	–	1.14	1.99
1595	1600	cedrol	nd	nd	nd	nd	1.46	1.47	2.67	–	–	1.11
1655	1653	α-eudesmol	nd	nd	nd	nd	–	–	1.71	–	–	–
1699	1709	*cis*-thujopsenal	nd	nd	nd	nd	5.53	–	4.23	–	–	–
1902	1905	isopimara-9,15-diene	nd	nd	nd	nd	1.11	1.81	–	–	nd	nd
1929	1938	cembrene	–	–	–	–	1.68	2.69	–	–	9.98	4.07
1978	1974	sclarene	nd	–	nd	–	1.07	–	–	–	nd	–
2054	2043	*ent*-16-kaurene	2.70	1.26	–	–	1.57	–	2.44	–	nd	–
monoterpenoids			17.43	14.24	11.39	14.09	8.85	7.21	2.17	4.73	53.02	46.05
sesquiterpenoids			72.72	76.18	78.75	76.43	72.82	73.44	83.31	74.47	18.10	30.71
diterpenoids			3.14	1.73	0.70	0.75	5.43	5.83	2.92	0.90	10.78	5.35
others			1.46	0.72	0.99	0.75	1.63	1.33	0.14	0.04	0.83	0.00
identified			94.76	92.88	91.84	92.02	88.72	87.82	88.54	80.15	82.73	82.11

[a] Data are shown as averages ($n = 3$), and standard errors are all less than 10%. [b] Kovats indices determined relative to *n*-alkanes (C_9–C_{21}) on the DB-5ms column. [c] Reference Kovats indices based on the work of Adams.[13] [d] The relative content was less than 1%. [e] Not detected.

25 apparent peaks were detected and that there were significant differences among the BVOC patterns of these three *Chamaecyparis* species. The major constituents of BVOCs of *C. formosensis* leaves were germacrene D (peak 15) and α-pinene (peak 1), while those of *C. obtusa* var. *formosana* leaves were thujopsene (peak 12) and compound A (peak 16). In addition, two major constituents, α-terpinyl acetate (peak 9) and L-bornyl acetate (peak 8), were found in the BVOCs from *C. obtusa* leaves.

Seventy-five compounds of BVOCs from three *Chamaecyparis* leaves were identified, and those with relative contents over 1% are listed in Table 1 in order of their KIs from a DB-5ms column. These identified compounds were divided into four categories,

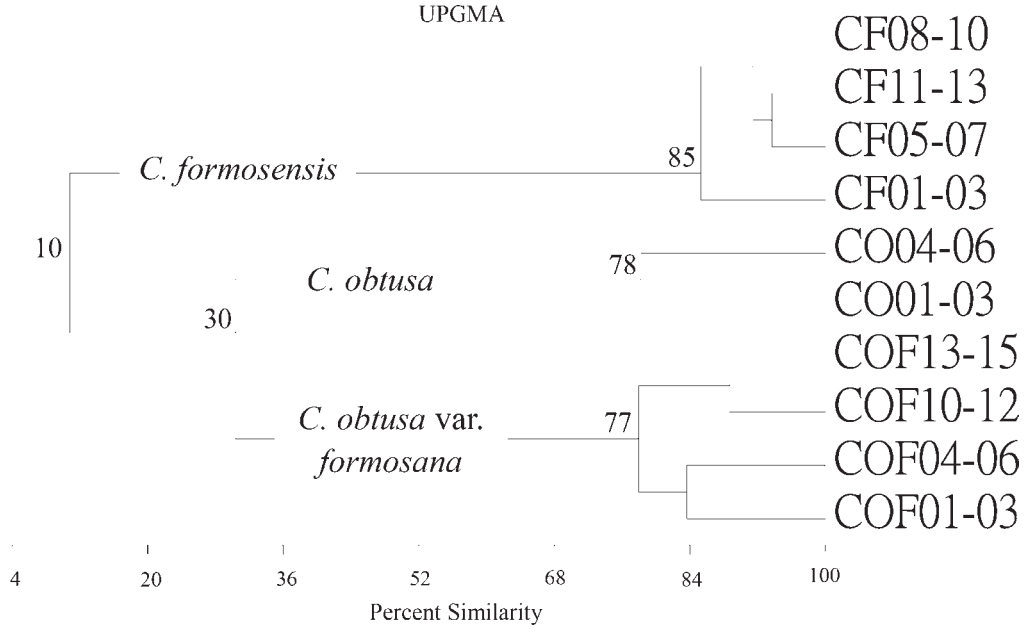

Figure 2. Dendrogram of BVOC compositions emitted from three *Chamaecyparis* species.

