JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Molecular Genetic and Chemical Assessment of Rhizoma Curcumae in China

QUAN XIA,[†,§] KUI J. ZHAO,[†] ZHAO G. HUANG,[§] PING ZHANG,[§] TINA T. X. DONG,[†] SHAO P. LI,[‡] AND KARL W. K. TSIM*,[†]

Department of Biology, The Hong Kong University of Science and Technology, Clear Water Bay Road, Hong Kong, Institute of Chinese Medical Sciences, University of Macau, Taipa, Macau, and Department of Pharmacy, The First Affiliated Hospital of Anhui Medical University, Hefei, China

Rhizoma Curcumae (Ezhu) is a traditional Chinese medicine that has been used in removing blood stasis and alleviating pain for over a thousand years. Three species of *Curcuma* rhizomes are being used, which include *Curcuma wenyujin*, *Curcuma phaeocaulis*, and *Curcuma kwangsiensis*. In China, the production of Rhizoma Curcumae largely depends on agricultural farming. The essential oils are considered as active constituents in Rhizoma Curcumae, which include curdione, curcumol, and germacrone. On the basis of the yield of curdione, curcumol, and germacrone in an orthogonal array design, the optimized extraction condition was developed. The amounts of these compounds within essential oils in Rhizoma Curcumae varied according to different species and their regions of cultivation. Chemical fingerprints were generated from different species of *Curcuma*, which therefore could serve as identification markers. In molecular genetic identification of Rhizoma Curcumae, the 5S-rRNA spacer domains of 5 *Curcuma* species, including the common adulterants of this herb, were amplified, and their nucleotide sequences were determined. Diversity in DNA sequences among various species was found in their 5S-rRNA spacer domains. Thus, the chemical fingerprint together with the genetic distinction could serve as markers for quality control of *Curcuma* species.

KEYWORDS: *Curcuma*; authentic identification; HPLC analysis; TCM; GAP farming; 5S-rRNA spacer domain

INTRODUCTION

Rhizoma Curcumae (rhizome of *Curcuma*; Ezhu) is a common traditional Chinese medicine that has been used for more than a thousand years; this herb could also be prepared as a decoction in many Chinese families as health food supplements (*1*). The usage of Rhizoma Curcumae was first recorded in *Yao Xing Lun* by Su Song in 627∼649 B.C.; the described functions were to remove blood stasis and to alleviate pain (*2*). *Curcuma* belongs to the Zingiberaceae family and in a genus of about 70 species of rhizomateous herbs distributed worldwide. About 20 *Curcuma* species occur in China, of which a few of them are being used as herbal medicine and food. The Chinese Pharmacopoeia (*2*) recorded that Rhizoma Curcumae should be the dry rhizomes derived from *Curcuma wenyujin* Y. H. Chen et C. Ling, *Curcuma phaeocaulis* Val., and *Curcuma kwangsiensis* S. G. Lee et C. F. Liang. However, different parts of the plants derived from the three *Curcuma* species, as well as other closely related species of *Curcuma*, are also used for herbal medicine or food but have different therapeutic usages. Radix Curcumae (Turmeric Root Tuber; Yujin) is the dry root tubers of *C. wenyujin*, *Curcuma longa* L., *C. phaeocaulis*, and *C. kwangsiensis* (*2*); it is commonly used in the treatment of hepatitis, menstrual disorders, and epilepsy (*3*). Rhizoma Curcumae Longae (Turmeric; Jianghuang) is the dry rhizome of *C. longa*; it is used as an analgesic in the treatment of menstrual disorders, rheumatism, and traumatic diseases (*3*). In addition, the root tuber and rhizome of *Curcuma chuanyujin* C. K. Hsieh et H. Zhang are also used as substitutes of Radix Curcumae and Rhizoma Curcumae, respectively, in the Sichuan province of China (*4*).

Although the aforementioned five *Curcuma* species belong to the same genus, the chemical composition of these plant extracts, either from root or rhizome, are different. Phenolic pigment and essential oil are main constituents in *Curcuma* plants; however, their amounts showed variation among different species (*5*, *6*). The rhizome of *C. longa* contains the highest amount of the pigments, which has been known for its coloring, flavoring, and digestive properties since ancient times. Indeed, it is a major constituent of curry powder in Chinese and Indian cooking. The essential oils are considered as active constituents, which have been reported to possess antitumor and antiviral activities (*7*, *8*). Accounting for most of the known pharmacological properties of essential oils in Rhizoma Curcumae, the

* Corresponding author. Tel: (852) 2358-7332; fax: (852) 2358-1559; e-mail: BOTSIM@UST.HK.
[†] The Hong Kong University of Science and Technology.
[‡] Institute of Chinese Medical Sciences, University of Macau.
[§] The First Affiliated Hospital of Anhui Medical University.

10.1021/jf0508495 CCC: $30.25
Published on Web 06/23/2005

Table 1. The Contents of Curdione, Curcumol, and Germacrone from Different Populations of Rhizoma Curcumae

				content (mg/kg)[c]		
no.[a]	voucher	species	origin[b]	germacrone	curdione	curcumol
1	ESS-005	C. wenyujin	Leqing, Zhejiang	5342	15088	78
2	ESS-006	C. wenyujin	Yongjia, Zhejiang	6766	17959	-[d]
3	ESS-007	C phaeocaulis	Xinjiang, Sichuan	1639	967	-
4	ESS-008	C. phaeocaulis	Jiangyuan, Sichuan	1658	307	-
5	ESS-009	C phaeocaulis	Sanjiang, Sichuan	1298	321	-
6	ESS-010	C phaeocaulis	Zhoudu, Sichuan	1349	360	-
7	ESS-011	C phaeocaulis	Wangdang, Sichuan	1336	335	-
8	ESS-012	C phaeocaulis	Shuangliu, Sichuan	1477	1588	-
9	ESS-016	C. kwangsiensis	Wuming, Guangxi	395	337	-
10	ESS-017	C. kwangsiensis	Wenshan, Yunnan	880	204	-
11	ESS-019	C. kwangsiensis	Fujian market	204	968	-
12	ESS-020	C. kwangsiensis	Yuling, Guangxi	139	450	-
13	ESS-021	C. kwangsiensis	Wenshan, Yunnan	98	238	-
14	ESS-025	C. kwangsiensis	Anhui market	468	1764	-
15	ESS-022	C. longa	Anhui market	318	179	-
16	ESS-023	C. longa	Jianli, Sichuan	65	315	-
17	ESS-024	C. longa	Hongkong market	-	193	-
18	ESS-013	C. chuanyujin	Chengdu, Sichuan	569	329	-
19	ESS-014	C. chuanyujin	Malipo, Yunnan	571	348	-

