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FULL PAPER

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Chemical Constituents of Species in the Genus *Pleione* (Orchidaceae) and the Implications from Molecular Phylogeny

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Pleiones are popular ornamental orchids and different species of *Pleione* are long being used as traditional medicine in many Asian countries. However, previous chemical investigations of the genus *Pleione* are restricted to only a few species. In the present study, high performance liquid chromatography (HPLC) fingerprint of *Pleione* plants was established, which in particular, eight common peaks were confirmed in 16 species/hybrids. Three of the compounds corresponding to the chromatographic peaks were identified by electrospray ionization tandem mass spectrometry (ESI-tandem-MS). HPLC analysis confirmed the studied taxa shared most of chemical compounds but the content of chemical compounds was significantly different between species. Comparison of hierarchical clustering result with phylogenetic tree revealed that closely related species have higher similarities in chemical constituents. In consideration of low chemical similarity between spring-flowering and autumn-flowering species, we suggest a discrimination of these two groups during medicinal use of the genus *Pleione*. Species with a large pseudobulb and with high content of a certain compound should be given priority in future artificial cultivation and medicinal cultivar breeding. We hope our findings will contribute to the quality control and promote conservation of such endangered plant group.

Keywords: *pleione*, shan-ci-gu, medicinal orchid, HPLC fingerprint, phylogeny.

Introduction

Pleione D.Don is a medium-sized genus (~20 species and several natural hybrids) of the family Orchidaceae found from central Himalayas eastwards to China and southwards to central Indo-China Peninsula.[1,2] Members in this genus are fairly popular ornamental plants in Europe and the USA and widely used as traditional medicine in Asian countries.[3] Pseudobulbs of *P. maculata* (Lindl.) Lindl., *P. praecox* (Smith) D.Don and *P. humilis* (Smith) D.Don have commonly been applied in the northeast India for the treatment of cuts, wound, or liver complaints.[4] The latter two species are also used as tonic and energizer in Nepal.[5] In China, two species of *Pleione*, *P. bulbocodioides* (Franch.) Rolfe and *P. yunnanensis* (Rolfe) Rolfe, as well as *Cremastra appendiculata* (D.Don) Makino, are listed in *Pharmacopoeia of the People's Republic of China* as sources of the traditional medicine 'shan-ci-gu', which is used to remove heat, counteract toxicity, dissipate phlegm, and resolve masses, or for other symptoms such as furuncles, snake, and insect bites.[6] Other *Pleione* species are also used regionally in China for similar symptoms.[7,8]

More than 180 highly diverse compounds, including phenanthrenes, bibenzyls, glucosyloxybenzyl suc-

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cinate derivatives and flavonoids, have been isolated from pseudobulbs of *Pleione*, and these compounds have been demonstrated to play a significant role in antitumor, anti-neurodegenerative and anti-inflammatory biological activities and improving immunity.[9] However, previous chemical investigations of the genus *Pleione* are restricted to only a few species (mainly *P. bulbocodioides*, *P. yunnanensis* and *P. formosana* Hayata),[9] and there are still large proportion of species in the genus that remains unexplored. The Hengduan Mountains Region of southwest China has the highest species diversity of *Pleione*, where several species have overlapping geographical distributions. For example, over 15 species or natural hybrids have been recorded in Yunnan Province in southwest China.[10–12] Based on our field and traditional medicine market investigations in the biodiversity hotspot region in west Yunnan, the medicinal use of *Pleione* by indigenous people is undoubtedly indiscriminate. For example, there are at least seven species and natural hybrids (*P. albiflora* P.J.Cribb & C.Z.Tang, *P. aurita* P.J.Cribb & H.Pfennig, *P. bulbocodioides*, *P. forrestii* Schltr., *P. yunnanensis*, *P.* ×*taliensis* P.J.Cribb & Butterf. and *P.* ×*confusa* P.J.Cribb & C.Z.Tang) recorded on the Cangshan mountain range near the city of Dali according to specimen record and our field investigation, and our samples obtained from the local traditional medicine market are usually the mix of the above mentioned species. In fact, without seeing the flower it is almost impossible to distinguish most *Pleione* species just by vegetative characters such as the shape leaf and pseudobulb[2] (see *Figure 1*).

