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Evaluation on Genetic Relationships Among China's Endemic *Curcuma* Herbs by SRAP Markers

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Abstract: *Curcuma* is historically problematic to identify and clear their relationships for cultivar and variety appearance commonly in China. Sequence-Related Amplified Polymorphism (SRAP) marker was used to detect genetic relationships among 10 China's endemic *Curcuma* herbs including three Chinese rarely species firstly (*C. amarissima*, *C. flaviflora* and *C. yunnanensis*). The 2-35 polymorphic bands were amplified by each pair of primers, and a total of 1084 polymorphism bands with an average of 11.78 bands were produced by 93 out of 100 pair of primers within 29 accessions. The data were used to construct a dendrogram by means of COMPLETE in NTSYS–pc2.1. The results shown that the genetic distance coefficient varied from 0.12 to 0.53, *Curcuma* were divided into three groups, the placement of three rarely species were firstly achieved, and partial species might be muti-origin within Chinese *Curcuma*.

Keywords: *Curcuma*, Rare Species, Genetics, SRAP

1. Introduction

Curcuma L. (Zingiberaceae), is native to tropical areas of southeast Asia and south Asia with a few of which scattering in China, comprises approximately 120 species with many allopolyploids in the world [1, 2]. Numerous *Curcuma* species were exclusively used as herbs in Asia from ancient. The curcuminoids, extracted from *Curcuma* rhizomes, has been reported on vital medicinal qualities, including antioxidant, anti-cancer, anti-Alzheimer's, and anti-tumor effects [3–5]. About 12 species, *C. longa*, *C. sichuanensis*, *C. amarissima*, *C. yunnanensis*, *C. phaeocaulis*, *C. kwangsiensis*, *C. aromatica*, *C. wenyujin*, *C. flaviflora*, *C. exigua*, *C. viridiflora* and *C. zanthorrhiza*, including three rare species (*C. amarissima, C. flaviflora and C. yunnanensis*) were officially recorded in the Flora of China [6, 7]. Among those *Curcuma*, *C. viridiflora* located in Taiwan and have not been sampled by the present authors which is suspected of its existence, *C. exigua* was extinct, and actually *C. zanthorrhiza* is major distribution in Indonesia, Malaysia and Thailand [8–10]. Four (*C. longa*, *C. wenyujin*, *C. phaeocaulis* and *C. kwangsiensis*) of which are recorded as folk herbs officially in Chinese Pharmacopoeia named as Radix Curcumae (also named Yujin), Rhizoma Curcumae Longae (also named Jianghuang) and Rhizoma Curcumae (also named Ezhu) [11]. However, *C. sichuanensis* and *C. chuanhuangjiang* are frequently appeared as substitutes of the aforementioned four *Curcuma* species in cultivation, herb trading and curing in Traditional Chinese Medicine (TCM), and the remainings are also used

as herbs at rural areas in China [12, 13].

As the intra- and inter-species morphological characters of rhizomes and leaves are very similar, confusion persists on the identification of these *Curcuma* species, their florescence varies from April to October, and the color always shows diversity within intra-species, and the similarities of growth habit, leaf-shapes, and the flowers among these *Curcuma* species are similar. It is difficult to distinguish them at both vegetative and reproductive stages [14, 15]. In hence, it is necessary to involve various methods to identify these herbs and their genetic relationships [16, 17].

In China, previous researchers have studied on morphological characteristics of *Curcuma* L., and difficult to identify each species clearly for lacking of species coverage [14, 15, 18, 19]. Xiao et al. [14] firstly analyzed the lateral root of *Curcuma* L. on oil droplets, starch grains and crystals, using stereological and computer image quantitative system method to classify Chinese *Curcuma* into six species, one complex species and one cultivar. Zheng et al. [20] enlarged sampling size and morphological characters (31 accessions, 28 qualitative and 19 quantitative indicators) to re-check their systematic relathionships, and the clustering result was roughly the same as Xiao's classification.