[a] Nineteen populations of Rhizoma Curcumae were collected in fresh and dried under vacuum. Extraction by ultrasonication was optimized as described in **Figure 3**. [b] About 10–15 individual samples from each population were analyzed. All samples were collected in September to October, and they were all about 3-years-old. [c] Values are in mg/kg dry rhizome and in means, $n = 10–15$. The SD values are not shown for clarity, which are less than 10% of the means. [d] Not detectable.

major components within the essential oils are curdione, curcumol, and germacrone; these components are commonly used as quality control markers (*9*−*11*). The production of Rhizoma Curcumae depends on agricultural farming in China; however, the quality of the herb varies greatly. For instance, the level of essential oils within Rhizoma Curcumae could be varied according to the origin of the species, regions of farming, farming technique, and extraction methods. Among different members of Rhizoma Curcumae, *C. wenyujin* from Zhejiang China is considered to be the best source. On the other hand, different methods in extracting essential oils have been described (*12*), and hydrodistillation is a conventional one (*11*). Thus, the aforementioned parameters cause serious problems in the quality control of Rhizoma Curcumae.

To ensure the quality of Rhizoma Curcumae, we determined the chemical and genetic distinctions of rhizomes derived from *C. phaeocaulis*, *C. kwangsiensis*, and *C. wenyujin*, as well as from its common adulterants. Chemically, we developed the optimized extraction method in recovering curdione, curcumol, and germacrone, and the quality of Rhizoma Curcumae in different parts of China was also presented. Genetically, the 5S-rRNA spacer domains, the unique intronic sequences that have been used commonly for species authentication (*13*−*15*), were sequenced and compared among different species of *Curcuma*.

MATERIALS AND METHODS

Acquisition of Plant Materials. Fresh plants and dried crude drugs were collected from the agricultural farms in China. The voucher specimens were deposited in the Department of Biology, The Hong Kong University of Science and Technology, Hong Kong. For DNA analysis, entire plants of *C. wenyujin* (03-04-01) were obtained from Zhejiang; *C. longa* (03-04-05), *C. chuanyujin* (03-04-15), and *C. phaeocaulis* (03-04-09) were collected from Sichuan; and *C. kwangsiensis* (03-04-11) was obtained from Guangxi. The botanical origins of the *Curcuma* plants in forms of whole plants were identified morphologically by ourselves during the field collection. Samples were kept in silica gel during field collection and were dried under vacuum. For chemical analysis, the rhizomes of different *Curcuma* species were collected from various regions of China and were dried under vacuum; their notations are listed in **Table 1**. The chosen area for the herbal collection was based on the popularity of Rhizoma Curcumae that was cultivated. The plant materials were collected in April of 2003; they were ∼2−3 years of growth. About 10 batches of individual species having similar but not identical geographical properties at the same region were tested. Individual samples were prepared from ∼500 g of powder (0.10−0.15 mm) that was grounded from ∼20 plants of the same population. These grinding processes were done during the field collection before they were delivered to the laboratory. The collected powder was stored with silica gel, which stabilized the chemical constituents.

Optimization of Extraction Condition. Exactly 0.5 g of ground powder was tested under different methods of extraction. In hydrodistillation, the powder was placed in a flask of Clevenger extractor and was extracted with 100 mL of water for 6 h. In ultrasonic extraction, the powder was placed in a 50-mL centrifuge tube containing 20 mL methanol, and the capped tube was sonicated for 30 min at room temperature in an ultrasonic bath (150 W, 50 Hz). The supernatant was collected by centrifugation at 5000*g*. In reflux extraction, the powder was suspended in 20 mL of methanol in a 50-mL conical flask. Heat reflux extraction using a water bath was performed for 2 h. A mechanical stirrer was used during the extraction. When the extraction finished, the suspension was transferred into a centrifuge tube for centrifugation, 10 min at 5000*g*. The supernatant was collected. In Soxhlet extraction, the powder was extracted in 150 mL petroleum ether for 6 h. The Soxhlet extracts were evaporated with nitrogen until they reached about 2 mL in volume. The concentrated extract was brought up to 25 mL with methanol. The supernatant was collected and filtered through a Millipore filter unit (0.45 μm). Ten microliters of the sample was injected for reverse-phase high-performance liquid chromatography (HPLC).

Quantitative Analysis of Curdione, Curcumol, and Germacrone. Curdione and curcumol were purchase from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and germacrone was from Sigma (St. Louis, MO). HPLC grade reagents were from Fischer and Labscan (Dublin, Ireland). Chemical standards were weighed and dissolved in 1 mL of methanol to give serial concentrations, and three injections were performed for each dilution. The standard curve was calibrated by using the linear least-squares regression equation derived from the peak area; the concentration was calculated according to the regression parameters derived from the standard curves. HPLC analysis was performed on an Agilent Series 1100 liquid chromatograph, equipped with a vacuum degasser, a quaternary pump, an auto-sampler, and a DAD detector, connected to an Agilent ChemStation software. A ZORBAX Eclipse XDB-C18

column (4.6 × 250 mm i.d., 5 μm) and a ZORBAX Eclipse XDB-C18 guard column (4.6 × 12.5 mm i.d., 5 μm) were used. The solvents of mobile phase were A (methanol) and B (water). A linear gradient elution was applied from 24 to 28% B starting from 0 to 25 min. The flow rate was 0.8 mL/min, and the injection volume was 10 μL. The system operated at 35 °C, and peaks were detected at 214 nm.

