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Full Length Research Paper

Authentication of three related herbal species (*Curcuma*) by DNA barcoding

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The *psb*A-*trn*H intergenic region was studied for authenticating *Curcuma longa* and its two related species (*Curcuma sichuanensis* and *Curcuma chuanhuangjiang*). The sequences were analyzed by means of Neighbor-Joining to improve the phylogenetic resolution of these three *Curcuma* species. The genetic diversity of these three species is 0.009 to 0.014 (< 0.05). The results show that partial population specimens of *C. sichuanensis* originate from the cultivated mutation of *C. longa*; and retain the *C. chuanhuangjiang* as an individual species. The differentiation is engendered between the wildness and cultivated specimens within *C. longa* species.

Key words: *Curcuma*, Radix Curcumae, related species, DNA barcoding, *psb*A-*trn*H, phylogeny.

INTRODUCTION

Curcuma L. (Zingiberaceae) is a geograhyically wide-spread group, comprising approximately 70 species. About 10 *Curcuma* species are distributed in China (Xiao et al., 1997; Li et al., 2001; Ye et al., 2008) of which 6 species were used as Chinese folk herbal medicine more than a thousand years ago, and an extract of rhizomes exhibits anti-inflammatory, anticancer and HIV-1 protease inhibitory activity (Moussavi et al., 2006). Radix Curcumae (also named Yujin) and Rhizoma Curcumae Longae (also named Jianghuang) which derived from *Curcuma* are traditional Chinese medicines. The dried rhizomes of *C. longa* L., was named Radix Curcumae in Traditional Chinese Medicine (TCM), are officially recorded in Chinese Pharmacopoeia (2010). However, the radix of *C. sichuanensis* C. K. Hsich et H. Zhang and *C. chuanhuanjiang* Z. Y. Zhu also can be used as Radix Curcumae in folk therapeutic uses (Chen, 1981; Zhu, 1992). The three species were always mixed in systematization and in TCM. The morphological features of these three species are very common. The natural flowering seasons vary from April to October, and the same species have flowers with different colors as usual.

The similarities of their growth habit, leaf-shapes, and the flowers among these *Curcuma* species are so common that it is generally difficult to distinguish the species at both vegetative and reproductive stages. Such phenotypic plasticity of the species can lead to wrong taxonomic treatment of individuals. Meanwhile, in TCM, the same Chinese materia medica can be produced from several species (*Curcuma*) of which one can be used as different Chinese materia medica. These problems have been troublesome in phylogenetic analysis and accurate on clinic. Therefore, it is necessary to adopt various methods to identify this three species and evaluate their genetic relationship for taxon and pharmacognosy.

Due to the fact that DNA barcoding provided a potential effectiveness in the identification and evaluation of quality for medicinal plants (Newmaster et al., 2006; Chen et al., 2007; Taberlet et al., 2007; Valentini et al., 2009; Chen et al., 2010), the DNA barcode has showed some advantages on phylogeny analysis. Kress et al. (2005) studied the whole angiosperm group of classes with 9 chloroplast genes and suggested *rbc*L combineing *psb*A-*trn*H and ITS to assess genetic relationships.

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

S/N	Taxon	Origins	Gene bank accession	Notes
1	*Curcuma longa*	Dayi, Sichuan	JF730221	Cultivated
2	*C. longa*	Chendu, Sichuan	JF730222	Wildness
3	*C. longa*	Qianwei, Sichuan	JF730223	Cultivated
4	*C. longa*	Shuangliu, Sichuan	JF730224	Cultivated
5	*C. longa*	Xinjin, Sichuan	JF730226	Cultivated
6	*C. longa*	Muchuan, Sichuan	JF730227	Cultivated
7	*C. longa*	Muchuan, Sichuan	JF730228	Cultivated
8	*C. longa*	Qianwei, Sichuan	JF730229	Wildness
9	*C. longa*	Yibin, Sichuan	JF730231	Cultivated
10	*C. longa*	Yibin, Sichuan	JF730233	Wildness
11	*C. longa*	Leshan, Sichuan	JF730234	Cultivated
12	*C. longa*	Muchuan,Sichuan	JF730235	Cultivated
13	*C. longa*	Yibin, Sichuan	JF730236	Wildness
14	*C. longa*	Medicinal Botanical Garden, Guangxi	JF730238	Cultivated
15	*C. sichuanensis*	Chongzhou, Sichuan	JF730240	Cultivated
16	*C. sichuanensis*	GAP land, Chongzhou, Sichuan	JF730241	Cultivated
17	*C. sichuanensis*	Chongzhou, Sichuan	JF730242	Wildness
18	*C. sichuanensis*	Yibin, Sichuan	JF730243	Wildness
19	*C. sichuanensis*	Weiyuan, Sichuan	JF730244	Cultivated
20	*C. sichuanensis*	Chongzhou, Sichuan	JF730245	Wildness
21	*C. sichuanensis*	GAP land, Chongzhou, Sichuan	JF730246	Cultivated
22	*C. chuanhuangjiang*	Jianyang, Sichuan	JF730250	Cultivated
B-3	*C. longa*	Chongzhou, Sichuan	JF730253	Cultivated
B-10	*C. longa*	Muchuan, Sichuan	JF730254	Cultivated
B-18	*C. longa*	Cuiping, Sichuan	JF730255	Cultivated