Early in 1961, Ramachandran [21] found the chromosome base of *Curcuma* L. was 21, and supposed *Curcuma* originated from the species which has X=12 and 9 in the Zingiberaceae. The majority of *Curcuma* L. are diploids (e.g. *C. exigua*, 2n=2x=42), a few are triploids (e.g. *C. aromatica*, 2n=3x=63; *C. xanthofacia*, 2n=3x= 63; *C. yunnanensis*, 2n=3x=63), and very few are tetraploids (e.g. *C. kwangsiensis*, 2n=4x= 84) [22–24]. Also, some studies showed that the chromosome base number was X=8-25, and the chromosome number divergence existed in different populations [25, 26]. At present, most studies agree that the chromosome basic number of *Curcuma* is 2n=18-105, most of *Curcuma* L. are polyploids, and there are some deviations in chromosome number of different populations from same species [27, 28]. It is difficult to study *Curcuma* phylogenetic relationship only using chromosome number.

For chemical taxonomy, Xia et al. [29] determined the total curcumin, and curcumin of several Chinese *Curcuma* species and found that the compound content from different populations and species were totally discrepancies. Wang et al. [30] used HPLC to determine main chemical constituents of 9 accessions and found that the chemical constituent in *C. longa* were higher than those in *C. wenyujin*, *C. kwangsiensis* and *C. phaeocaulis*. The determination and analysis of the main chemical constituents of the genus *Curcuma* can provide an effective reference for the identification and classification of the genus *Curcuma*; however, the method is not specific and accuracy, easy to be interfered by other factors such as individuals of the same species from different locations or different individuals plants from same region [31, 32]. At present, there is no uniform standard for *Curcuma* identification based on effective components.

In order to solve the problem of classification of related *Curcuma* plants in China, Chen et al. [33] used RAPD combining chemical and morphological methods to study 8 accessions represented three species of *Curcuma* plants, the results showed that *C. wenyujin* and *C. aromatic* were closely related, *C. sichuanensis* should be merged into *C. wenyujin*. Xiao et al. [34] classified 10 species of *Curcuma* by RAPD and suggested mergeing *C. wenyujin* into *C. aromatic*, or treated *C. wenyujin* as the cultivar of *C. aromatic*; and *C. kwangsiensis* should be named as *Curcuma kwangsiensis* S. G. Lee & C. F. Liang complex. Tang et al. [35] analyzed POD and EST isozymes on 39 samples represented six species, showed that *C. longa* and *C. sichuanensis* could not be distinguished, and considered *C. sichuanensis* should be a cultivar of *C. longa*, *C. chuanhuangjiang* was regarded as an individual species. Studies by RAPD, isozymes (SOD, PPO, MDH, COD), and DNA barcodes held identical views that *C. sichuanensis* is a cultivar/or variety of *C. longa* [36–38].

The molecular markers, have shown some advantages on genetic relationship, identification among medicinal plants [39, 40]. Due to polyploidy arising commonly within *Curcuma* and hybrid origins [27, 41], the genetic relationships within *Curcuma* were poorly resolved using some classic universal genes, including *rbc*L, *mat*K, and ITS [17, 28, 42, 43]. Clearly, more DNA markers should be tested on the identification, genetic relationship, and phylogeny of *Curcuma* species.

This is the first study to test genetic relationships among 10 related Chinese *Curcuma* herbal species involving three rare species (*C. amarissima*, *C. flaviflora* and *C. yunnanensis*) by using 100 pairs of SRAP markers.

2. Material and Methods

2.1. Taxon Sampling

The 29 accessions represented 10 species including three rare species were sampled in this study (Table 1).

