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Full Length Research Paper

Relationships among six medicinal species of *Curcuma* assessed by RAPD markers

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Random amplified polymorphic DNA (RAPD) markers were applied to detect the genetic relationships and diversity among 33 accessions of *Curcuma* species in China, which involved six medicinal species. A total of 115 products were amplified by 21 primers, among which 106 products (92.17%) were found to be polymorphic. A total of 3 to 8 polymorphic bands were amplified by each polymorphic primer, with an average of 5.48 bands. The data of 115 RAPD bands were used to generate Jaccard's similarity coefficients and to construct a dendrogram by means of UPGMA. The results show that the genetic similarity coefficient of these six species is relatively large, from the cluster diagram, while the genetic relationships are not associated with their geographical distributions.

Key words: *Curcuma*, cluster analysis, genetic diversity, random amplified polymorphic DNA, similarity coefficient.

INTRODUCTION

The genus *Curcuma* is a member of the ginger family (Zingiberaceae), which comprises over 70 species of rhizomatous perennial herbs. It has a widespread occurrence in the tropics of Asia and extends to Africa and Australia. It is endowed with widespread adaptation from sea level to an altitude as high as 2000 m in the Himalayas. More than 10 species are distributed in China, out of which 6 species can be used as herbal drugs. *Curcuma* is gaining importance worldwide as a potential source of new drug(s) to combat a variety of ailments as the species contain molecules credited with anti-inflammatory, hypocholestraemic, choleratic, antimicrobial, insect repellent, antirheumatic, antifibrotic, antivenomous, antiviral, antidiabetic and antihepatotoxic properties, as well as anticancerous properties (Sasikumar, 2005). Three traditional Chinese medicines, *Radix Curcumae* (also named Yujin), *Rhizoma Curcumae Longae* (also named Jianghuang) and *Rhizoma Curcumae* (also named Ezhu), are derived from *Curcuma*. In the 2010 edition of the Chinese Pharmacopoeia, *R. curcumae* came from the radix of *Curcuma wenyujin* (Chen et al., 1999), *Curcuma longa* L., *Curcuma kwangsiensis* S. G. Lee et C. F. Liang and *Curcuma phaeocaulis* Valeton; *R. Curcumae Longae* came from the rhizome of *C. longa* L.; and R. *Curcumae* came from the rhizome of *C. phaeocaulis*, *C. kwangsiensis* and *C. wenyujin*. However, the radix of *Curcuma sichuanensis* C. K. Hsich et H. Zhang and *Curcuma chuanhuanjiang* (Zhu, 1992) can also be used as *R. Curcumae* (Cheng, 1984; Zhu, 1992), and the rhizome of *C. chuanhuanjiang*, *C. sichuanensis* and *C. wenyujin* are always used as *R.*

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Curcumae Longae in folk therapy.

As various parts of the same species can be used as different drugs, and as the same drug comes from several species, confusion persists on the taxonomy of these *Curcuma* species. Furthermore, by distributing this species in a large area, phenotypic variation of morphological characters of rhizomes and leaves are very common in this species, in that such phenotypic plasticity of the species can lead to wrong taxonomic treatment of individuals. However, the correct identity is important to confirm the sources of origin of herbal drugs within the genus *Curcuma* (Cao et al., 2001; Sasaki et al., 2002). It is necessary to adopt various methods to identify the sources of origin of different *Curcuma* species and evaluate their genetic relationship.

Molecular markers have proved to be valuable tools in the characterization and evaluation of genetic diversity within and between species and population. The random amplified polymorphic DNA (RAPD) technique (Williams et al., 1990) is a popular tool in genetic studies. RAPD markers provide a rapid, inexpensive and effective system for studying plant genetic relationships. RAPD have been used to detect some *Curcuma* plants (Chen et al., 1999; Xiao et al., 2000; Syamkumar et al., 2007). The objectives of this paper are to estimate the genetic diversity of six medicinal species of *Curcuma* distributed in China by using RAPD analysis and provide a basis for identifying these species.

MATERIALS AND METHODS

Plant materials

Thirty-three accessions of genus *Curcuma*, distributed in six species, were analyzed in this study (Table 1). As Sichuan and Guangxi belong to the famous regions of these species, the materials studied here were collected from the two provinces. Twenty-six accessions were collected from different localities in Sichuan, while 7 accessions were collected from Guangxi Medicinal Botanical Garden of Guangxi. All the materials are currently grown in the farmland of Sichuan Agricultural University, China.