MeanwhileKress and Erickson (2007) believed that combining *rbc*L and *psb*A-*trn*H were better choice on the analysis of genetic relationships. Zheng and Xia (2010) studied the phylogeny of tribe Zingibereae (Zingiberaceae) based on nrDNA ITS and cpDNA *mat*K sequence data and confirmed that the two genes were poorly identified. The objectives of this paper based on the study of *psb*A- *trn*H, were to evaluate the phylogenetic relationships among this three related species; to explore the taxonomic status of *C. sichuanensis* and *C. chuanhuangjiang* species and then to provide helpful information on clinic of TCM.

MATERIALS AND METHODS

Plant materials

The materials were analyzed in this study (Table 1). As Sichuan is the geo-herbalism habitat of *C. longa*, *C. sichuanensis* and *C. chuanhuangjiang* in China (Hu, 1998). Most of specimens were collected from different localities in Sichuan, of which were found nowhere else. The remaining 1 specimen was collected from Guangxi Medicinal Botanical Garden, which was introduced from Sichuan Province.

DNA extraction and PCR amplification

Total DNA isolation was carried out on fresh leaves by modified CTAB method (Doyle and Doyle, 1987). The primers used for the amplification were 1F forward primer (5′-CTT GGT ATG GAA GTA ATG CA -3′) and 1R reverse primer (5′- ATC CAC TTG GCT ACA TCC G -3′) (Techaprasan et al., 2006). The PCR reactions were conducted in a final volume of 25 µl containing 9.5 µl 2 × Taq MasterMiX (CWBIO), 1.5 µl DNA, 12.5 µl ddH_2O, 1 µl primer on a GeneAmp PCR System 9700 thermocycler and amplification condition consisted of pre denaturation at 94℃ for 5 min, denaturation at 94℃ for 1 min, annealing at 55℃ fo r 1 min, extension at 72℃ for 1 min and final extension at 72℃ for 7 min. The integrity of target loci was checked using electrophoresis on a 1.5% agarose gel and purified by QIAquick kit (invitrogen^{TM}). Sequencing was conducted by BGI Company (Peking, China).

*Psb*A-*trn*H data analysis

We took one species from Gene Bank (*Curcuma zedoaria*, FJ687417) into analysis as number C in the materials table. The DNA sequences were minimally edited and manually aligned in Geneious 4.7.4 (Drummond et al., 2006). The analysis of DNA sequence was conducted by Neighbor Joining to assess topology with MEGA version 4 (Tamura et al., 2007). All positions containing gaps and missing data were eliminated. Both the analysis of per

Table 2. Estimates of evolutionary divergence over sequence pairs between groups. Standard error estimate(s) are shown in the last column.

Species 1	Species 2	Dist	Std. Err
Curcuma longa	*C. sichuanensis*	0.014	0.003
C. longa	*C. chuanhuangjiang*	0.014	0.004
C. sichuanensis	*C. chuanhuangjiang*	0.009	0.003
C. longa	*C. zedoaria*	0.027	0.005
C. sichuanensis	*C. zedoaria*	0.021	0.005
C. chuanhuangjiang	*C. zedoaria*	0.018	0.005

site from averaging over all sequence pairs between groups and the number of base substitutions per site from between sequences were conducted using the Kimura 2-parameter model, are shown in Tables 2 and 3, respectively. The phylogenetic tree (Figure 1) was conducted by the method of Neighbor-Joining (NJ), which was tested with Kimura 2-parameter for evolutionary distances in MEGA4.