2.2. DNA Extraction, Amplification

DNA was extracted from dried leaf tissue using the CTAB procedure (Doyle & Doyle, 1987). PCR amplifications were performed using 100 pair primers in Table 2. Each 25 μl of polymerase chain reaction (PCR) contained 1 μl of DNA solution (20 ng), 2.5 μl of PCR reaction buffer, 2.5 μl dNPT mix (0.2 mM), 0.5 μl of each primer, and 1.5 U *Taq*DNA polymerase (Takara, Japan). PCR amplification procedure consisted of pre-denaturation at 94°C for 2 mins, denaturation at 94°C for 1 min, annealing at 36°C for 1 min, extension at 72°C for 1 min with 30 cycles, and final extension at 72°C for 7 min.

Table 1. *Species and location information used in this study.*

NO.	NAME	LOCATION
YJ1-YN	*Curcuma aromatica* Salisb.	Daluo, Yunnan
YJ2-YN	*C. aromatica* Salisb.	Yiwu, Yunnan
YJ3-GX	*C. aromatica* Salisb.	Medicinal Botanical Garden, Guangxi
YJ4-SC	*C. aromatica* Salisb.	Meishan, Sichuan
JH1-YN	*C. longa* L.	Yiwu, Yunnan
JH2-SC	*C. longa* L.	Longquan, Sichuan
JH3-SC	*C. longa* L.	Leshan, Sichun
JH4-GX	*C. longa* L.	Nanning, Guangxi
CYJ1-GX	*C. sichuanensis* X. X. Chen	Medicinal Botanical Garden, Guangxi
CYJ2-SC	*C. sichuanensis* X. X. Chen	GAP Base, Sichuan
CYJ3-SC	*C. sichuanensis* X. X. Chen	Chongzhou, Sichuan
CYJ4-SC	*C. sichuanensis* X. X. Chen	Yibin, Sichuan
CYJ5-SC	*C. sichuanensis* X. X. Chen	Neijiang, Sichuan
WYJ1-GX	*C. wenyujin* Y, H. Chenet C. Ling	Medicinal Botanical Garden, Guangxi
WYJ2-GX	*C. wenyujin* Y, H. Chenet C. Ling	Medicinal Botanical Garden, Guangxi
WYJ3-GD	*C. wenyujin* Y, H. Chenet C. Ling	Gaoxian, Guangdong
WYJ4-ZJ	*C. wenyujin* Y, H. Chenet C. Ling	Taoshan, Zhejiang
PEZ1-GX	*C. phaeocaulis* Valeton	Medicinal Botanical Garden, Guangxi
PEZ2-GZ	*C. phaeocaulis* Valeton	Xingyi, Guizhou
PEZ3-SC	*C. phaeocaulis* Valeton	Shuangliu, Sichuan
HH1-YN	*C. flaviflora* S. Q. Tong	Meng'a, Yunnan
HH2-YN	*C. flaviflora* S. Q. Tong	Menglun, Yunnan
WJK1-YN	*C. amarissima* Roxb.	Meng'a, Yunnan
WJK2-YN	*C. amarissima* Roxb.	Mengkang, Yunnan
DH1-YN	*C. yunnanensis* N. Liu et S. J. Chen	Mengxing, Yunnan
DH2-YN	*C. yunnanensis* N. Liu et S. J. Chen	Meng'a, Yunnan
DH3-GD	*C. yunnanensis* N. Liu et S. J. Chen	Zhongkai University, Guangdong
CHJ-SC	*C. chuanhuangjiang* Z. Y. Zhu	Jianyang, Sichuan
GXEZ-GX	*C. kwangsiensis* S. G. Lee et C. F. Liang	Hengxian, Guangxi

2.3. Data Analysis

Photographs (Gel Imaging) were used to score the SRAP data. For each material of the x primers 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 final data was used to produce genetic distances with Jaccard's similarity coefficient and cladogram with COMPLETE in NTSYS–pc2.1 [44].