According to our interviews with several Chinese traditional medicine dealers from Yunnan and Sichuan provinces, it is estimated at least 60 tons of fresh *Pleione* pseudobulbs were harvested annually in this

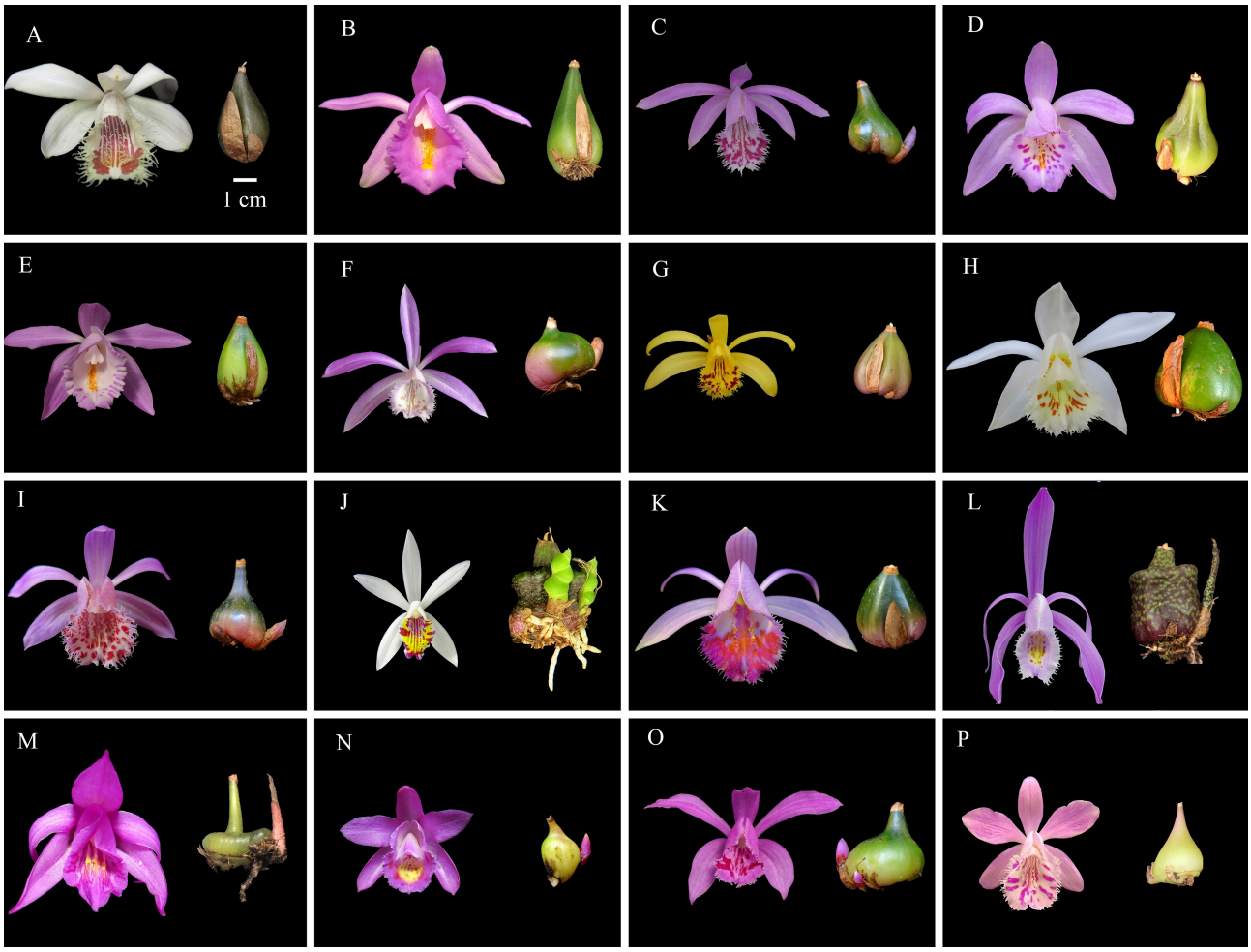

Figure 1. Flower and pseudobulb of 16 samples involved in the present study. A) *Pleione albiflora*; B) *P. aurita*; C) *P. bulbocodioides*; D) *P.* Burnsall; E) *P. chunii*; F) *P. formosana*; G) *P. forrestii*; H) *P. grandiflora*; I) *P. limprichtii*; J) *P. maculata*; K) *P. pleionoides*; L) *P. praecox*; M) *P. saxicola*; N) *P. scopulorum*; O) *P.* × *taliensis*; P) *P. yunnanensis.*