DNA extraction and polymerase chain reaction (PCR) amplification

Fresh tender leaves of *Curcuma* species were used for the isolation of DNA. The genomic DNA was isolated by CTAB method (Doyle and Doyle, 1987). The extraction buffer contains 2% CTAB, 1.5 M NaCl, 100 mM Tris-HCl pH 8.0 and 20 mM EDTA. RAPD reaction was carried out in 25 µl reaction volume containing 20 ng genomic DNA, 1 U Taq DNA polymerase, 200 M dNTPs, 2 mM $MgCl_2$ and 10 p-moles of random decamer primer (Boracker, Chengdu). Nonetheless, the amplification condition consisted of pre-denaturation at 94°C for 4 min, denaturation at 94°C for 1 min, annealing at 36°C for 1 min, extension at 72°C for 1 min with 40 cycles, and final extension at 72°C for 10 min. DNA amplification was performed by the use of a MJ Research Inc., while PCR products were separated on 1.0% agarose gel and visualized by ethidium bromide staining.

RAPD data analysis

Photographs were used to score the RAPD data. For each material of the x primer combination, the presence (1) or absence (0) of an amplified fragment was treated as an independent character without considering the quantitative aspects of the results, that is, band intensity. The data matrix was entered into the NTSYS-pc program (Rohlf, 1993). Genetic similarities (GS) among the 33 germplasm materials were calculated based on Jaccard's coefficient using the Simqual (similarity for qualitative data) method. GS was used to construct a dendrogram via the unweighted pair group method with arithmetic average (UPGMA) and the SHAN (sequential, hierarchical, agglomerative, and nested clustering) routine in the NTSYS program.

RESULTS

Thirty 10-arbitrary primers were tested to select those that produced polymorphic DNA bands. Of the 30 primers tested, 21 (70%) produced polymorphic fragments. Various primers produced different fragments from the same template DNA. Figure 1 shows the results of amplification from primer 3.

The 21 primers produced 115 products, ranging from 3 to 8 bands per primer (Table 2), with 5.48 bands per primer. Of the 115 bands, only 9 (7.83%) fragments amplified were present in all the 33 accessions of genus *Curcuma*. A total of 106 products (92.17%) were found to be polymorphic, and about 3 to 8 polymorphic bands were amplified by each polymorphic primer (Table 2), with an average of 5.48. It indicated that there was considerable RAPD variation among species of genus *Curcuma*.

All the 115 bands were used to calculate the GS value among the 33 accessions. Although the GS value varied from 0.425 to 0.83, there was little genetic difference among the different accessions in the same species, while the genetic difference among the different species was distinct. The highest GS value existed between the 2 cultivated accessions of *C. sichuanensis*, which originated from Guangxi Medicinal Botanical Garden and Chongzhou respectively, and the 2 wild accessions of *C. phaeocaulis*, which originated from Xinjin and Chongzhou.

The GS values were used to generate a dendrogram (Figure 2) with UPGMA method. As seen from the dendrogram, at the GS value of 0.64, the 33 accessions were divided into three groups. Group I was composed of 27 accessions - 19 accessions of *C. longa*, 4 accessions of *C. sichuanensis* and 4 accessions of *C. phaeocaulis*; group II was composed of 4 accessions including 3 species, namely: *C. kwangsiensis*, *C. chuanhuangjiang* and *C. wenyujin*; and group III contained 2 accessions of *C. sichuanensis*. At the GS value of 0.66, group I can be divided into three subgroups. Ia contained 13 accessions, distributed in species of *C. longa* (5 accessions), *C. phaeocaulis* (4 accessions) and *C. sichuanensis* (4 accessions); Ib contained 12 accessions of *C. longa*, which came from Leshan, Qianwei, Shuangliu, Chongzhou,

Table 1. The origin of materials used in this study.

