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Relationships among six herbal species (*Curcuma*) assessed by four isozymes

Relaciones entre seis especies herbáceas (*Curcuma*) utilizando cuatro isoenzimas

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Abstract. Four isozymes, superoxide dismutase (SOD), polyphenol oxidase (PPO), malate dehydrogenase (MDH) and cytochrome oxidase (COD) were studied for identification of six herbal species (*Curcuma* L.). All the 37 study specimens produced a total of 168 polymorphism isozyme bands. The genetic distance coefficients (GS) varied from 0.08 to 0.54. The dendrogram, obtained according to the polymorphism isozyme bands by the UPGMA method with the software NTSYS–pc2.1, contributed to improve the resolution of phylogeny. From the dendrogram, it was possible to differentiate between the wild and cultivated specimens of *C. longa*, and within *C. sichuanensis* species.

Keywords: *Curcuma*; Isozymes; Genetic relationship; Phylogeny.

Resumen. Se estudiaron cuatro isoenzimas [superóxido dismutasa (SOD), polifenol oxidasa (PPO), málico deshidrogenasa (MDH) y citocromo oxidasa (COD)] para identificar seis especies herbáceas de *Curcuma* L. Los 37 especimenes estudiados produjeron un total de 168 bandas de isoenzimas polimórficas. Los coeficientes de distancia genética (GS) variaron entre 0.08 y 0.54. El dendrograma, obtenido de acuerdo a las bandas de isoenzimas polimórficas por el método UPGMA con el software NTSYS-pc2.1, contribuyó a resolver la filogenia. A partir del dendrograma, fue posible diferenciar entre los especímenes silvestres y cultivados de *C. longa*, y dentro de las especies de *C. sichuanensis*.

Palabras clave: *Curcuma*; Isoenzimas; Relación Genética; Filogenia.

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INTRODUCTION

Curcuma L. (Zingiberaceae) is widespread in the tropics of Asia, Africa and Australia, and it is composed of approximately 70 species (Purseglove, 1974). About 10 *Curcuma* species are distributed in China (Xiao et al., 1997; Li et al., 2001; Ye et al., 2008). Six of those species have been used as Chinese herbal medicine for more than a thousand years. For example, an extract of their rhizomes exhibits anti-inflammatory, anticancer activity (Moussavi et al., 2006). There are three traditional Chinese medicines [Radix Curcumae (also named Yujin), Rhizoma Curcumae Longae (also named Jianghuang) and Rhizoma Curcumae (also named Ezhu)] derived from these six *Curcuma* species. Roots of *C. longa* L.; *C. wenyujin* Y. H. Chen et C. Ling; *C. kwangsiensis* S. G. Lee et C. F. Liang, and *C. phaeocaulis* Valeton (used as herbal species) are officially recorded in Chinese Pharmacopoeia (2010). However, roots of *C. sichuanensis* C. K. Hsich et H. Zhang, and *C. chuanhuanjiang* Z. Y. Zhu can also be used as Radix Curcumae; also, the rhizomes of *C. chuanhuanjiang*, *C. sichuanensis* and *C. wenyujin* can be used as Rhizoma Curcumae Longae in folk therapeutic uses (Chen, 1981; Zhu, 1992).

In Traditional Chinese Medicine (TCM) the same medicinal substances can be produced from these six *Curcuma* species, although one of them can be used as a different medicinal substance. Morphological characteristics are very large for rhizomes and leaves, both intra- and inter-species. The similarities of the growth habit, leaf-shapes, and flowers among the study *Curcuma* species are so great that it is generally difficult to distinguish the species at both the vegetative and reproductive stages. The *Curcuma* flowering season vary from April to October, and it is common that the same species has flowers with different colors. These problems have been troublesome in phylogenetic analysis, and made the clinic of TCM inaccurate. However, the correct identity is important to confirm the sources of origin of herbal drugs within the genus *Curcuma* (Sasaki et al., 2002; Cao & Katsuko, 2003).

Different techniques have different advantages and disadvantages. It is then necessary to confirm the origin of and the genetic relationships among these six herbal species by various methods. Because of the effectiveness of molecular markers in plant systematics (Crawford, 1991), the isozymic technique has been widely applied to (1) deal with evolvement of botany, (2) identify idioplasm resources (Apavatrut et al., 1999; Monireh, 2007), and (3) investigate genetic relationships (Fang et al., 1993; Guo & Li, 2000; Wu et al., 2002; Arzate-Fernandez et al., 2005).