DNA Extraction. Genomic DNA was extracted from the ground powder derived from crude herbs by using DNA extraction buffer consisting of 25 mM Tris-HCl pH 8.0, 50 mM EDTA, 0.5% SDS, 10 μg/mL RNase, and 0.2% β-mercaptoethanol. The mixed solution was incubated for 15 min at 58 °C and then was centrifuged at 12 000*g* for 10 min. The supernatant was extracted by an equal volume of water-saturated phenol:chloroform (1:1), mixed, and then centrifuged. The aqueous phase was collected and added to 0.1 volume of 3 M sodium acetate and 2 volumes of 100% ethanol. The resulting pellet was collected after centrifugation and was dissolved in 10 mM Tris-HCl pH 8.0, 5 mM EDTA (Tris-EDTA buffer).

PCR Amplification and Sequencing. A 50-μL PCR reaction mix consisted of 5 μL 10X reaction buffer, 1 μL each of 10 mM dNTPs stock, 2.5 μL containing 0.3 μg forward and reverse primers (synthesized by Invitrogen Life Technologies, Carlsbad, CA), and 1 unit of *Taq* polymerase (Roche, Mannheim, Germany). The primers used for the amplification were 5S-P1 forward primer (5′-GGA TTC GTG CTT GGG CGA GAG TAG TA-3′) and 5S-P2 reverse primer (5′-ACG CTA GTA TGG TCG TGA TTC CTA GG-3′). These primers are flanking the spacer domain of 5S-rRNA (*13*−*15*). Approximately 50 ng of genomic DNAs was used as a template for the reaction. The reaction mix was overlaid with mineral oil and placed in a GeneAmp PCR System (Applied Biosystems, Foster City, CA). Cycling conditions consisted of an initial 5 min at 94 °C followed by 1 min denaturing at 94 °C, 2 min annealing at 53 °C, and 3 min elongation at 72 °C repeated for 30 cycles with 10 min extension at 72 °C. The PCR products were subjected to ∼1% agarose gel electrophoresis and were visualized by ethidium bromide staining under UV. DNAs were purified by Concert Rapid gel extraction system (Invitrogen Life Technologies).

The PCR products were subcloned into a TA cloning vector pTAg (Promega, Madison, WI). Competent *Escherichia coli* JM 109 cells were transformed with the ligated products, and the minipreparation of plasmid DNAs from the ligator cell was performed using alkaline lysis (*16*). The correct DNA inserts were verified by restriction analysis, and the plasmid DNAs, verified by colonies, were isolated with the Concert nucleic acid purification system (Invitrogen Life Technologies). ABI Prism, BigDye Terminator, and cycle Sequencing Ready Reaction Kit were used for sequencing reaction with T7 or SP6 primers. Sequences were detected by an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Both strands of DNA clones were sequenced at least twice, and the sequences were aligned by MacVector software (Kodak, New Heven, CT). Comparisons of entire sequences of test samples were aligned by a software Clustal X having a gap opening of 10 and a gap extension of 0.05. Polygenetic tree based on the hierarchical clustering of the alignments of the 5S-rRNA spacers was assessed by the neighbor-joining (UPGMA) method.

Other Assays. The statistical software package was SPSS 10.0 for windows (SPSS Inc, Chicago, IL), which comprise many "procedures"—graphical, statistical, reporting, processing, and tabulating procedures—that enable simple and rapid data evaluation. Statistical tests were made by the PRIMER program, version 1: differences from basal or control values (as shown in the plots) were classed as significant [*] where $p < 0.05$ and as highly significant [**] where $p < 0.001$.

RESULTS

The major constituents of essential oils within Rhizoma Curcumae are curdione, curcumol, and germacrone (**Figure 1**), which could be determined by HPLC and resolved in a single chromatography (**Figure 2**). These chemical markers are chosen because they represent over 15% of essential oils in the rhizome. The HPLC calibration curves of curdione, curcumol, and germacrone exhibited good linearity in a range from 10 to ∼1000 μg/mL. The correlation coefficients of these essential

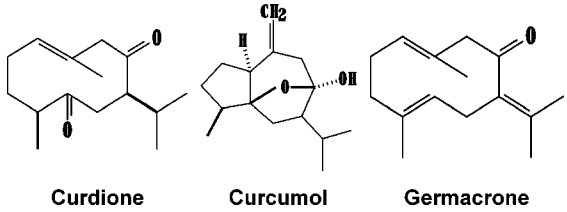

Figure 1. Chemical structures of the major constituents in the essential oils of Rhizoma Curcumae: curdione, curcumol, and germacrone.

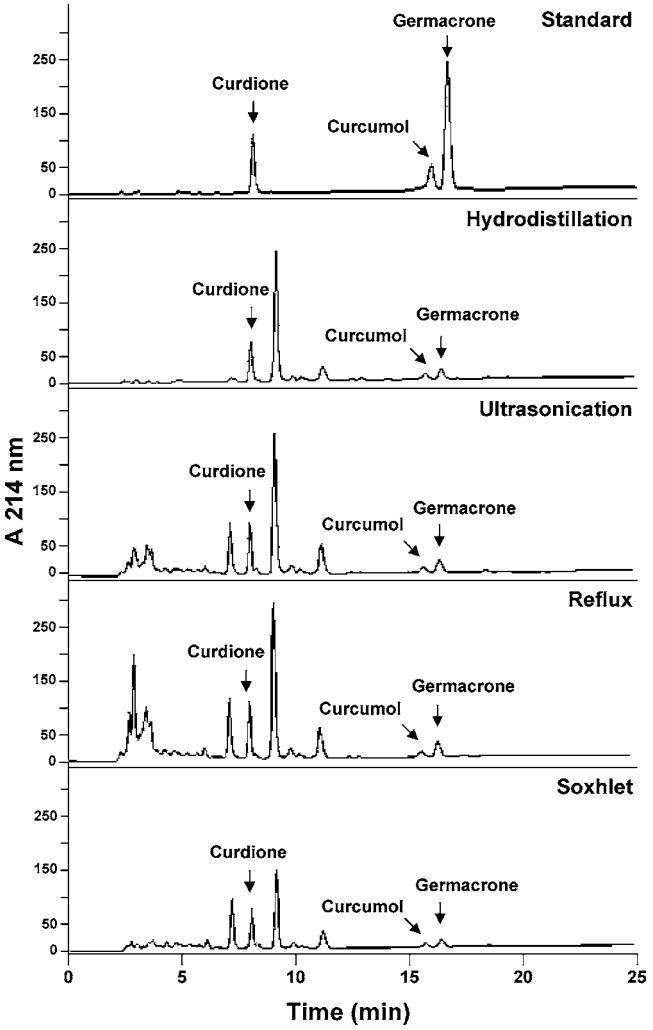

Figure 2. Determinations of curdione, curcumol, and germacrone from Rhizoma Curcumae. By using a C18 column in HPLC analyses, the amount of essential oils was determined in extracts from hydrodistillation, ultrasonication, reflux, and Soxhlet extractions. Details of the extraction and HPLC running condition are stated in Materials and Methods. Absorbance at 214 nm was recorded. Pure markers for curdione, curcumol, and germacrone are indicated. Typical chromatograms are shown.