region recent years. All these individuals, without exception, were from wild collection. Several species assessed by IUCN as vulnerable or endangered[13,14] are included in these wild collections. Therefore, artificial cultivation and obtainment of good germplasm resources are critically needed to promote conservation and sustainable utilization of this endangered plant group.[15] Different species of *Pleione* are long being used as traditional medicine in many Asian countries, however, chemical investigation of these species is seriously lagging behind. Extensive investigations on more species are necessary in order to obtain germplasm with high concentrations of effective compounds for future artificial cultivation.

Chromatographic fingerprint provides a comprehensive and quantifiable identification method to reveal the chemical information of herbal medicines with chromatogram, spectrograms and other graphs.[16] In this study, we use the method of chromatographic fingerprint based on high-performance liquid chromatography (HPLC), combined with principal component analysis (PCA), hierarchical cluster analysis (HCA) and chemometric approaches of similarity analysis (SA) to assess 16 pseudobulb samples of different *Pleione* species. The aims of the present study were to identify and distinguish different species based on the HPLC method, and try to find out replacement of medicinal resources with high concentrations of effective compounds for future artificial cultivation.

Results and Discussion

HPLC Analysis

The chromatographic fingerprints of these 16 samples (*Table 1*) were obtained using HPLC technique and their fingerprints profiles were presented in *Figure 2*. According to the HPLC fingerprint, eight common characteristic peaks were found between 0.5 and 25 min, indicating that these *Pleione* samples share some components (*Figure 2*). The means and values of relative standard deviation (RSDs) of the RTT and RPA were showed in *Table S1* and *Table S2* in the *Supporting Information.* All of the RSDs of RPA were $<4.0\,\%$.

Compounds Characterization by LC/MS

Peaks V5, V6 and the peak around 23.42 min were identified as dactylorhin A, militarine and batatasin III, respectively, by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry based on previous study on *P. Yunnanensis* and *P. bulbocodioides*[17,18] (*Figure 2*, *Figure S1*).

Table 1. Sample information.

Sample No.	Species/hybrid	Origin	Fresh weight of pseudobulb (g, mean ± SE, $n=10$)	Voucher specimen (bar code number)
S_1	*Pleione* Burnsall	Artificial hybrid between *P. aurita* and *P. yunnanensis*	8.55 ± 0.24	–
S_2	*P. albiflora*	Longling, Yunnan	3.77 ± 0.29	1498257
S_3	*P. chunii*	Xing'an, Guangxi	2.97 ± 0.27	1498258
S_4	*P. grandiflora*	Wenshan, Yunnan	9.25 ± 0.67	1498259
S_5	*P.* × *taliensis*	Dali, Yunnan, natural hybrid between *P. bulbocodioides* and *P. yunnanensis*	4.92 ± 0.13	1498260
S_6	*P. bulbocodioides*	Zhaotong, Yunnan	5.32 ± 0.33	1498261
S_7	*P. scopulorum*	Fugong, Yunnan	2.61 ± 0.11	1498262
S_8	*P. forrestii*	Yunlong, Yunnan	5.25 ± 0.36	0025899
S_9	*P. pleionoides*	Ziyuan, Guangxi	3.83 ± 0.13	1498263
S_{10}	*P. limprichtii*	Luding, Sichuan	2.92 ± 0.09	1498264
S_{11}	*P. formosana*	Tongshan, Hubei	4.87 ± 0.75	1498265
S_{12}	*P. saxicola*	Fugong, Yunnan	5.51 ± 0.61	1498266
S_{13}	*P. aurita*	Yangbi, Yunnan	9.17 ± 0.87	1498267
S_{14}	*P. praecox*	Fugong, Yunnan	9.61 ± 1.23	1498268
S_{15}	*P. maculata*	Longling, Yunnan	9.31 ± 0.91	1498269
S_{16}	*P. yunnanensis*	Baoshan, Yunnan	5.61 ± 0.85	0025813