Table 2. *Primers of SRAP used in this study. We combined the primers of Me and Em into 100 (10 × 10 = 100) pairs, and excluded 7 combination pairs of primers (M3E1, M3E2, M3E10, M4E2, M6E2, M6E5 and M7E1) for no band amplified in 29 accessions.*

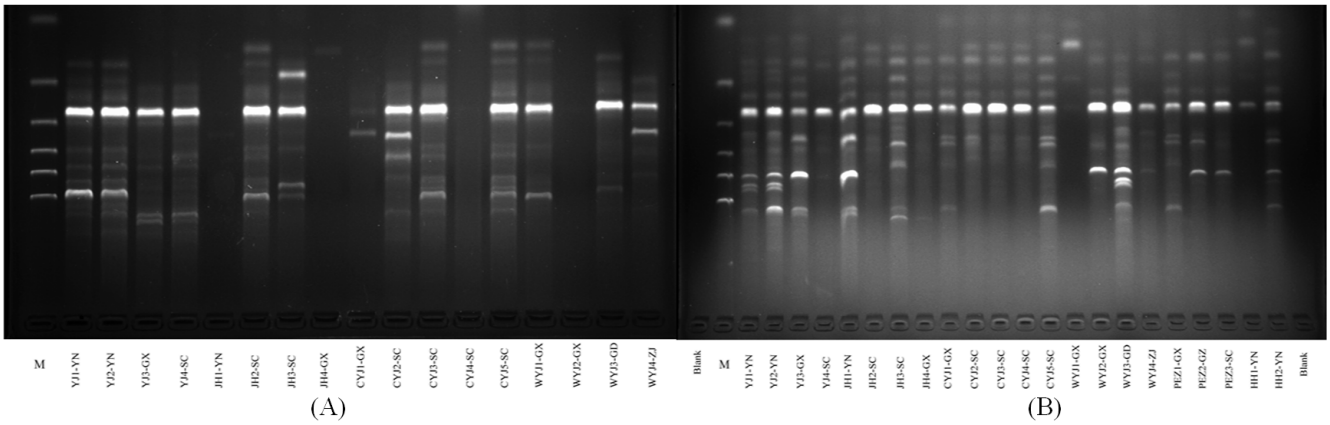
Primer NO.	Sequence (5'to3')	Primer NO.	Sequence (5'to3')
Me1	TGAGTCCAAACCGGATA	Em1	GACTGCGTACGAATTGAC
Me2	TGAGTCCAAACCGGAGC	Em2	GACTGCGTACGAATTAAC
Me3	TGAGTCCAAACCGGTGC	Em3	GACTGCGTACGAATTGCA
Me4	TGAGTCCAAACCGGACC	Em4	GACTGCGTACGAATTCAA
Me5	TGAGTCCAAACCGGACA	Em5	GACTGCGTACGAATTCTG
Me6	TGAGTCCAAACCGGAGA	Em6	GACTGCGTACGAATTTGA
Me7	TGAGTCCAAACCGGACG	Em7	GACTGCGTACGAATTGAG
Me8	TGAGTCCAAACCGGAAA	Em8	GACTGCGTACGAATTGCC
Me9	TGAGTCCAAACCGGAAC	Em9	GACTGCGTACGAATTCAT
Me10	TGAGTCCAAACCGGTCA	Em10	GACTGCGTACGAATTTCA

3. Results

93 out of 100 primers were picked up to evaluate genetic relationships among *Curcuma* species (Partial Gel Imaging see Figure 1). Totally, 1084 polymorphic bands were produced by such 93 pairs of primers. The 2-35 polymorphic bands were amplified by each pair of primers, with an average of 11.78 bands. The genetic distance coefficient varied from 0.12 to 0.53.