oils were 0.9998 (curdione), 0.9997 (curcumol), and 0.9993 (germacrone). The precision test within the same sample of chemical markers ($n = 5$) showed the relative standard deviation (RSD) values of 1.4% (curdione), 2.1% (curcumol), and 2.2% (germacrone). The short-term (12 h) and long-term (24 h) repeatabilities of curdione, curcumol, and germacrone were calculated for five determinations ($n = 5$). The RSD of short (or long) term repeatability for curdione, curcumol, and germacrone was 1.1% (2.2%), 2.5% (3.1%), and 2.6% (2.8%), respectively. The recovery experiment was carried out to evaluate the accuracy of the method. Known amounts of curdione, curcumol, and germacrone were added to the herbal

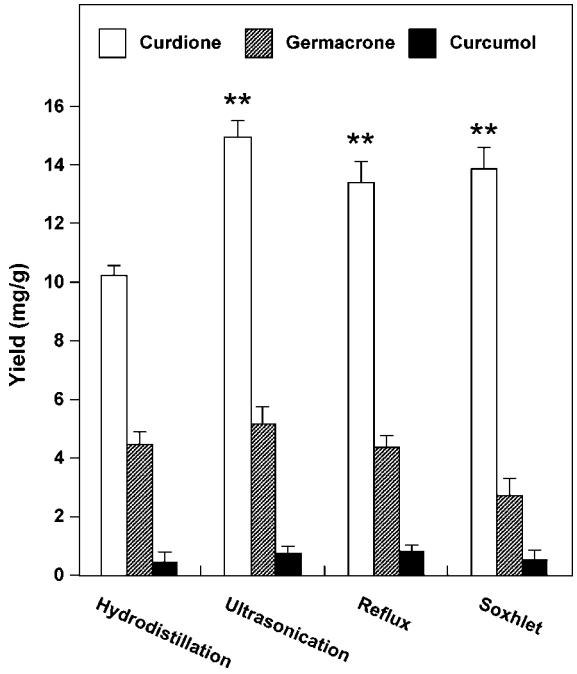

Figure 3. Amounts of curdione, curcumol, and germacrone in different extracts of Rhizoma Curcumae. The optimized conditions in each extraction method are described in the text. The starting material, 0.5 g rhizome of *C. wenyujin*, was used. The amounts of curdione, curcumol, and germacrone determined as in **Figure 2**. The yield values of mg/g are in means ±SD, $n = 5$, where ** $p < 0.001$ by comparing to hydrodistillation.

sample and were extracted by using the ultrasonication method accordingly; the extracted material was subjected to analysis, and the contents of these compounds were calibrated. The average recoveries of the tested chemicals were from 95.7 to 98.9% ($n = 5$).

By determining the contents of curdione, curcumol, and germacrone, the optimized extraction condition was revealed for Rhizoma Curcumae. Four extraction methods were tested: hydrodistillation, ultrasonication, reflux, and Soxhlet. In different extractions, an orthogonal array design was used to determine the effects of particle size (0.1−0.21 mm), solvent nature (water, petroleum ether, ethyl acetate, methanol), solvent volume (10−150 mL), and extraction time (1−10 h) in recovering curdione, curcumol, and germacrone (data not shown). The optimized condition of individual method in extracting 0.5 g sample was hydrodistillation of having 0.1-mm particle size in 100 mL water for 6 h, ultrasonication of having 0.1-mm particle size in 20 mL methanol for 0.5 h, reflux extraction of having 0.1-mm particle size in 20 mL methanol for 2 h, and Soxhlet extraction of having 0.1-mm particle size in 150 mL petroleum ether for 6 h. **Figure 2** shows typical HPLC profiles of Rhizoma Curcumae (*C. wenyujin*) under different extraction methods. The peaks of curdione, curcumol, and germacrone were distinct, which were identified by two means: (i) by comparing the retention times of the unknown peaks with those of the standards eluted under the same conditions and (ii) by spiking the sample with stock standard solutions of chemical markers (data not shown).

On the basis of the yield of three chemicals, one-way ANOVA analysis was performed to compare the extraction efficiency of four methods. Overall speaking, the order of extraction efficiency was ultrasonication > reflux extraction > Soxhlet extraction > hydrodistillation (**Figure 3**). Hydrodistillation was significantly lower in terms of the chemical composition among the four methods. Although the efficiency of ultrasonication in extracting curdione and germacrone was higher than that of reflux or Soxhlet extraction, the differences were small. A previous study showed that curdione was unstable during heating (*17*). As expected, a rise in the temperature of extraction could lead to a decrease of the oil yield (*5*, *18*). Here, the yields of chemical markers were decreased in general with the extended extraction time at high temperature, which was in accordance with those of previous results. The extraction by ultrasonication was performed at room temperature and at a shorter time, which avoided the influence of heat on the oil contents of Rhizoma Curcumae, and therefore it was a desirable extraction method for further analysis.