RESULTS AND DISCUSSION

The distance of interspecific (0.009 to 0.021) is < 0. 05. *Psb*A-*trn*H sequences were suitable for identification of *C. longa*, *C. sichuanensis* and *C. chuanhuangjiang*. All the specimens were divided into three groups: *C. chuanhuangjiang* formed Group 1; group 2 included most of *C. longa*; and the rest of specimens formed group 3. In group 2, 3/4 wildness specimens (number 8, 10, and 13) united together as a clade firstly; only one wildness specimen clustered with other cultivated specimens. The specimen (number 14) which was collected from Medicinal Botanical Garden of Gungxi was not included in group 2.

The genetic distance between *C. sichuanensis* and *C. longa* is 0.014. The relationship between *C. longa* and *C. sichuanensis* is close and complex (Xiao et al., 1997, 1999, 2000). Xiao et al. (2001) inferred that *C. sichuanensis* was the cultivated variety of *C. longa*. The histological and morphological study of leaves and rhizomes, as well as numerical taxonomy analysis (Xiao et al., 2004a, b, c) indicated that both *C. sichuanensis* and *C. chuanhuangjiang* were the cultivated varieties of *C. longa*.

This view had some self-contradiction tergiversate, (i) on the study of leaves, *C. longa* was far away from *C. sichuanensis*; (ii) and on the study of rhizomes, *C. longa* and *C. sichuanensis* clustered together firstly. Xia et al. (2005) studied the contents of curdione, curcumol by means of HPLC and 5sRNA sequence analysis, and showed *C. longa* was on close terms with *C. sichuanensis*. Tang et al. (2008) believed that *C. sichuanensis* was the cultivated mutation species of *C. longa* by isozymes patterns of POD and EST. Dai (2009) studied the chromosome numbers of these two species (2n = 3 × = 63).

In our study, most specimens of *C. longa* were clustered together firstly as Group 2. Only three cultivated specimens of *C. longa* (number 7, 9, and 14) were mixed together with *C. sichuanensis* in phyletic trees, of which number 14 was collected from Guangxi Medicinal Botanical Garden. Combining the study of morphology (Xiao et al., 1998, 2001, 2004a, b, c), medicinal ingredients (Xie et al., 2004; Xia et al., 2005), RAPD (Xiao et al., 2000), isoenzymes (Tang et al., 2008), *trn*K gene (Cao and Katsuko, 2003), and 5S-rRNA spacer (Xia et al., 2005). We discovered that partial *C. sichuanensis* originated from the cultivated mutation of *C. longa*; confirmed that *C. sichuanensis* is not the cultivated mutation of *C. longa*. *C. sichuanensis* should be an individual species retained and study the relationship with the cultivated species of *C. longa*.

Liu and Wu (1999) pointed out that *C. chuanhuangjiang* should be merged into *C. kwangsiensis*. The chromosome numbers of them are 2n = 3 × = 63 (*C. chuanhuangjiang*) (Dai, 2009) and 2n = 4 × = 84 (*C. kwangsiensis*) (Chen et al., 1988). However, Xiao et al. (2004b) thought *C. chuanhuangjiang* was the cultivated mutation of *C. longa*. The distance between *C. chuanhuangjiang* and *C. sichuanensis* is 0.009 and 0.018 with *C. zedoaria*. The rhizome of *C. chuanhuangjiang* had different rosin smell and leaf epidermis with pubescence compared to other Radix Curcumae species (Zhu, 1992); we confirmed that *C. chuanhuangjiang* is an individual species; this view is consistent with Cao and Katsuko (2003) and Tang et al. (2008).

Conclusion

The current study represents an improvement of our understanding of evolution within the three related Radix Curcumae species. The differentiation of *C. longa* has been engendered between the wildness and cultivated specimens.

Some population specimens of *C. sichuanensis* originate from the cultivated mutation of *C. longa*, and *C. chuanhuangjiang* are separate species. We strongly suggest paying attention to the origin complexity of *C. sichuanensis*, and investigation the distinction between