Number	Taxon	Origins	Notes wild
1	*Curcuma longa*	Ziyang, Sichuan	Cultivated
2	*C. longa*	Leshan, Sichuan	Wild
3	*C. longa*	GAP land, Chongzhou, Sichuan	Cultivated
4	*C. longa*	Chongzhou, Sichuan	Cultivated
5	*C. longa*	Guangxi Medicinal Botanical Garden, Guangxi	Wild
6	*C. longa*	Leshan, Sichuan	Cultivated
7	*C. longa*	Leshan, Sichuan	Cultivated
8	*C. longa*	Qianwei, Sichuan	Cultivated
9	*C. longa*	Yibin, Sichuan	Wild
10	*C. longa*	Guangxi Medicinal Botanical Garden, Guangxi	Cultivated
11	*C. longa*	Leshan, Sichuan	Cultivated
12	*C. longa*	Qianwei, Sichuan	Cultivated
13	*C. longa*	Leshan, Sichuan	Cultivated
14	*C. longa*	Chengdu, Sichuan	Cultivated
15	*C. longa*	Qianwei, Sichuan	Cultivated
16	*C. longa*	Shuangliu, Sichuan	Wild
17	*C. longa*	Guangxi Medicinal Botanical Garden, Guangxi	Cultivated
18	*C. longa*	Yibin, Sichuan	Wild
19	*C. longa*	Yibin, Sichuan	Wild
20	*C. phaeocaulis*	Xinjin, Sichuan	Wild
21	*C. phaeocaulis*	Shuangliu, Sichuan	Cultivated
22	*C. phaeocaulis*	Chongzhou, Sichuan	Wild
23	*C. phaeocaulis*	Qianwei, Sichuan	Cultivated
24	*C. sichuanensis*	Chongzhou, Sichuan	Cultivated
25	*C. sichuanensis*	Guangxi Medicinal Botanical Garden, Guangxi	Cultivated
26	*C. sichuanensis*	Chongzhou, Sichuan	Wild
27	*C. sichuanensis*	GAP land, Chongzhou, Sichuan	Cultivated
28	*C. sichuanensis*	Chongzhou, Sichuan	Cultivated
29	*C. sichuanensis*	Weiyuan, Sichuan	Cultivated
30	*C. kwangsiensis*	Guangxi Medicinal Botanical Garden, Guangxi	Wild
31	*C. kwangsiensis*	Guangxi Medicinal Botanical Garden, Guangxi	Cultivated
32	*C. wenyujin*	Guangxi Medicinal Botanical Garden, Guangxi	Cultivated
33	*C. chuanhuangjiang*	Jianyang, Sichuan	Cultivated

Chengdu and Yibin of Sichuan; moreover, all the places belong to the famous region of *C. longa*. Ic contained 2 accessions of *C. longa* that came from Yibin and Guangxi Medicinal Botanical Garden. In group II, the 2 accessions of *C. kwangsiensis* clustered together at the GS value of 0.746, while *C. chuanhuangjiang* and *C. wenyujin* clustered together at the GS value of 0.725, after which the 2 species were converged with *C. kwangsiensis* at the GS of 0.669. The 2 accessions of *C. sichuanensis* that came from Chongzhou were formed from group III. However, *C. longa*, *C. phaeocaulis* and *C. sichuanensis* were clustered in group I, which revealed that the genetic relationship of the three species was very close. The GS of *C. longa* and *C. phaeocaulis* ranged from 0.478 to 0.791, while the GS of *C. longa* and *C. sichuanensis* ranged from 0.513 to 0.783, which demonstrated that the genetic relationship between *C. longa* and *C. sichuanensis* was more closer than *C. longa* to *C. phaeocaulis*.

DISCUSSION

In this study, the dendrogram shows that the genetic relationship between *C. longa* and *C. sichuanensis* is very close, as *C. sichuanensis* always cohabits with *C. longa* and they have much similarity in floral, vegetative and