The objectives of this paper were to (1) evaluate the phylogenetic relationships among *Curcuma* herbal species; (2) explore the taxonomic status of *C. sichuanensis* and *C. chuanhuangjiang* species; and (3) identify the origin of traditional medicine in China.

MATERIALS AND METHODS

Plant materials. Thirty seven specimens of the genus *Curcuma*, which were divided into six species, were analyzed in this study (Table 1). Thirty one specimens were collected from different localities in the Sichuan province, and the other 6 specimens were gathered from the Guangxi Medicinal Botanical Garden. Sichuan and Guangxi belong to well-known regions of these species, and Sichuan is the geo-herbalism habitat of *C. longa*, *C. sichuanensis*, *C. phaeocaulis* and *C. chuanhuangjiang* in China (Hu, 1998).

Protein extraction and isozyme analysis. For protein extraction, 0.5 g of tender leaves were powdered in liquid nitrogen. Flour of the leaves was first homogenized in the PBS (0.5 mol, pH 7.8) using 1:1 (v/v) ratio, and then centrifuged at - 4 °C for 12 minutes at 12000 rpm. The supernatant was collected and stored at - 20 °C. PAGE was performed according to a modified method of using vertical slab gels (1.5 mm thick) and was set up forming a discontinuous system of two layers. These two layers were: (i) resolving gel: 13.5 cm layer of 7.5% polyacrylamide, and (ii) stacking gel: 1.5 cm layer of 3% polyacrylamide. Four isozymes were tested and the staining protocols followed Wendel & Weeden (1989). Only clear isozyme bands were scored (numbered beginning with the running closer to the origin) and enzymatic schema diagrams painted according to RF values (relative mobility) (Kuhns & Fretaz, 1978). The frequency distribution of isozyme bands was calculated according to enzymatic schema diagram (Table 2 – 5). Different patterns occurring in each zone of activity (not single bands data) were scored as discrete variables, using '1' to indicate presence, and '0' to indicate absence of a unique pattern. A dendrogram, that depicts the degree of relationships among the taxa, was produced using hierarchical cluster analysis [NTSYS–pc2.1 software (Rohlf, 2000), UPGMA method (Sneath & Sokal, 1973)].

RESULTS

Isozyme bands. All 37 specimens produced a total of 168 polymorphism isozyme bands; of these, there were 58 PPO, 47 COD, 28 SOD and 35 MDH. Portion pictures of electrophoresis and schema graphs are shown in Figs 1 to 5. Variation of isozyme bands in each specimen were 6–13 (PPO), 6–12 (COD), 2–8 (SOD), and 0–8 (MDH). The isozyme bands showed polymorphism. Consequently, the four isozymes patterns were suitable for fingerprints to distinguish different species of the genus *Curcuma*.

The bands presented large diversities between species. Two of the *C. longa* specimens (number 18 and 11) had 6 PPO isozyme bands, and *C. phaeocaulis* (number 33) produced 13 PPO bands; *C. sichuanensis* (number 29) had 6 COD bands, and number 12

Table 1. Origin of the study materials in this research.
Tabla 1. Origen de los materiales estudiados en esta investigación.

Number	Taxon	Origin	Notes
1	*Curcuma longa*	Dayi, Sichuan	cultivated
2	*Curcuma longa*	Longquan, Sichuan	wild
3	*Curcuma longa*	Chongzhou, Sichuan	cultivated
4	*Curcuma longa*	Qianwei, Sichuan	cultivated
5	*Curcuma longa*	Shuangliu, Sichuan	cultivated
6	*Curcuma longa*	Qianwei, Sichuan	cultivated
7	*Curcuma longa*	Xinjin, Sichuan	cultivated
8	*Curcuma longa*	Muchuan, Sichuan	cultivated
9	*Curcuma longa*	Muchuan, Sichuan	cultivated
10	*Curcuma longa*	Muchuan, Sichuan	cultivated
11	*Curcuma longa*	Qianwe, Sichuan	wild
12	*Curcuma longa*	Shiling, Sichuan	cultivated
13	*Curcuma longa*	Cuiping, Sichuan	wild
14	*Curcuma longa*	Yibin, Sichuan	wild
15	*Curcuma longa*	Baihua, Sichuan	wild
16	*Curcuma longa*	Fulu,Sichuan	wild
17	*Curcuma longa*	Muchuan, Sichuan	cultivated
18	*Curcuma longa*	Cuiping, Sichuan	wild
19	*Curcuma longa*	Cuiping, Sichuan	wild
20	*Curcuma longa*	Medicinal Botanical Garden, Guangxi	cultivated
21	*Curcuma longa*	Medicinal Botanical Garden, Guangxi	cultivated
22	*Curcuma longa*	Medicinal Botanical Garden, Guangxi	cultivated
23	*Curcuma sichuanensis*	Chongzhou, Sichuan	cultivated
24	*Curcuma sichuanensis*	GAP Base,Sichuan	cultivated
25	*Curcuma sichuanensis*	Sanjiang, Sichuan	wild
26	*Curcuma sichuanensis*	Cuiping, Sichuan	wild
27	*Curcuma sichuanensis*	Weiyuan, Sichuan	cultivated
28	*Curcuma sichuanensis*	Chongzhou, Sichuan	cultivated
29	*Curcuma sichuanensis*	GAP Base, Sichuan	cultivated
30	*Curcuma phaeocaulis*	Chongzhou, Sichuan	cultivated
31	*Curcuma phaeocaulis*	Qianwei, Sichuan	cultivated
32	*Curcuma phaeocaulis*	Chengdu, Sichuan	cultivated
33	*Curcuma phaeocaulis*	Shuangliu, Sichuan	cultivated
34	*Curcuma phaeocaulis*	Medicinal Botanical Garden, Guangxi	cultivated
35	*Curcuma chuanhuangjiang*	JianyangSichuan Province	cultivated
36	*Curcuma kwangsiensis*	Medicinal Botanical Garden, Guangxi	cultivated
37	*Curcuma wenyujin*	Medicinal Botanical Garden, Guangxi	cultivated