When the coefficient was at 0.49-0.43, the 29 accessions were fallen into three groups (Group I, Group II and Group III) in the cladogram (Figure 2). All the samples of *C. aromatica* were interlaceed with three accessions of *C. wenyujin* (WYJ1-GX, WYJ2-GX, WYJ3-GD) in Group I. The *C. sichuanensis* and *C. longa* were mixed together in Group II, and WYJ4-ZJ (*C. wenyujin*) clustered with CYJ3-SC (*C. sichuanensis*) belonged to this group. In Group III, the relationships were very clearly. The populations of each single species, *C. kwangsiensis*, *C. chuanhuangjiang*, *C. flaviflora*, *C. phaeocaulis*, *C. amarissima*, *C. yunnanensis*, were firstly togethered, then clustered with other species.

In Group I, two subgroups were divided obviously. Two *C. aromatica* accessions (Yunnan) and one *C. wenyujin* accession (Guangxi), fallen into one subgroup; and the remaining four accessions, two *C. aromatica* accessions (Guangxi and Sichuan) and two *C. wenyujin* accessions (Guangxi and Guangdong), were clustered together in another subgroup. In Group II, all the *C. longa* and *C. sichuanensis* were included. The phylogeny is very clear presented in Group III with the topology of (((*C. kwangsiensis*, *C. chuanhuangjiang*), *C. flaviflora*), ((*C. phaeocaulis*, *C. amarissima*), *C. yunnanensis*)).



(A) (B)

Figure 1. *Gel Imaging of amplified results by primers of M1E5 (A) and M10E8 (B).*