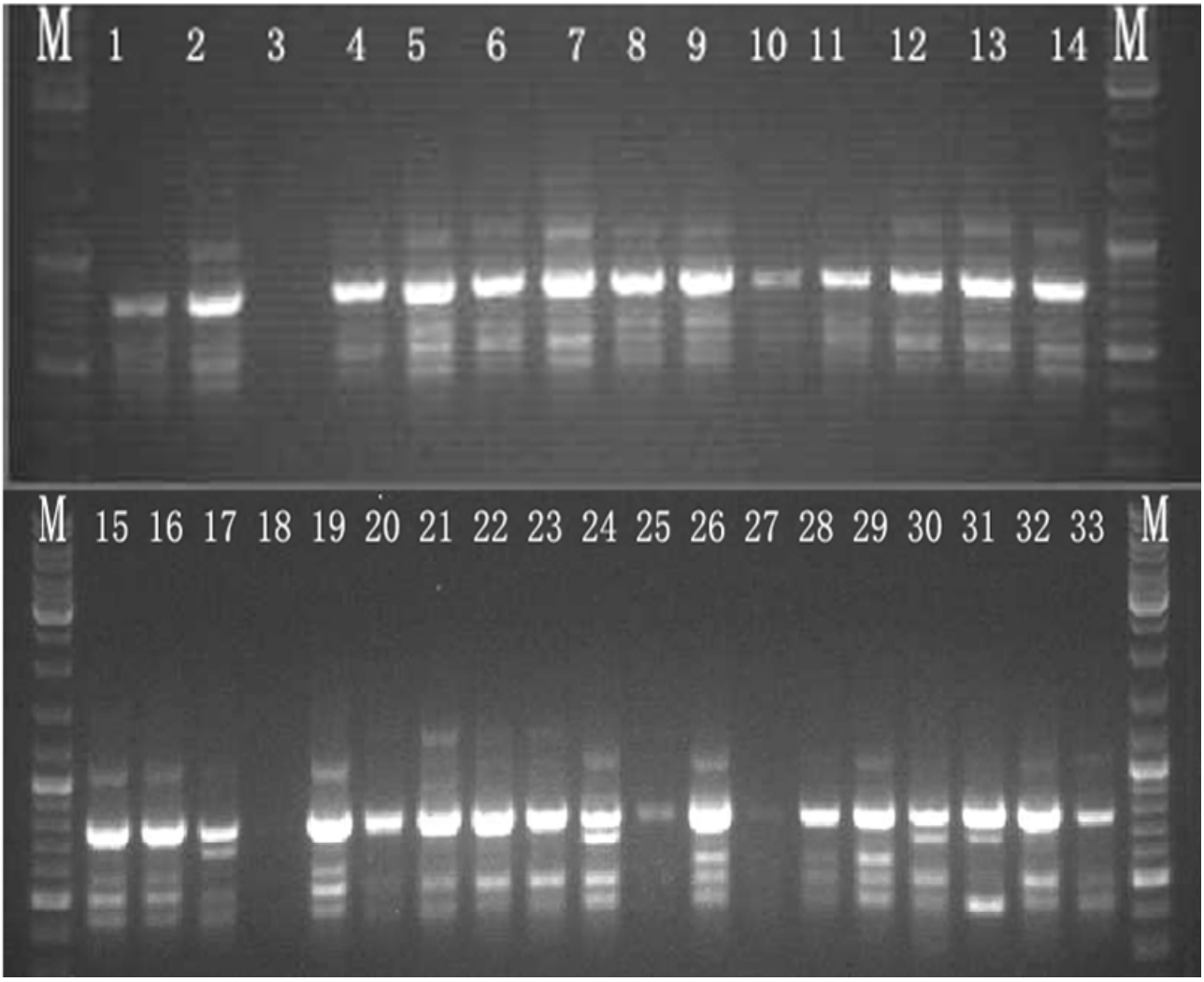

Figure 1. RAPD polymorphism in 33 *Curcuma* accessions with primer 3. Accessions 1 to 33 are described in Table 1. M - GeneRuler DNA Ladder Mix (Fermentas).

Table 2. RAPD primer and amplified results of the tested material.

Primes	Sequences 5'-3'	Total bands	Polymorphic bands	Primers	Sequences 5'-3'	Total bands	Polymorphic bands
1	TTCCCCGCGC	5	4	12	GGGCACGCGA	7	7
2	TGCCCCGAGC	7	6	13	GAGGGCGAGG	4	4
3	TTCCGGGTGC	5	5	14	GGGCACGCGA	8	7
4	TTGGCCGAGC	4	4	15	GAGCACCTGA	7	6
5	TTCCCCGTCG	4	3	16	GAGGTCCAGA	5	5
6	TTCCCCGCCC	4	4	17	GAGCACCAGG	8	8
7	GAGGGCGGGA	6	6	18	CCTGGGCTTT	5	5
8	AGGGGCGGGA	3	3	19	GGGGGGTTGG	5	4
9	GAGGGCGTGA	4	3	20	ATCCTGCCAG	8	8
10	GAGGGCGAGC	4	4	21	ATCGGGTCCG	7	6
11	GAGGGCAAGA	5	4	Total		115	106