monoterpenoids, sesquiterpenoids, diterpenoids, and others, based on their chemical composition. The BVOCs of *C. formosensis* leaves collected from four locations consisted of 11.39−17.43% monoterpenoids and 72.72−78.75% sesquiterpenoids, showing that there are slight variations in the composition of BVOCs from the four locations. A similar variation of BVOCs is also observed for *C. obtusa* var. *formosana* and *C. obtusa* leaves collected from different locations. This is likely due to differences in the growing environments (temperature, sunshine, humidity, etc.) of each tree. The chemical composition of *C. obtusa* var. *formosana* (2.17−8.85% monoterpenoids and 72.82−83.31% sesquiterpenoids) is slightly similar to that of *C. formosensis*, whereas that of *C. obtusa* (46.05−53.02% monoterpenoids, 18.10−30.71% sesquiterpenoids, and 5.35−10.78% diterpenoids) is significantly different from those of *C. formosensis* and *C. obtusa* var. *formosana*.

The BVOCs emitted from *C. obtusa* var. *formosana* leaves primarily contained 17.14−23.49% thujopsene, 9.71−21.88% compound A, 6.12−7.14% β-himachalene, and 3.37−7.20% β-cedrene, while the BVOCs of *C. obtusa* leaves were mainly composed of α-terpinyl acetate (21.70−25.79%), L-bornyl acetate (11.31−13.28%), thujopsene (6.00−9.25%), and cembrene (4.07−9.98%). These results are similar to previous findings,[4,11] in which the major compounds of leaf essential oils from *C. obtusa* var. *formosana* and *C. obtusa* are thujopsene and α-terpinyl acetate, respectively, indicating that the leaf compositions of these two species extracted using the SPME technique resemble those from hydrodistillation (i.e., essential oils). However, in this study the BVOCs of *C. formosensis* leaves were mainly composed of germacrene D (35.10−41.20%), α-pinene (8.07−13.33%), α-humulene (4.38−8.66%), δ-cadinene (6.30−10.34%), and γ-muurolene (5.82−9.09%). These data are not in good agreement with those of Su et al.,[14] who reported that the leaf essential oil of *C. formosensis* was predominantly composed of 71.6% α-pinene, followed by δ-2-carene (4.6%) and β-myrcene (4.1%). An explanation for this result may be that germacrene D was released extensively from the leaves due to wounding.[15]

Multivariate Data Analyses. To investigate the differences in the compositions of BVOCs among three *Chamaecyparis* species, the data in Table 1 were examined using CA. The resulting dendrogram shows the existence of three major clusters (Figure 2). The first group, *C. formosensis*, was composed of CF01-03, CF05-07, CF08-10, and CF11-13, and the similarity within this group was 85%. The second group (*C. obtusa*) included CO01-03 and CO04-06, and the similarity between two samples was 78%. The third group (*C. obtusa* var. *formosana*) was formed by COF01-03, COF04-06, COF10-12, and COF13-15, and the similarity within this group was 77%. There was only a 30% similarity between *C. obtusa* and *C. obtusa* var. *formosana*, showing that these two species were considerably different. Moreover, the similarity between *C. formosensis* and these two species was extremely low, only 10%. These results are not only in good agreement with our previous findings[11] but also in accordance with morphological and genetic classification.[6]

In addition, we also applied PCA to differentiate the data listed in Table 1. The results of the PCA score plot (Figure 3) indicated that PC1 and PC2 explained 75.0% and 21.5%, respectively, of the total variance. Three main clusters, including *C. formosensis* (CF01-03, CF05-07, CF08-10, and CF11-13), *C. obtusa* var. *formosana* (COF01-03, COF04-06, COF10-12, and COF13-15), and *C. obtusa* (CO01-03 and CO04-06) were clearly differentiated. Both CA and PCA led to the same differentiation of the three species, revealing that an ideal differentiation of the three species could be achieved via analysis of their BVOC compositions using SPME−GC/MS.