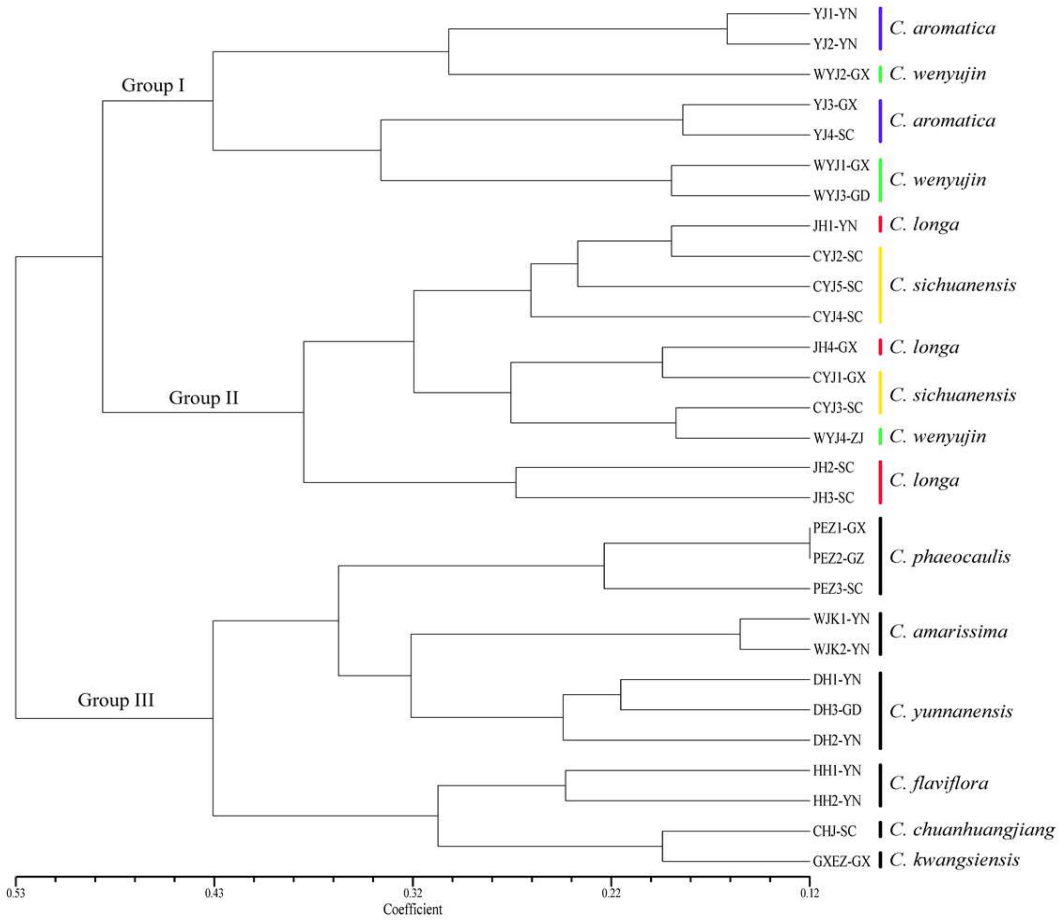


Figure 2. *The cladogram of Curcuma produced by all the 1084 polymorphic bands.*

4. Discussion

The efforts in this study have improved the understanding of genetics among Chinese *Curcuma* herbs. Species (*C. wenyujin*, *C. aromatica*, *C. longa*, *C. sichuanensis*) in Group I and Group II, were commonly used as same medicine in TCM. Their relationships, classifications, have long been confused by both botanist and doctor of TCM for their similarity of morphological characters, low genetic

variability, and commonly polyploidy/hybrid origins [10, 14, 15, 17, 19, 27, 34, 35, 41].

The *C. wenyujin* was an individual species in the Flora of China. But, it was described as *C. aromatica* 'Wenyujin' (i.e., a cultivar) in Flora Republicae Popularis Sinicae (http://frps.eflora.cn/), and was a synonym of *C. aromatica* in the Plant list (http://www.theplantlist.org/). Based on our study, both *C. wenyujin* and *C. aromatica* were not monophyly in the phylogeny, combining with other studies, we concluded that *C. wenyujin* was not supposed to be an individual species. However, one *C. wenyujin* (Zhejiang) clustering with one *C. sichuanensis* (Sichuan) in Group II, with some similarity to Xiao et al. [15] studied on leaf morphology of *C. wenyujin* and *C. sichuanensis*.

The *C. sichuanensis* is also a confused species for pretty close relationship to *C. longa*. In an early study, *C. sichuanensis* was identified as a variety of *C. longa* by using RAPD markers [34]. This identification was recovered by numerical taxonomy, histological and morphological characteristics of *Curcuma* leaves and rhizomes [14, 15, 19]. Isozyme analyses also inferred that *C. sichuanensis* was a variety/or cultvar of *C. longa* [35, 36]. In this study, two accessions of *C. longa* were as the first branch within this Group, then was sister to the remainings. We also supposed that *C. sichuanensis* was a cultivar or variety of *C. longa* as well as some conclusions in the previous studies.

The *C. chuanhuangjiang*, is an endemic species and exclusively located in Jianyang, Sichuan province, was not included in Flora of China [7], was supposed as an individual species in some previous studies [10, 13, 17, 35]. It was suggested to be a variety species of *C. longa* based on leaf morphological characteristics [15]. Liu & Wu [24] sorted *C. chuanhuangjiang* as *C. kwangsiensis* without considering their diversities of chromosome numbers. The chromosome number of *C. chuanhuangjiang* was 2n=3x=63, and *C. kwangsiensis* was 2n=4x=84 [12, 25]. In this study, the clade of ((*C. chuanhuangjiang* + *C. kwangsiensis*) + *C. flaviflora*) was as a single branch in Group III. Moreover, the rhizome of *C. chuanhuangjiang* had different rosin smell and leaf epidermis with pubescence compared to other *Curcuma* species [13]. *C. chuanhuangjiang* is an independent species, which was same with most previous views.

5. Conclusion

Our analyses indicated that (1) *C. wenyujin*, might be supposed as a cultivar/variety of *C. aromatica* and/or *C. longa*; *C. sichuanensis* is more likely a cultivar or variety of *C. longa*; *C. chuanhuangjiang* should be considered as an independent species. (2) the placement of three Chinese rare species, *C. amarissima*, *C. flaviflora* and *C. yunnanensis*, were clearly achieved. (3) Partial species might be muti-origin in Chinese *Curcuma*.

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