Rhizoma Curcumae is the rhizomes from *C. phaeocaulis*, *C. kwangsiensis*, and *C. wenyujin*. Historically, the best quality of Rhizoma Curcumae is derived from *C. wenyujin* or *C. phaeocaulis*. Having the improvement of the farming method, Rhizoma Curcumae (mainly *C. wenyujin* rhizome) produced in the Zhejiang province of China had a better reputation than other species. By using the optimized ultrasonic extraction as described in **Figure 3**, the contents of curdione, germacrone, and curcumol were determined by HPLC from different members of Rhizoma Curcumae; these rhizomes were also from different cultivated regions in China as listed in **Table 1**. Nineteen populations of Rhizoma Curcumae from five *Curcuma* species and different geographical regions were collected; each chosen population contained 10−15 different batches of samples. The chosen five species represent the major population of *Curcuma* in China. Among all tested samples, only *C. wenyujin* rhizome cultivated from Leqing, Zhejiang contained a detectable amount of curcumol (**Table 1**). In addition, rhizome from *C. wenyujin* also contained, significantly, the highest amount of germacrone and curdione (on average, from 3 to 5-fold difference) than the other two species. The two subregions, Leqing and Yongjia of Zhejiang, showed similar amounts of the essential oils, except for curcumol. *C. phaeocaulis* is mainly cultivated in the Sichuan province, and its amount of the essential oils was higher than that of *C. kwangsiensis*. Despite the different subregions where *C. phaeocaulis* could be cultivated, they had comparable amounts of the constituents. Rhizome from *C. kwangsiensis*, either from Guangxi or Yunnan, contained the least amount of germacrone and curdione. Not only the low levels of the analyzed markers but also the variation among different subregions of farming was rather high. On the other hand, the common adulterants, or substitutes, of Rhizoma Curcumae including *C. longa* and *C. chuanyujin* showed a lower amount of germacrone and curdione (**Table 1**).

The quality of crude drugs is closely related to their chemical constituents, and thus the identification of herb could be assessed by a chemical pattern recognition method. By using HPLC fingerprinting, different samples of Rhizoma Curcumae were analyzed and their profiles were compared (**Figure 4A**). Within the same HPLC analytic condition, 53 constituents including curdione (at retention time 18.22 min), curcumol (25.45 min), and germacrone (31.44 min) were identified in different herbal samples. These 53 constituents were calibrated for their relative retention times by using the peak at ∼11 min retention time as a reference point of $t_R' = 1$; this peak was common to all samples (**Figure 4A**). By using hierarchical clustering analysis, a matrix of 19 (number of samples) × 53 (number of chosen HPLC peaks) was obtained, which gave the differences among the tested 19 populations, as listed in **Table 1**. Besides the known peaks for curdione, germacrone, and curcumol, the following peaks, although not known for their identities, are unique to different *Curcuma* species: $t_R' = 1.43$, 1.65, 4.25,

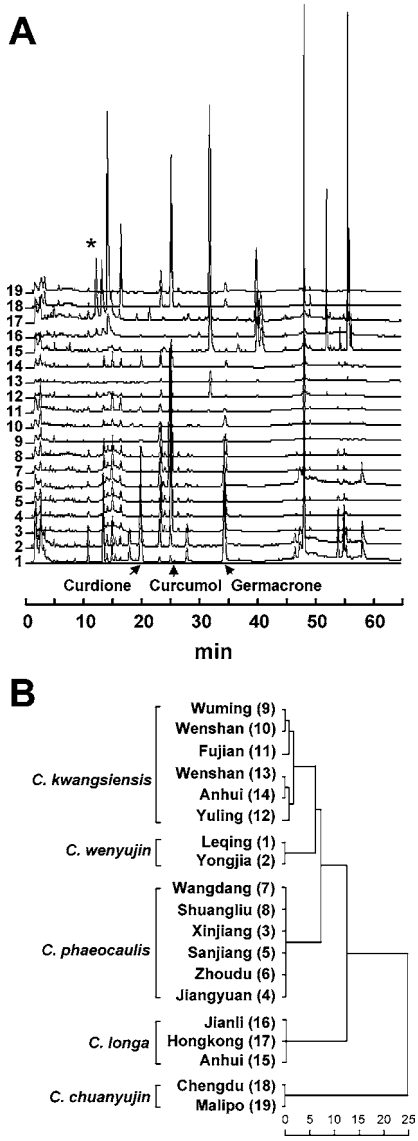

Figure 4. Chemical fingerprinting and cluster analysis of *Curcuma* rhizomes. (**A**) Superimpose of HPLC fingerprints from the 19 samples including five different species of *Curcuma* rhizomes. The HPLC condition was similar to that of essential oil determination as in **Figure 2** except for the mobile phase. Solvents were A: acetonitrile and B: water. The gradient of A:B was 0∼3 min, 45:55; 3∼30 min, 45:55 gradient up to 65:35; 30∼38 min, 65:35; 38∼45 min, 65:35 gradient up to 90:10; 45∼55 min, 90:10 gradient up to 100:0; 55∼65 min, 100:0. These samples were chosen randomly from various populations, one sample from each population, as listed in **Table 1**. The relative retention times (t_R') at 1 are denoted by an ∗. Peaks for curdione, curcumol, and germacrone are indicated. (**B**) The hierarchical clustering analysis of different *Curcuma* rhizomes. The clustering was done by SPSS software from 53 HPLC peaks analyzed from the 19 *Curcuma* rhizomes; these samples matched with the HPLC fingerprints as in **A**. Rescale cluster distance is indicated.

and 4.34 for *C. wenyujin*; $t_R' = 2.19$ and 2.40 for *C. phaeocaulis*; $t_R' = 0.68$ and 0.94 for *C. kwangsiensis*; $t_R' = 0.47$, 0.70, 1.12, 1.96, 2.90, 3.63, and 3.69 for *C. longae*; $t_R' = 0.53$ for *C. chuanyujin*. Besides $t_R' = 1.00$ peak, the peak at $t_R' = 1.38$ was also revealed in all five species.

A method named as the average linkage between groups was applied, and Pearson correlation was selected as the measurement, and the result is shown in **Figure 4B**. From the cluster analysis, the tested 19 populations of Rhizoma Curcumae could be grouped into five main clusters: samples 9−14 (compromising *C. kwangsiensis* only) as cluster one, samples 1−2 (compromising *C. wenyujin* only) as cluster two, samples 3−8 (compromising *C. phaeocaulis* only) as cluster three, samples 15−17 (compromising *C. longa* only) as cluster four, and samples 18−19 (compromising *C. chuanyujin* only) as cluster five. The cluster analysis agrees very well with the results of classic taxonomy for genus *Curcuma*. This analysis therefore could be used as a mean in finding out the botanical source of Rhizoma Curcumae.