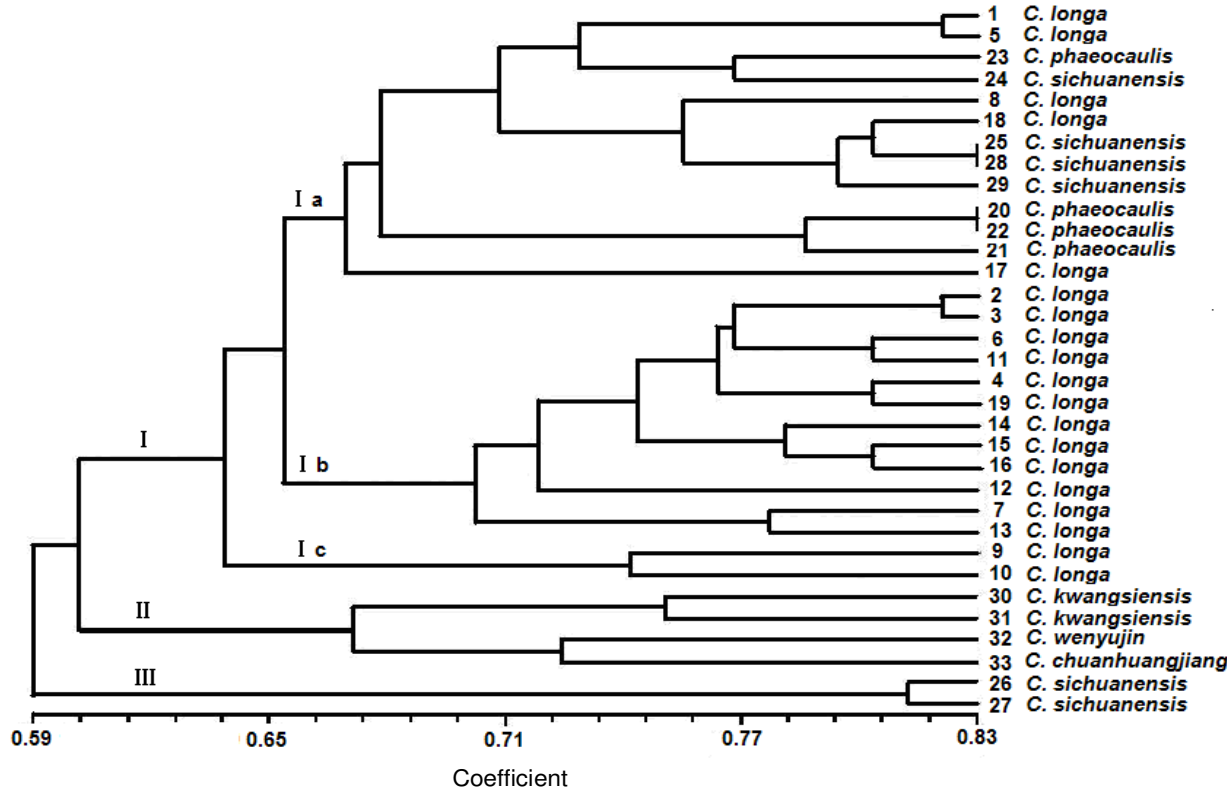

Figure 2. Dendrogram for *Curcuma* based on the GS of RAPD markers.

rhizome characters. The leaf blade is glabrous on the surfaces of both species, in that their inflorescences are terminal on pseudostems and their flowering occurs in autumn. Moreover, their cross sections colour of radix and rhizome is also similar to each other. Thus, we conclude that C. *sichuanensis* is probably a cultivated variety of C. *longa*. This speculation is consistent with previous studies of isozyme analysis (Tang et al., 2008). In the analysis of EST isozyme (Tang et al., 2008), the two species have the same zymogram. The RAPD (Xiao et al., 2000) histological and morphological analysis (Xiao et al., 2004) also suggested that they were very close to each other. However, the 2 accessions of *C. sichuanensis* in group III were far from the same species in group I. As such, there is need to further study the reason behind this.

The 2 accessions of *C. kwangsiensis,* numbered 30 and 31, have distinct morphologic variations, while the stem and leaf mid rib colour of the *C. kwangsiensis* numbered 30 was blue, and the other was mauve. Moreover, the natives thought that the *C. kwangsiensis* numbered 30 was a variety of *C. kwangsiensis*. So, Chen (1981) agreed with this view, but isozyme analysis indicated that they should belong to the same species (Tang et al., 2008). Thus, it is consistent with previous studies (Xiao et al., 2004; Liu and Wu, 1999). The 2 accessions of *C. kwangsiensis* clustered together at the similarity coefficient value of 0.746 in this dendrogram, revealed a rather low genetic variability between them, and the differences in the morphological characters might be caused by their growing environment.

Besides a detailed study depicted at the Flora of Sichuanica by Zhu (1992), *C. chuanhuangjiang* was not mentioned at the Flora of China. Zhu (1992) assumed that this species was just a form of *C. sp*. (Chen, 1981). Liu and Wu (1999) classified *C. sp* as *C. kwangsiensis*, although the isozyme analysis revealed that it should be an independent species (Tang et al., 2008). In the present study, *C. chuanhuangjiang* was first clustered with *C. wenyujin*, after which it was clustered with *C. kwangsiensis*, which revealed that it was more closely related to *C. wenyujin*. Thus, further study is needed for the classification of *C. chuanhuangjiang*.

In this study, the same species that came from different localities clustered together in the dendrogram reveal that the genetic relationships of these *Curcuma* species are not associated with their geographical distribution, and there is no separation of cultivated populations from wild populations.

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