The loading plot of PCA was carried out to further understand the influence of the BVOC components on differentiation of the three species. Those components that explain maximum variance in the data are given higher loading values, while others that do not play an important role are given loading values near 0. Figure 4 shows that the components with the greatest differentiation for PC1 were germacrene D (peak 15), thujopsene (peak 12), and compound A (peak 16), with loading values of 0.80, −0.36, and −0.27, respectively. This result shows that the germacrene D

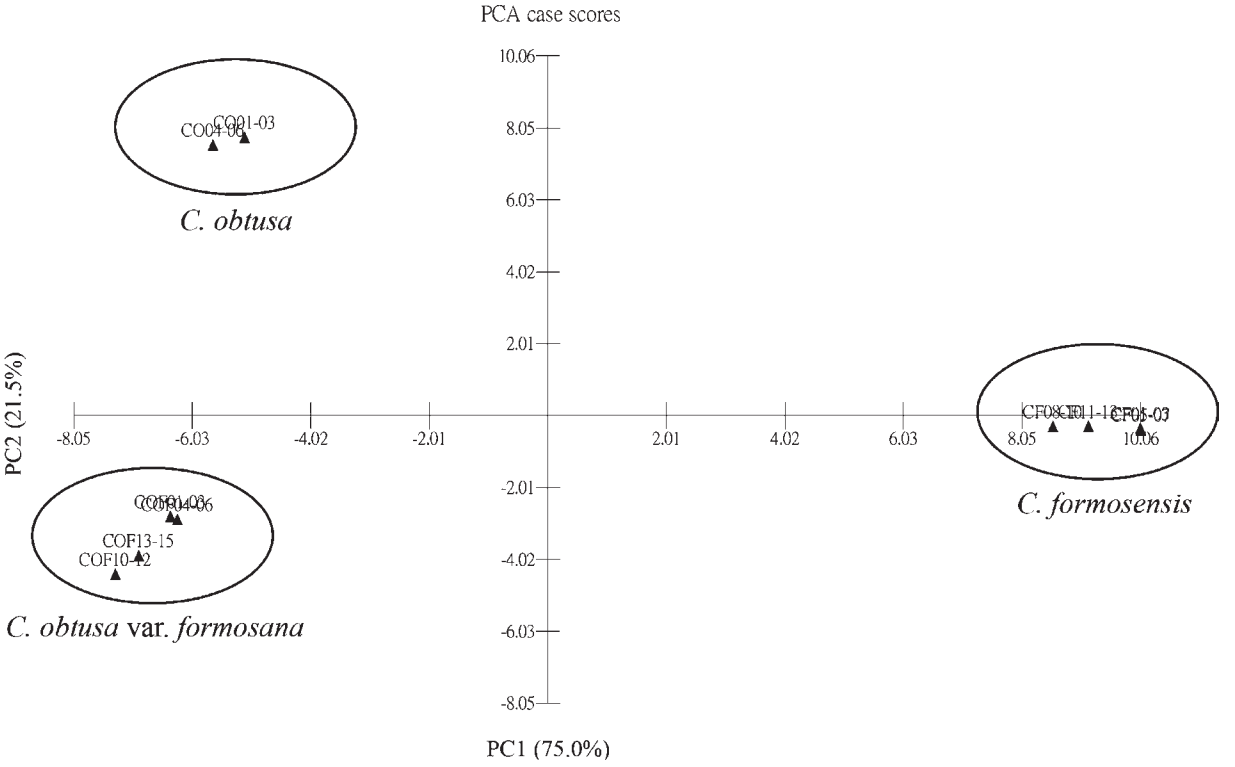

Figure 3. PCA analysis of BVOC compositions emitted from three *Chamaecyparis* species.