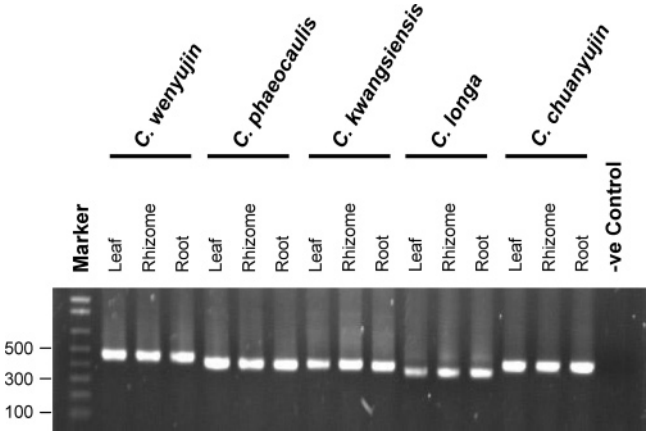

Figure 5. PCR analysis of five *Curcuma* species. PCR products were generated by primers (5S-P1 and 5S-P2) on the DNAs isolated from *C. wenyujin*, *C. longa*, *C. phaeocaulis*, *C. kwangsiensis*, and *C. chuanyujin*. The PCR products were separated in a 1% agarose gel and were visualized by ethidium bromide staining. The negative control is the one without genomic DNA. DNA markers are indicated.

The genetic characteristic of the five *Curcuma* plants was also determined. The 5S-rRNA spacer domain was selected for analysis; this spacer represents the unique intronic sequences that have been commonly used for species authentication (*13*−*15*). Primers flanking the spacer domain of 5S-rRNA were used in PCR analyses of genomic DNAs isolated from plant tissues of *C. wenyujin*, *C. phaeocaulis*, *C. kwangsiensis*, *C. longa*, *and C. chuanyujin*. *C. longa and C. chuanyujin* are common substitutes or adulterants of Rhizoma Curcumae, and therefore they were selected for comparison. In *C. wenyujin*, *C. phaeocaulis*, *C. kwangsiensis*, and *C. chuanyujin*, PCR products having ∼450 bp were detected in agarose electrophoresis. However, an ∼350 bp PCR product was revealed in *C. longa*. Over 10 individual plants from the same species were analyzed, and the same PCR product was generated from either leaf, or rhizome, or root (**Figure 5**). The PCR products were subcloned and sequenced. Several individual clones of the same PCR product were sequenced as to avoid any mutation introduced by *Taq* polymerase. The respective lengths of 5S-rRNA spacer domains in different species are *C. wenyujin* 361 bp (GenBank accession number is AF548125), *C. phaeocaulis* 352 bp (AY962526), *C. kwangsiensis* 343 bp (AY962527), *C. longa* 267 bp (AF548126), and *C. chuanyujin* 342 bp (AY962528) (**Figure 6A**). The DNA fragments were sequenced in both directions several times. A consensus sequence was obtained by aligning different individual clones from the same species. An identical sequence was obtained when the amplification was done with DNA isolated from leaf, rhizome, or root of the same species (data not shown). Each species had a unique sequence in the 5S-rRNA spacer domain; they could be easily discriminated at the DNA level. In addition, **Figure 6B** shows a phylogenetic tree obtained by comparing the sequence identity of 5S-rRNA spacer among the five *Curcuma* species. Significantly, the members of Rhizoma Curcumae, that is, *C. wenyujin*,