of *C. longa* had 12 COD bands; *C. phaeocaulis* (number 31) had 2 SOD bands, and *C. longa* (number 1, 2, 5, 7, 8, and 19) had 8 SOD bands; *C. wenyujin* (number 37) had 8 bands of MDH, and *C. phaeocaulis* (number 34) had no bands.

Genetic relationships analysis. The Jaccard's similarity coefficients were calculated with the four isozymes data and the genetic distance coefficients (GS) varied from 0.08 to 0.54 among the 37 specimens. The phylogenetic tree (Fig. 6) was constructed following the UPGMA method according to RF values.

When GS was 0.52, the 37 specimens were largely divided into two groups. The first group contained 18 specimens of *C. longa*, all of which were collected from the Sichuan province. The second group included four specimens of *C. longa*, and all specimens of the other five species. In the first group, most of the cultivated *C. longa* specimens assembled together as a subgroup and some of the wild *C. longa* clustered as another subgroup. In the second group, four cultivated *C. longa* specimens and five *C. sichuanensis* specimens (numbers 23–27) belonged to the same subgroup; another subgroup included all the specimens of *C. phaeocaulis*, *C. chuanhuangjiang*, *C. kwangsiensis*, *C. wenyujin* and two cultivated *C. sichuanensis* species (numbers 28 and 29).

Table 2. Rf value and frequency distribution of PPO bands.
Table 2. Valores Rf y frecuencia de distribución de bandas PPO.

Bands	Rf value	Frequency %	Bands	Rf value	Frequency %	Bands	Rf value	Frequency %
P1	0.07	2.7	P21	0.32	5.4	P41	0.56	2.7
P2	0.1	2.7	P22	0.33	16.2	P42	0.57	18.9
P3	0.11	27.0	P23	0.35	5.4	P43	0.58	37.8
P4	0.13	35.1	P24	0.36	24.3	P44	0.59	27
P5	0.14	16.2	P25	0.37	18.9	P45	0.6	8.1
P6	0.15	16.2	P26	0.38	10.8	P46	0.63	2.7
P7	0.16	29.7	P27	0.39	16.2	P47	0.68	2.7
P8	0.18	29.7	P28	0.4	21.6	P48	0.71	2.7
P9	0.19	2.7	P29	0.42	24.3	P49	0.72	18.9
P10	0.2	10.8	P30	0.43	29.7	P50	0.75	2.7
P11	0.21	13.5	P31	0.44	40.5	P51	0.76	8.1
P12	0.22	13.5	P32	0.45	2.7	P52	0.77	37.8
P13	0.23	43.2	P33	0.47	10.8	P53	0.78	24.3
P14	0.24	2.7	P34	0.48	32.4	P54	0.79	10.8
P15	0.25	8.1	P35	0.49	2.7	P55	0.8	2.7
P16	0.26	8.1	P36	0.5	2.7	P56	0.81	18.9
P17	0.27	5.4	P37	0.51	2.7	P57	0.83	16.2
P18	0.28	8.1	P38	0.53	16.2	P58	0.85	2.7
P19	0.29	8.1	P39	0.54	2.7			
P20	0.3	2.7	P40	0.55	29.7			

Table 3. Rf value and frequency distribution of COD bands.
Tabla 3. Valores Rf y frecuencia de distribución de bandas COD.