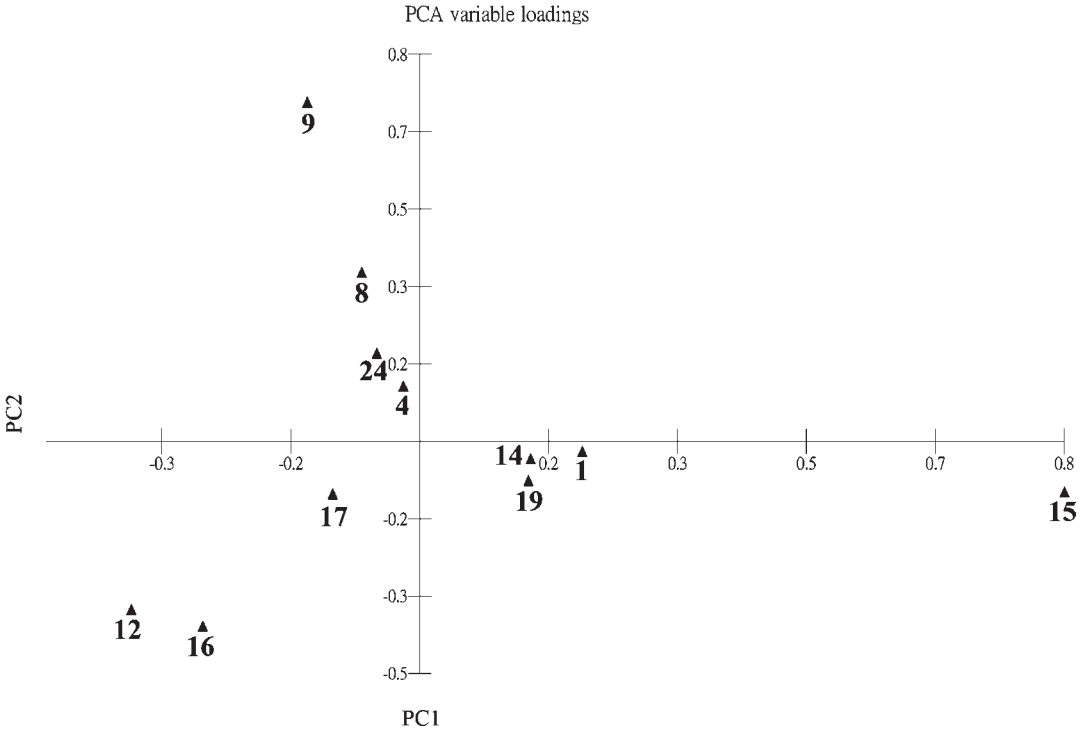

Figure 4. Loading plot of PCA analysis of BVOC compositions emitted from three *Chamaecyparis* species: 1, α-pinene; 4, limonene; 8, L-bornyl acetate; 9, α-terpinyl acetate; 12, thujopsene; 14, γ-muurolene; 15, germacrene D; 16, compound A; 17, β-himachalene; 19, δ-cadinene; 24, cembrene.

content can be used as an indicator to differentiate *C. formosensis* from *C. obtusa* var. *formosana* and *C. obtusa*, either of which contains no germacrene D. The loading values of α-terpinyl acetate (peak 9), thujopsene (peak 12), and compound A (peak 16) were 0.70, −0.34, and −0.38, respectively, and they were the most differentiating components on PC2. This result shows that

the differentiation of *C. obtusa* var. *formosana* from *C. obtusa* was due to higher levels of thujopsene and compound A. Many research reports have demonstrated that the volatile composition of leaves could be used to discriminate different genera, especially when they are morphologically hard to distinguish.[5,16] These traditional methods normally take a longer time (>3 h) and require a larger amount of leaves (>100 g) for hydrodistillation. In the current study, it merely takes 20 min of extraction time and 0.15 g of leaf sample to achieve species differentiation. This result clearly suggests that using SPME to extract BVOCs is a rapid and feasible method to differentiate these three *Chamaecyparis* species.

In conclusion, the BVOCs of leaves from three *Chamaecyparis* species (*C. formosensis*, *C. obtusa*, *C. obtusa* var. *formosana*) were analyzed using SPME−GC/MS in this study. The major constituents of BVOCs from *C. formosensis*, *C. obtusa*, and *C. obtusa* var. *formosana* leaves were germacrene D, α-terpinyl acetate, and thujopsene, respectively. According to CA, the three species were clearly separated. In addition, the results of PCA indicated that the three species were distinguished by the major components of their leaf BVOCs. Therefore, our findings provide a simple and useful method, the combination of SPME−GC/MS, CA, and PCA, to rapidly classify three *Chamaecyparis* species using only a small amount of leaves.

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