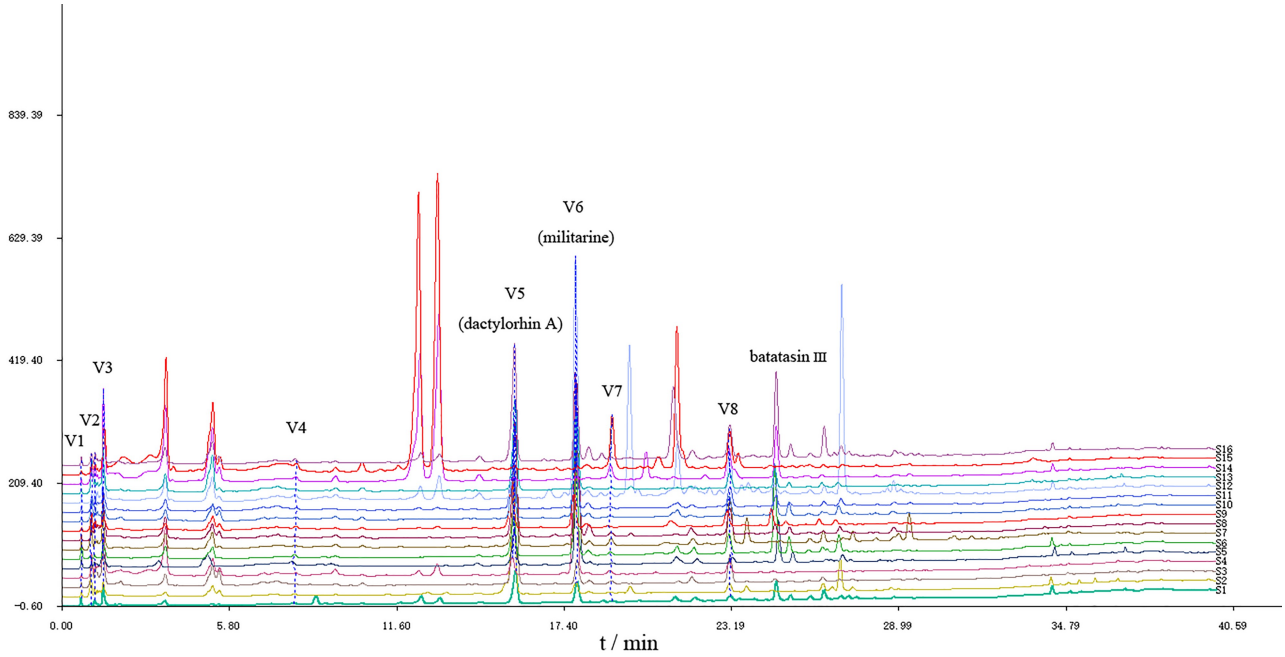

Figure 2. HPLC fingerprints of16 *Pleione* samples (S1 – S16) under the same condition.

Similarity Evaluation of 16 Pleione Samples

Similarity values of HPLC fingerprints of 16 samples of *Pleione* were shown in *Table 2*. According to *Table 2*, the correlation coefficient of similarity between the autumn-flowering *P. praecox* and *P. maculata* (S_{14} and S_{15}) and the spring-flowering group (all other samples except S_{12}) are relatively low, while those values within the spring-flowering group are relatively higher. The autumn-flowering *P. saxicola* (S_{12}) has higher similarity values with spring-flowering species than autumn-flowering species. Remarkably, the lowest value for correlation coefficient of similarity was 0.041 and found between S_2 and S_{15}, and such parameter between *P. bulbocodioides* and *P. yunnanensis* (S_6 and S_{16}), two species that listed in the *Pharmacopoeia of the People's Republic of China*, was 0.779. Both the values of correlation coefficient of similarity between the artificial hybrid *P.* Burnsall (S_1) and each parent (S_{13} and S_{16}) were higher than 0.8, while the natural hybrid

Table 2. Correlation coefficient of similarity of HPLC fingerprints of 16 samples of *Pleione*.