Table 3. Estimates of evolutionary divergence between sequences.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	B-3	B-10	B-18	C
1																										
2	0.01																									
3	0.00	0.00																								
4	0.01	0.00	0.00																							
5	0.00	0.00	0.00	0.00																						
6	0.02	0.01	0.01	0.01	0.01																					
7	0.02	0.02	0.02	0.02	0.02	0.02																				
8	0.01	0.01	0.00	0.01	0.00	0.02	0.02																			
9	0.02	0.01	0.01	0.01	0.01	0.02	0.01	0.02																		
10	0.01	0.00	0.00	0.00	0.00	0.01	0.02	0.01	0.02																	
11	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.02	0.01																
12	0.01	0.00	0.00	0.00	0.00	0.01	0.02	0.01	0.01	0.00	0.01															
13	0.00	0.00	0.00	0.00	0.00	0.01	0.02	0.00	0.01	0.00	0.01	0.00														
14	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.02	0.01	0.02	0.02	0.02	0.02													
15	0.02	0.01	0.01	0.01	0.01	0.02	0.01	0.02	0.00	0.02	0.02	0.01	0.01	0.01												
16	0.02	0.01	0.01	0.01	0.01	0.02	0.01	0.02	0.00	0.01	0.02	0.01	0.01	0.01	0.00											
17	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.02	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01										
18	0.02	0.02	0.01	0.02	0.01	0.02	0.01	0.02	0.01	0.02	0.02	0.02	0.01	0.01	0.01	0.00	0.01									
19	0.02	0.02	0.02	0.02	0.02	0.03	0.01	0.02	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01								
20	0.02	0.01	0.01	0.01	0.01	0.02	0.01	0.02	0.00	0.01	0.02	0.01	0.01	0.01	0.00	0.00	0.01	0.01	0.01							
21	0.02	0.01	0.01	0.01	0.01	0.02	0.01	0.02	0.01	0.02	0.02	0.01	0.01	0.01	0.01	0.00	0.01	0.00	0.01	0.00						
22	0.02	0.01	0.01	0.01	0.01	0.02	0.01	0.02	0.01	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01					
B-3	0.01	0.00	0.00	0.00	0.00	0.01	0.02	0.01	0.01	0.00	0.01	0.00	0.00	0.02	0.01	0.01	0.02	0.02	0.02	0.01	0.01	0.01				
B-10	0.01	0.00	0.00	0.00	0.00	0.01	0.02	0.01	0.01	0.00	0.01	0.00	0.00	0.02	0.01	0.01	0.02	0.02	0.02	0.01	0.01	0.01	0.00			
B-18	0.00	0.01	0.00	0.01	0.00	0.02	0.02	0.01	0.02	0.01	0.01	0.01	0.00	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.01		
C	0.03	0.02	0.03	0.02	0.03	0.04	0.02	0.03	0.02	0.03	0.03	0.02	0.03	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.03	

the wildness and cultivated specimens within species of *C. longa* in TCM.

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REFERENCES

Cao H, Katsuko K (2003). Molecular identification of six medicinal *Curcuma* plants produced in Sichuan: Evidence from plastid trnK gene sequences. Acta Pharm. Sin., 38(11): 871-875.

Chen SL, Yao H, Han JP, Liu C, Song JY, Shi LC, Zhu YJ, Ma XY, Gao XH, Luo K, Li Y, Li XW, Jia XC, Lin YL, Leon C (2010). Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. PLoS ONE, 5(1): e8613.

Chen SL, Yao H, Song JY, Li XW (2007). Use of DNA barcoding to identify Chinese medicinal materials. World. Sci. Technol/Mod. Trad. Chin. Med. Mater. Med., 9(3): 7-12.

Chen ZY, Chen SJ, Huang XX (1988). A Report on Chromosome Number on Chinese Zingiberaceae Guibai, 8(2): 143-147.