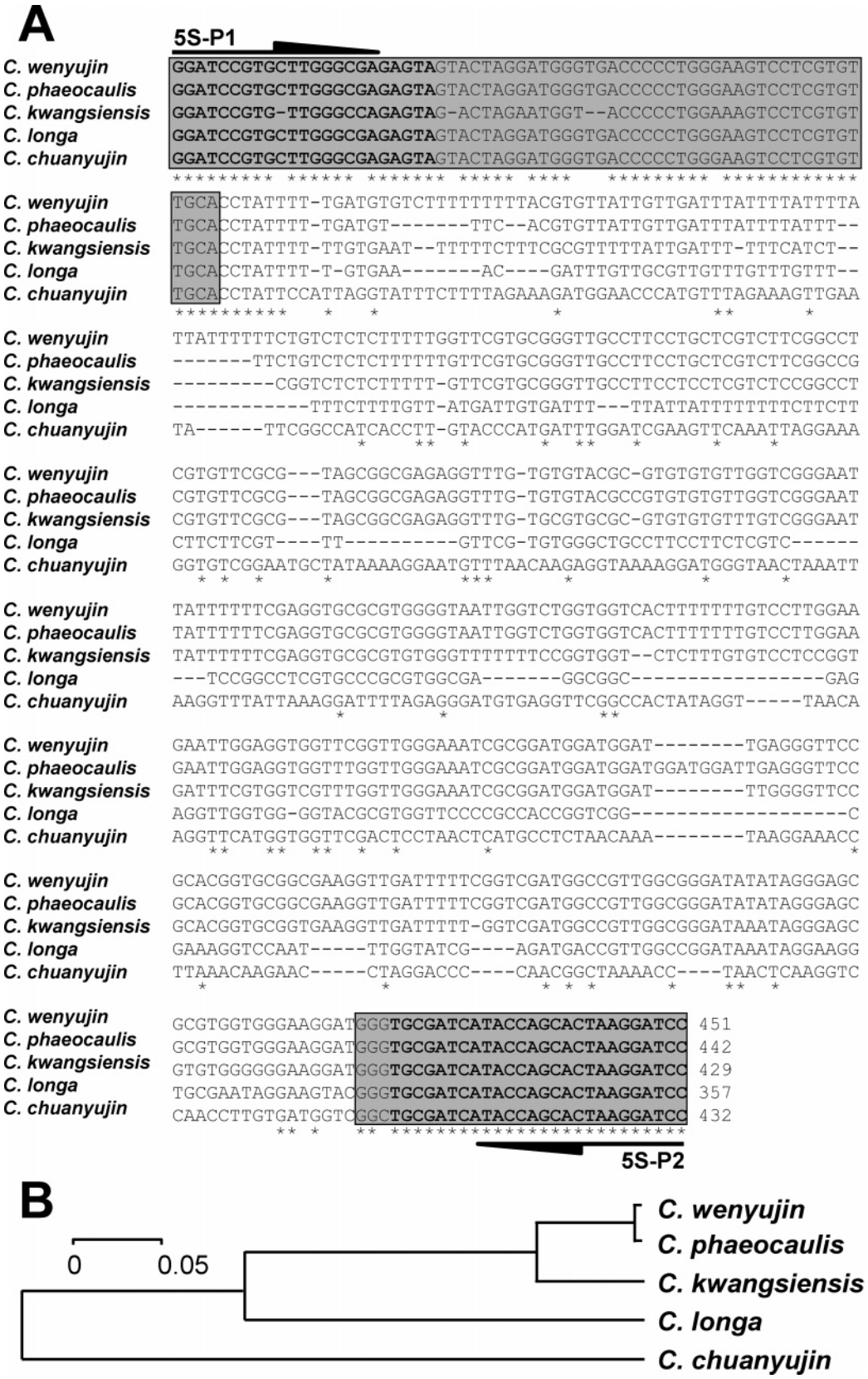

Figure 6. Sequence analysis of the spacer domains of 5S-rRNA genes from *Curcuma* species. (**A**) Sequence alignments of the 5S-rRNA spacer domains. The coding regions are boxed. Primers used for amplification are indicated by an arrow. Identical sequences are indicated by an ∗. Gaps (–) are introduced for the best alignment. (**B**) Phylogenetic tree for the five species of *Curcuma* plants assessed by the UPGMA method. The highest identity is between *C. wenyujin* and *C. phaeocaulis*. The distance corresponding to 0.05 sequence divergence is indicated by a bar.

C. phaeocaulis, and *C. kwangsiensis*, are in a distinct group of which *C. wenyujin* and *C. phaeocaulis* show the closest DNA homology of ∼95% identity. In contrast, *C. longa* and *C. chuanyujin* show a unique 5S-rRNA spacer, and they have only 50−55% DNA identity as compared to the other three members of Rhizoma Curcumae. Interestingly, the phylogentic result by

DNA analysis is similar to the clustering result generated by chemical fingerprints.

DISCUSSION

Precise identification of crude drugs is a prerequisite for chemical and pharmacological investigations of traditional Chinese medicine and for their clinical applications. Besides, this requirement is also important in achieving good agricultural practice (GAP) farming of herbal medicine in China. In case of Rhizoma Curcumae here, both chemical fingerprint and genetic analysis are used for identification, and the results show that *C. wenyujin*, *C. phaeocaulis*, and *C. kwangsiensis* are closely related. Indeed, these three species are being used as Rhizoma Curcuma. In contrast, *C. longa* and *C. chuanyujin* are rather separated from the members of Rhizoma Curcumae. Although chemical and genetic methods are two rather distinct analyses, their results are in line with each other suggesting the unique characteristic of the herb. Thus, both chemical and genetic methods could be used as tools in identifying the correct species of *Curcuma*.

The root of *Curcuma* plant, instead of rhizome, could also be used as herbal medicine, which is known as Radix Curcumae. According to Chinese Pharmacopoeia (*2*), Radix Curcumae should be the dry root tubers of *C. wenyujin*, *C. longa*, *C. phaeocaulis*, and *C. kwangsiensis*. Radix Curcumae was recorded in *Tang Ben Cao* by Su Jing (659 A.D.) for the first time but no description of the plant morphology was given. Subsequently, Tang Shen Wei (1108 A.D.) in *Zheng Lei Ben Cao* and Li Shi Zhen (1596 A.D.) in *Ben Cao Gang Mu* described the morphology of Radix Curcumae and provided detailed drawings of the plant. The described species, at that time, was referring to *C. longa* but not to other *Curcuma* plants, and the pharmaceutical part was the rhizome and not the root. Only in the 17th century were other *Curcuma* plants such as *C. wenyujin*, *C. phaeocaulis*, *C. kwangsiensis*, and *C. chuanyujin* used as Radix Curcumae, and the pharmaceutical part was also gradually changed from the rhizome into the root tuber.

The taxonomic positions of *C. wenyujin* and *C. chuanyujin* have been a controversial issue for a long time. *C. chuanyujin* is mainly cultivated by large-scale farming in Sichuan China, and its rhizome is commonly used in Sichuan as a substitute of Rhizoma Curcumae. This plant is also named as *Curcuma sichuanensis* X.X. Cen. *C. chuanyujin* blooms after leaves grow in autumn and inflorescence develops from the center of leaf thickets. On the other hand, *C. wenyujin* is cultivated primarily in the Zhejiang province, which blooms before leaves grow in spring and inflorescence develops from the lateral side of the rhizome. In classical taxonomy, the growing position of inflorescence is used as an important taxonomical marker. Because of this morphological difference, *C. wenyujin* and *C. chuanyujin* were named as two distinct species (*19*). However, a recent report using random amplified polymorphic DNA (RAPD) analysis has suggested that *C. wenyujin* and *C. chuanyujin* could be deriving from the same species (*20*). According to our results, the differences of 5S-rRNA spacer between *C. wenyujin* and *C. chuanyujin* are quite larger; the genetic distance between them (0.355) is the longest among the five species (**Figure 6B**). The interpretation by using RAPD could be an error by using a pair of primers that is commonly flanking the same fragment between the genomic DNAs of *C. wenyujin* and *C. chuanyujin*. Indeed, this is a common limitation of using RAPD instead of sequence analysis. In parallel to the 5S-rRNA spacer analysis, the hierarchical clustering analysis on the chemical constituents in rhizomes of *C. wenyujin* and *C. chuanyujin* also suggests that they are two distinct species. Thus, our result strongly supports the naming of *C. wenyujin* and *C. chuanyujin* as two independent species.

The sequences of 18S rRNA and *trn*K genes have been reported in *Curcuma* species (*21*). Similar to our conclusion revealed by using 5S-rRNA spacer domain, the species distinction could be clearly resolved genetically, in particular, the resolution between *C. wenyujin* and *C. longa*. However, the genetic variation of the 5S-rRNA spacer is superior than that of 18S rRNA and *trn*K sequences; the genetic variation of 18S rRNA and *trn*K genes in *Curcuma* species is very small, for example, only one base difference among the six tested *Curcuma* species in the ~1.8 kb 18S rRNA sequence (*21*).