	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9	S_{10}	S_{11}	S_{12}	S_{13}	S_{14}	S_{15}	S_{16}
S_1	1															
S_2	0.755	1														
S_3	0.795	0.959	1													
S_4	0.524	0.199	0.271	1												
S_5	0.582	0.313	0.372	0.934	1											
S_6	0.781	0.463	0.507	0.757	0.782	1										
S_7	0.787	0.674	0.744	0.752	0.789	0.772	1									
S_8	0.818	0.697	0.756	0.816	0.857	0.829	0.938	1								
S_9	0.799	0.794	0.861	0.613	0.672	0.693	0.893	0.905	1							
S_{10}	0.729	0.442	0.508	0.897	0.913	0.941	0.851	0.914	0.769	1						
S_{11}	0.817	0.635	0.712	0.835	0.872	0.843	0.937	0.985	0.891	0.934	1					
S_{12}	0.498	0.225	0.262	0.734	0.738	0.621	0.629	0.653	0.511	0.712	0.696	1				
S_{13}	0.836	0.797	0.861	0.669	0.72	0.782	0.896	0.937	0.94	0.833	0.916	0.549	1			
S_{14}	0.339	0.066	0.111	0.184	0.055	0.173	0.153	0.112	0.183	0.143	0.141	0.17	0.186	1		
S_{15}	0.295	0.041	0.07	0.143	0.053	0.08	0.098	0.073	0.132	0.085	0.112	0.179	0.111	0.921	1	
S_{16}	0.876	0.717	0.768	0.466	0.548	0.779	0.753	0.769	0.772	0.722	0.78	0.511	0.811	0.237	0.264	1

P. ×*taliensis* (S_5) had relatively lower values compared with its potential parents (S_6 and S_{16}) for this parameter (*Table 2*).

Principal Component Analysis

Principal component analysis was performed on the RPA to make sure all the elements have an equal influence over the results. The correlation coefficient reflects the interaction between different components. With a correlation coefficient of 0.819, strong positive correlation was supported between V_5 and V_8 (*Table 3*). The first three PCs had the greatest eigenvalues and thus contained the chemically relevant variance, and the remaining PCs, which had eigenvalues that contained a significantly smaller portion of the total variance, were discarded. The eigenvalue for PC1 is 2.538, representing 31.722% of the total variance. The eigenvalue for PC2 is 2.238, representing 27.975% of the total variance, and the eigenvalue for PC3 is 1.438, representing 17.978% of the total variance. Cumulative percent variance for the first three factors is 77.675% (*Table 4*).

Hierarchical Clustering Analysis and Phylogenetic Tree

According to the HCA of the HPLC fingerprints, these 16 samples could be divided into two main clusters (*Figure 3A*). One cluster consists of *P. saxicola* (S_{12}) and all the spring-flowering samples, and the other consists of the two autumn-flowering species *P. praecox* and *P. maculata* (S_{14} and S_{15}). The tree obtained from our hierarchical clustering analysis of the HPLC fingerprints is consistent to the phylogenetic topology inferred from multi-loci DNA data and morphological characters[19] (*Figure 3B*). The two autumn-flowering species *P. praecox* and *P. maculata* clustered in a clade, and then, clustered with the clade consisting of all the spring-flowering species and the relatively isolated autumn-flowering species *P. saxicola* in both chemical tree and the phylogenetic tree (*Figure 3*).

Table 4. Eigenvalues of correlation matrix

PC	Eigenvalues (%)	Percentage variance	Cumulative percent variance
1	2.538	31.722	31.722
2	2.238	27.975	59.697
3	1.438	17.978	77.675
4	0.595	7.437	85.113
5	0.577	7.213	92.326
6	0.367	4.587	96.913
7	0.191	2.388	99.301
8	0.056	0.699	100.000

Discussion

Chemical components of herbal medicine are affected by various factors, such as geographic origin, growth environment and harvest time. For example, vitamin contents of *P. yunnanensis* were found significantly different between samples from different geographic regions.[20] In our study, all samples were from cultivated plants in one greenhouse and harvested at the same time. The chemical difference reflected by HPLC fingerprints can only be a result of genetic difference of these studied species. Various chemical compounds, such as phenanthrenes, bibenzyls, glucosyloxybenzyl succinate derivatives and flavonoids, have been found in pseudobulbs of previous studied *Pleione* species.[9] Dactylorhin A and militarine characterized in our study were demonstrated to have the effects of neuroprotection and cognitive impairment.[21–23] The determination of these two compounds therefore can be used for the quality control of *P. bulbocodioides* and *P. yunnanensis*.[24] Only three compounds corresponding to the chromatographic peaks were identified in our study. Never-

Table 3. Correlation coefficient between common peaks.