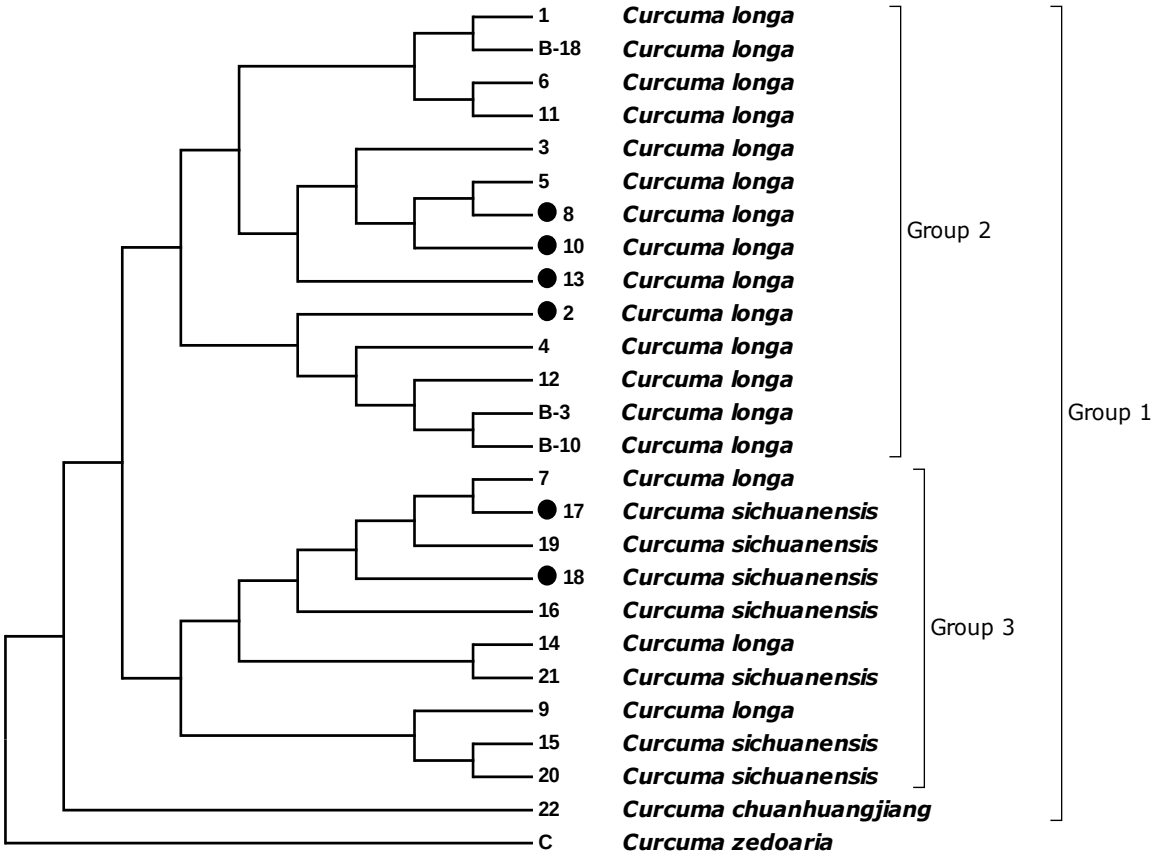

Figure 1. Neighbor-joining tree of, *psb*A-*trn*H representing the three species of Curcuma with 26 specimens. The evolutionary distances were computed using the Kimura 2-parameter method, and all positions containing gaps and missing data were eliminated. (black button means wildness specimens).

Chen YH (1981). Preliminary study of *Curcuma* in China. Plant appraisal. Acta Pharm. Sin., 16(50): 385-389.

China Pharmacopoeia Committee (2010). Pharmacopoeia of P. R. China. Beijing: Chinese Medicine and Technology Publishing House. Part. Ⅰ, pp. 193-194.

Dai ZJ (2009). Morphological, cytology and RAPD molecular marker Studies on the six medicinal materials of *Curcuma*. Pharmaceatical Botany Master paper. Yaan: Sichuan Agric Univ., pp. 19-29.

Doyle JJ, Doyle JL (1987). A rapid DNA isolation procedure from small quantities of fresh leaf tissue. Phytochem. Bull., 19: 1-15.

Drummond AJ, Ho SYW, Phillips MJ, Rambaut A (2006). Relaxed phylogenetics and dating with confidence. PLoS Biol,. 4(5): e88.

Hu SL (1998). Chinese genuine traditional Chinese unbleached illustrations. Jinan: Shandong Sci. Technol. Publ. Houseshi, 1: 250-252.

Kress WJ, Erickson DL (2007). A two-locus global DNA barcode for land plants: the coding *rbc*L gene complements the non-coding *trn*H-*psb*A spacer region. PloS one, 2(6): e508.

Kress WJ, Wurdack KJ, Zimmer EA, Weigt LA, Janzen DH (2005). Use of DNA barcodes to identify flowering plants. PNAS, 102(23): 8369-8374.

Li J, Zhang DZ, Gao LX (2001). The Overview Research of Chinese Radix Curcumae. Nei. Mong. J. Trad. Chin. Med., 1: 37-38.

Liu N, Wu TL (1999). Notes on *Curcuma* in China. J. Trop. Subtrop. Bot., 7(2): 146-150.

Moussavi M, Assi K, Go´mez-Mun˜ oz A, Salh B (2006). Curcumin mediates ceramide generation via the de novo pathway in colon cancer cells. Carcinogenesis, 27(8): 1636-1644.