Bands	Rf value	Frequency %	Bands	Rf value	Frequency %	Bands	Rf value	Frequency %
C1	0.11	32.4	C17	0.39	35.1	C33	0.62	5.4
C2	0.12	24.3	C18	0.40	13.5	C34	0.63	2.7
C3	0.13	18.9	C19	0.41	59.5	C35	0.64	5.4
C4	0.18	27.0	C20	0.43	10.8	C36	0.70	2.7
C5	0.19	21.6	C21	0.44	32.4	C37	0.71	2.7
C6	0.20	27.0	C22	0.45	21.6	C38	0.72	18.9
C7	0.21	13.5	C23	0.46	24.3	C39	0.73	10.8
C8	0.23	10.8	C24	0.47	24.3	C40	0.74	2.7
C9	0.27	8.1	C25	0.50	2.7	C41	0.75	2.7
C10	0.28	18.9	C26	0.51	10.8	C42	0.76	24.3
C11	0.29	2.7	C27	0.53	21.6	C43	0.78	27.0
C12	0.30	2.7	C28	0.55	27.0	C44	0.81	8.1
C13	0.33	5.4	C29	0.56	24.3	C45	0.83	62.2
C14	0.35	8.1	C30	0.59	64.9	C46	0.88	46.0
C15	0.36	5.4	C31	0.60	21.6	C47	0.89	2.7
C16	0.38	2.7	C32	0.61	5.4			

Table 4. Rf value and frequency distribution of SOD bands.
Table 4. Valores de Rf y frecuencia de distribución de bandas SOD.

Bands	Rf value	Frequency %	Bands	Rf value	Frequency %	Bands	Rf value	Frequency %
S1	0.17	5.4	S11	0.55	16.2	S21	0.73	24.3
S2	0.20	16.2	S12	0.56	18.9	S22	0.74	32.4
S3	0.21	24.3	S13	0.57	29.7	S23	0.76	24.3
S4	0.22	5.4	S14	0.58	24.3	S24	0.77	24.3
S5	0.23	10.8	S15	0.61	5.4	S25	0.78	51.4
S6	0.24	2.7	S16	0.63	40.5	S26	0.8	24.3
S7	0.28	21.6	S17	0.64	24.3	S27	0.83	24.3
S8	0.33	2.7	S18	0.66	8.1	S28	1	78.4
S9	0.51	24.3	S19	0.68	24.3			
S10	0.54	24.3	S20	0.71	32.4			

Table 5. Rf value and frequency distribution of MDH bands.
Table 5. Valores Rf y frecuencia de distribución de bandas MDH.

Bands	Rf value	frequency %	Bands	Rf value	frequency %	Bands	Rf value	frequency %
M1	0.06	21.6	M13	0.4	2.7	M25	0.54	27
M2	0.07	5.4	M14	0.41	16.2	M26	0.55	18.9
M3	0.08	8.1	M15	0.42	5.4	M27	0.56	18.9
M4	0.09	8.1	M16	0.43	27	M28	0.57	2.7
M5	0.11	43.2	M17	0.44	2.7	M29	0.58	24.3
M6	0.12	8.1	M18	0.46	24.3	M30	0.59	2.7
M7	0.14	2.7	M19	0.47	2.7	M31	0.6	2.7
M8	0.15	5.4	M20	0.48	13.5	M32	0.61	5.4
M9	0.16	2.7	M21	0.49	21.6	M33	0.62	24.3
M10	0.2	5.4	M22	0.5	10.8	M34	0.63	2.7
M11	0.23	2.7	M23	0.51	2.7	M35	0.65	8.1
M12	0.39	5.4	M24	0.52	21.6			

DISCUSSION

When GS was at 0.28, *C. kwangsiensis* clustered together with one specimen of *C. phaeocaulis* (number 33); thereafter, they grouped with the other four specimens of *C. phaeocaulis*. Results indicated that *C. kwangsiensis* and *C. phaeocaulis* had relatively closer genetic relationships. However, there might be an error due to the phylogeny tree methods, or this error may be the result of the long-term companion planting in the Medicinal Botanical Garden of Guangxi Autonomous Region. Further DNA analyses are needed to detect this error.