	V_1	V_2	V_3	V_4	V_5	V_6	V_7	V_8
V_1	1.000							
V_2	−0.327	1.000						
V_3	−0.236	0.452	1.000					
V_4	−0.174	0.322	0.697	1.000				
V_5	0.054	−0.157	0.092	0.352	1.000			
V_6	0.059	0.486	0.306	−0.004	−0.364	1.000		
V_7	0.542	−0.134	−0.071	−0.135	0.180	0.003	1.000	
V_8	−0.159	−0.130	0.149	0.535	0.819	−0.356	−0.246	1.000

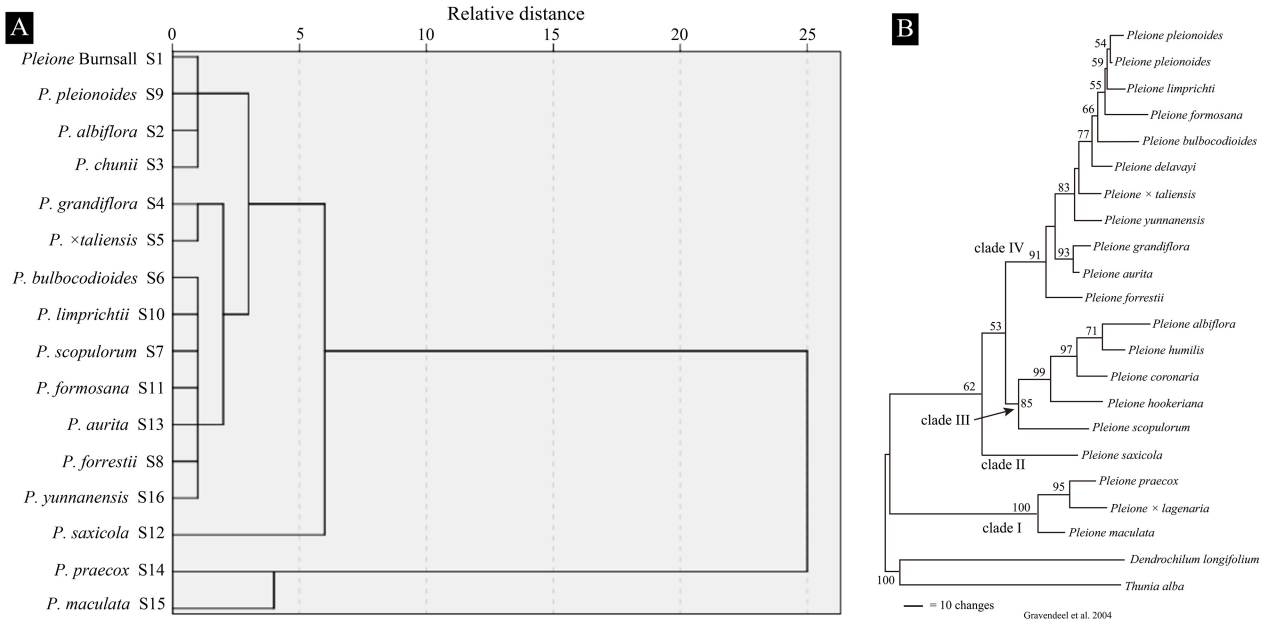

Figure 3. Hierarchical cluster result of 16 *Pleione* samples (A) and phylogeny of the genus *Pleione* based on nuclear, plastid DNA and morphological data (B; cited from Gravendeel et al., 2004). *Dendrochilum longifolium* and *Thunia alba* were chosen as outgroups in Plate B.

theless, we found all the studied species shared most of chemical compounds but the content of chemical compounds was significantly different between species. Species that are more closely related in phylogeny have higher similarity values in chemical constituent.

Many herbal medicines have multiple botanical origins.[25,26] Two species of *Pleione* (*P. bulbocodioides* and *P. yunnanensis*) are listed in *Pharmacopoeia of the People's Republic of China* as sources of the traditional medicine 'shan-ci-gu'.[6] In consideration of the high chemical similarity between these two species and allied species, we suggest all the spring-flowering species (*Pleione* Sect. *Humilies*), as well as the hybrids between them, may be used as substitutes of the above two pharmacopoeia recorded species, while a discrimination of the spring-flowering and autumn-flowering species should be regarded during medicinal use of the genus *Pleione*. Species with large pseudobulb (e.g., *P. aurita* and *P. grandiflora*, *Table 1* for detail) and with high content of a certain compound should be given priority in future artificial cultivation and medicinal cultivar breeding. More compounds corresponding to each chromatographic peak need to be addressed in future studies, and the understanding of how these effective compounds respond to environmental factors would largely contribute to the quality control and efficient use of these traditional medicines.