Newmaster SG, Fazekas AJ, Ragupathy S (2006). DNA barcoding in land plants: evaluation of *rbc*L in a multigene tiered approach. Botany, 84: 335-341.

Taberlet P, Coissac E, Pompanon F, Gielly L, Miquel C, Valentini A, Vermat T, Corthier G, Brochmann C, Willerslev E (2007). Power and limitations of the chloroplast *trn*L (UAA) intron for plant DNA barcoding. Nucleic Acids Res., 35(3): e14.

Tamura K, Dudley J, Nei M, Kumar S (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Boil. Evolut., 24(8): 1596-1599.

Tang JY, Li,QM, Yang RW, Liao JQ, Zhou YH (2008). Study on isozymes in six species of *Curcuma*. Chin. J. Chin. Mater. Med., 33: 1381-1386.

Techaprasan J, Ngamriabsakul C, Klinbunga S, Chusacultanachai S, Jenjittikul T (2006). Genetic Variation and Species Identification of Thai Boesenbergia (Zingiberaceae) Analyzed by Chloroplast DNA Polymorphism. J. Biochem. Mol. Biol., 39(4): 361-370.

Valentini A, Pompanon F, Taberlet P (2009). DNA barcoding for ecologists. Trends Ecol. Evol., 24: 110-117.

Xia Q, Zhao KJ, Huang ZG, Zhang P, Dong TTX, Li SP, Tsim KWK (2005). Molecular Genetic and Chemical Assessment of Rhizoma Curcumae in China. J. Agric. Food Chem., 53: 6019-6026.

Xia WJ, Xiao XH, Liu FQ, Su ZW, Qiao CZ (1999). Determination on Chemical Constitutents of *Curcuma* L. Produced in China. China. J.

Chin. Mater. Med., 24(7): 423-447.
Xiao XH, Liu FQ, Shi CH, Li LY, Qin SY, Qiao CZ, Su ZW (2000). RAPD polymorphism and authentication of medicinal plants from Turmeric (*Curcuma* L.) in China. China Trad. Herb Drugs, 31(3): 209-212.
Xiao XH, Qiao CZ, Su ZW, Yin GP, Fang QM, Su GM, Qin SY, Zhou Y, Li LY (1998). Recognition technique of the histomorphological images of Radix Curcumae. China J. Chin. Mater. Med., 2: 14-17.
Xiao XH, Shu GM, Li LY, Fang DQ, Xia WJ, Su ZW (2004a). Histological and morphological studies on the rhizomes of *Curcuma*. China. J. Chin. Mater. Med., 29(5): 395-399.
Xiao XH, Shu ZW, Qiao CZ, Luo ZY (1997). Advances in the study on medicinal of *Curcuma*. Chin. Trad. Herb Drugs, 28(2): 114-118.
Xiao XH, Xia WJ, Qin SY, Li JM, Fang DQ, Shu GM, Su ZW (2001). Pattern Recognition of Stereoscopic Features of the Leaves Epidermis of Medicinal *Curcuma* Plants in China by Image Analysis. China J. Chin. Mater. Med., 26(8): 523-528.
Xiao XH, Zhao YL, Jin C, Shu GM, Fang DQ, Su ZW (2004b). Histological and morphological studies on leaves of *Curcuma* in China. China J. Chin. Mater. Med., 29(3): 203-207.
Xiao XH, Zhong GY, Shu GM, Li LY, Fang QM, Chen SY, Su ZW (2004c). Numerical taxonomy of medicinal plants of *Curcuma* in China. China J. Chin. Mater. Med., 28(1): 15-25.
Xie CX, Gao SL, Zhang ZY, Huang XS (2004). Analysis of the chemical components and isomorphic amylase among difierent local cultivars of Dioscorea opposite. J. Plant Resour. Environ., 13(2): 21-24.
Ye XB, Chen J, Liu N (2008). *Curcuma nankunshanensis* (Zingiberaceae)- A New Species from China J. Trop. Subtrop. Bot., 16(5): 472-476.
Zheng ML, Xia YM (2010). A investigation on the phylogeny of tribe Zingibereae (Zingiberaceae) based on nrDNA ITS and cpDNA matK sequence data. J. Yunnan Univ., 32(S1): 426-432.
Zhu ZY (1992). Zingiberaceae, Flora Sichuanica. Chendu: Sichuan Minority Press. 10: 604-610.