Molecular genetic methods have several advantages over classical authentication by using morphological and chemical analyses. First, the genetic method requires genotype instead of the phenotype; therefore, it will not be changed according to the environment. Second, most of the traditional Chinese medicinal herbs require processes such as drying, heating, or long-term storage. Under some circumstances, the herbs have to be ground into powder before medication. This could make morphological identification impossible. Third, a small amount of rhizome is needed for DNA analysis, and it could be in different forms. Last, the molecular genetic approach provides a faster and more precise method to identify traditional Chinese medicinal herbs, which meets the needs of the present market.

ACKNOWLEDGMENT

This work is part of a project on the study of some common Chinese Material Medica in Hong Kong being undertaken by the Department of Health, Hong Kong SAR Government, China.

LITERATURE CITED

(1) Lang, W. J. *A thousand formulae of prepared decoctions for anti-cancer*; Chinese Medicinal Technology Press: Beijing, China, 1992; pp 119−123.

(2) In *Pharmacopoeia of the People's Republic of China*; Zheng, X. Y., Ed.; Chemistry Industry Press: Beijing, China, 2000; p 230.

(3) Xiao, P. G. *Modern Chinese Materia Medica*; Chemical Industry Press: Beijing, China, 2002; Vol. I, pp 772−773.

(4) Xu, G. J.; Xu, L. S. *Species Systematization and Quality Evaluation of Commonly Used Chinese Traditional Drugs, South-China Edition Volume I*; Science and Technology Press of Fujian: Fuzhou, China, 1994; pp 385−394.

(5) Chen, Y. H. The studies on the pharmaceutical plants of family *Zingiberaceae* in China. *J. Chin. Medicinal Mater.* **1986**, *4*, 20.

(6) Xia, W. J.; Xiao, X. H.; Liu, F. Q.; Su, Z. W.; Qiao, C. Z. Determination on chemical constituents of *Curcuma* L. produced in China. *China J. Chin. Mater. Med.* **1999**, *24*, 423−425.

(7) Nie, X. H.; Ao, Z. H.; Yin, G. Y.; Tao, W. Y. Effect of extraction techniques on the components and antitumor activity *in vitro* of volatile oil from *Curcuma wenyujin*. *Pharm. Biotechnol.* **2003**, *10*, 152−154.

(8) Xia, Q.; Huang, Z. G.; Li, S. P.; Zhang, P.; Wang, J.; He, L. N. Antivirus effects of Ezhu oil on influenza virus and respiratory syncytial virus. *Chin. Pharmacol. Bull.* **2004**, *20*, 357−238.

(9) Zheng, S.; Yang, H.; Zhang, S.; Wang, X.; Yu, L.; Lu, J.; Li, J. Initial study on naturally occurring products from traditional Chinese herbs and vegetables for chemoprevention. *J. Cell Biochem.* **1997**, *27*, (Suppl), 106−112.

(10) Wang, Y.; Wang, M. Z. Study on the quality of *Rhizoma Curcumae*. *Acta Pharm. Sin.* **2001**, *36*, 849−853.

(11) Manzan, A. C.; Toniolo, F. S.; Bredow, E.; Povh, N. P. Extraction of essential oil and pigments from *Curcuma longa* L. by steam distillation and extraction with volatile solvents. *J. Agric. Food Chem.* **2003**, *51*, 6802−6807.

(12) Vinatoru, M.; Toma, M.; Radu, O.; Filip, P. I.; Lazurca, D.; Mason, T. J. The use of ultrasound for the extraction of bioactive principles from plant materials. *Ultrason. Sonochem.* **1997**, *4*, 135−139.
(13) Gerlach, W. L.; Dyer, T. A. Sequence organization of the repeating units in the nucleus of wheat contains 5s-rRNA genes. *Nucleic Acids Res*. **1980**, *8*, 4851−4865.
(14) Cai, Z. H.; Li, P.; Dong, T. X.; Tsim, K. W. K. Molecular diversity of 5S-rRNA spacer domain in *Fritillaria* species revealed by PCR analysis. *Planta Med*. **1999**, *65*, 360−364.
(15) Zhao, K. J.; Tina, D. T. X.; Tu, P. F.; Song, Z. H.; Lo, C. K.; Tsim, K. W. K. Molecular genetic and chemical assessment of Radix Angelica (Danggui) in China. *J. Agric. Food Chem.* **2003**, *51*, 2576−2583.
(16) Sambrook, J.; Russell, D. W. *Molecular Cloning: a laboratory manual*, 3rd edition; Sambrook, J., Russell, D. W., Eds.; Cold Spring Harbor Laboratory Press: New York, 2001; Vol. 1.
(17) Hu, J. H.; Han, X. W.; Ji, T.; Yang, Z. Y.; Wu, X. Y.; Wen, J. H. A new curcumalactone and its structure. *Chin. Sci. Bull.* **1986**, *31*, 832−835.
(18) Began, G.; Goto, M.; Kodama, A.; Hirose, T. Response surfaces of total oil yield of turmeric (*Curcuma longa*) in supercritical carbon dioxide. *Food Res. Int.* **2000**, *33*, 341−345.
(19) Chen, X. X. A new species of genus *Curcuma*. *Guihaia* **1984**, *4*, 133.
(20) Chen, Y. H.; Bai, S. M.; Cheng, K. D.; Zhang, S.; Liu, N. RAPD analysis on *Curcuma wenyujin* and *C. chuanyujin*. *China J. Chin. Mater. Med.* **1999**, *24*, 131−133.
(21) Cao, H.; Yohei, S.; Hirotoshi, F.; Katsuko, K. Molecular analysis of medicinally-used Chinese and Japanese *Curcuma* based on 18S rRNA gene and *trn*K gene sequences. *Biol. Pharm. Bull.* **2001**, *24*, 1389−1394.

Received for review April 14, 2005. Revised manuscript received May 23, 2005. Accepted May 25, 2005. The research was supported by grants from the Area of Excellence Scheme established under the University Grants Committee of Hong Kong (AoE/B-10/01) to K.W.K.T. and National Natural Science Foundation of China (Grant# 30271592) to K.J.Z..

JF0508495