Conclusions

HPLC fingerprint of *Pleione* plants was established, which in particular, eight common peaks were confirmed in 16 species/hybrids. Three of the compounds corresponding to the chromatographic peaks, namely dactylorhin A, militarine and batatasin III, were identified by ESI-tandem-MS. We found that closely related species have higher similarities in chemical constituents. A discrimination of spring-flowering and autumn-flowering species during medicinal use of the genus *Pleione* was suggested. Suggestions and substitute species in future artificial cultivation and medicinal cultivar breeding were also proposed.

Experimental Section

Plant Materials

A total of 16 pseudobulb samples, including 14 species, one natural hybrid and one artificial hybrid of *Pleione* were investigated. All plant materials were grown in a medium containing a mix of seven parts

bark, two parts moss, and one part humus (by volume). Plants were grown in the same greenhouse in Kunming Institute of Botany for at least 2 years before our experiment in order to eliminate the impact of environment factors to chemical composition. The pseudobulbs were harvested in December 2018, when plants shed their leaves and went dormant (*Figure 1*), and then, oven dried at 60 °C for further use. Detail information of samples was shown in *Table 1*. All the samples were grown and identified by the first author, and voucher specimens are deposited in Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences (KUN).

Reagents

HPLC-grade methanol used for chromatography analysis was purchased from Merck Chemical Technology (Shanghai) Co., Ltd. Analytically pure methanol used for extraction was purchased from Chengdu Cologne Chemical Co., Ltd.

Sample Preparation

The dry samples were ground to fine powder and passed 80 mesh sieves. An accurately weighed pseudobulb powder (3.0 g) of each sample was added methanol (90 mL) and extracted at 80 °C for 3 h in a Soxhlet extractor. The extraction was filtered via vacuum and the filter cake was washed with methanol (15 mL × 2). The filtration was combined and condensed to provide the crude product that was solved in 50 mL of methanol, and well-shaken and filtered using sterile 0.45 μm filter before injecting into the HPLC system for the chromatographic analysis.

HPLC Analysis

Separation was operated on an Agilent 1290 Accurate-Mass QTOF (Agilent Technologies, Santa Clara, CA, USA). LC system equipped with a binary pump, micro degasser, and an injection valve. Eclipse Plus C18 column (4.6 mm × 100 mm, 3.5 μm) was used for chromatographic analysis. The mobile phase was composed of pure water (A) and methanol (B). Initial mobile phase was composed of 8% B and increased to 30% B in 5 min, moved to 55% B in 15 min, and continued to increase to 75% B in 10 min. After that, mobile phase B was changed to 0% in 5 min and kept for 5 min. The flow rate was 0.8 mL/min and detected at 224 nm and the injection volume was 5 μL.

Q-TOF MS Analysis

The mass spectrometer was operated in negative ion mode. The operation conditions were as follows: drying gas (N_2); flow rate, 8 L/min; drying gas temperature, 350 °C; nebulizer, 30 psig; fragmentor, 135 V; skimmer, 65 V; The collision energy was set at 30 V. The scan range was from 50 to 1700 *m/z*. The mass spectra were acquired in high sensitivity mode with a 1000 ms ion accumulation time

Data Analysis

Similarity analysis of HPLC fingerprints was performed on the basis of the relative retention time (RRT) and the relative peak area (RPA) using the software Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (2004A). Hierarchical clustering analysis and PCA were performed using SPSS software (SPSS, USA). Average linkage method was applied and Euclidean distance was selected as measurement. The peak areas of main characteristic peaks were selected as the clustering variable.

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Author Contribution Statement

Wei Zhang and Lin-Fei Zhang performed the experiments, analyzed the data, and wrote the article. Yu Deng and Jiao Qin analyzed the data. Shi-Bao Zhang and Jiang-Miao Hu conceived and designed the experiments.

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