On the study of RAPD marker analysis (Chen et al., 1999), 119 bands were produced with 12 primers, and the genetic distance was 0. 164 between *C. wenyujin* and *C. sichuanensis*; the intraspecific genetic distance was much larger than interspecific: they were 0.866 for *C. wenyujin* and 0.885 for *C. sichuanensis*. Chen et al. recognized that *C. wenyujin* was close to *C. sichuanensis*, and merged *C. sichuanensis* into *C. wenyujin*. It is difficult to identify these two species on the level of DNA. While the 18S rRNA and *trn*K gene sequences of *C. sichuanensis* and *C. longa* corresponded completely to the types either 1a or 1b (Sasaki et al., 2002), and the sequence of *C. wenyujin* belonged to type 5, great differences were shown between *C. wenyujin* and *C. sichuanensis* in the *trn*K and 18S rRNA sequences. *C. sichuanensis* and *C. wenyujin* clustered together after clustering study of leaf epidermal features (Xiao et al., 2000). However, Xiao et al. confirmed that *C. sichuanensis* was close to *C. longa* rather than *C. wenyujin* following investigation of the origin and the colour of tuber. It means that former studies of these two species had some different results. Based on our study, the isozymes patterns of PPO, COD, SOD, and MDH showed significant diversities within

C. wenyujin and *C. sichuanensis*; they were included into different groups in the phylogenetic tree. The genetic relationship was remote between *C. wenyujin* and *C. sichuanensis*, and *C. sichuanensis* was close to *C. longa*.

The relationship between *C. longa* and *C. sichuanensis* was complex (Xia et al., 1999; Xiao et al., 1997, 2000, 2001). On the morphological study of leaves and rhizomes, Xiao et al. (2004a, 2004b, 2004c) indicated that *C. sichuanensis* was the cultivated variety of *C. longa*. However, they contradicted themselves in their study of leaves and rhizomes: (i) on the morphological study of leaves, *C. wenyujin* and *C. sichuanensis* clustered together firstly, and *C. longa* was far away from them; (ii) on the morphological study of rhizomes, *C. longa* and *C. sichuanensis* got together at first. Quan et al. (2005) examined the contents of curdione and turmerol by means of HPLC and 5sRNA sequence on five species (*C. kwangsiensis*, *C. wenyujin*, *C. phaeocaulis*,

Fig. 1. Isozyme band photos of some SOD, PPO, COD and MDH in plants of *Curcuma* L. A, B: SOD photos of 19-27 and 28-37 materials; C, D: PPO photos of 9-18 and 29-37 materials; E, F: COD photos of 10-18 and 29-37 materials; G, H: MDH photos of 9-18 and 19-27 materials.
Fig. 1. Fotos de las bandas de izoenzimas de algunas SOD, PPO, COD y MDH en plantas de *Curcuma* L. A, B: Fotos de SOD de los materiales 19-27 y 28-37; C, D: Fotos de PPO de los materiales 9-18 y 29-37; E, F: Fotos de COD de los materiales 10-18 y 29-37; G, H: Fotos de MDH de los materiales 9-18 y 19-27.

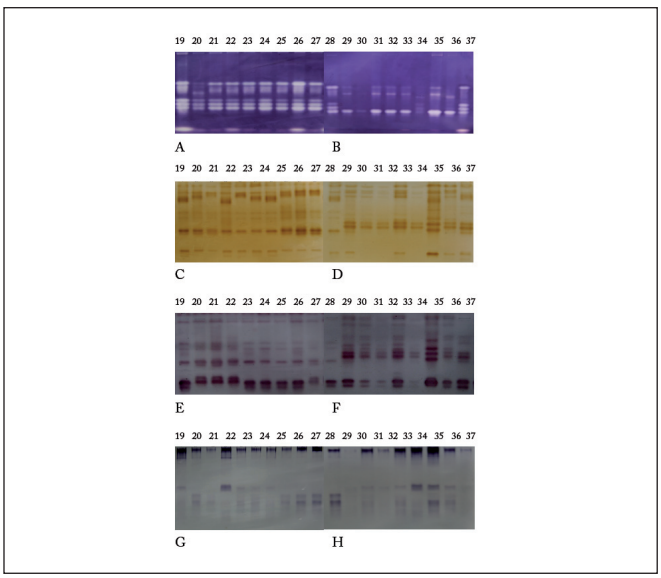


Fig. 2. Isozyme patterns of PPO enzyme to identify the 37 specimens of six *Curcuma* species. (Numbers from 1 to 37 refer to the materials listed in Table 1).
Fig. 2. Modelo de distribución de las isoenzimas de la enzima PPO para identificar los 37 especímenes de las seis especies de *Curcuma*. (Los números 1 a 37 se refieren a los materiales listados en la Tabla 1).

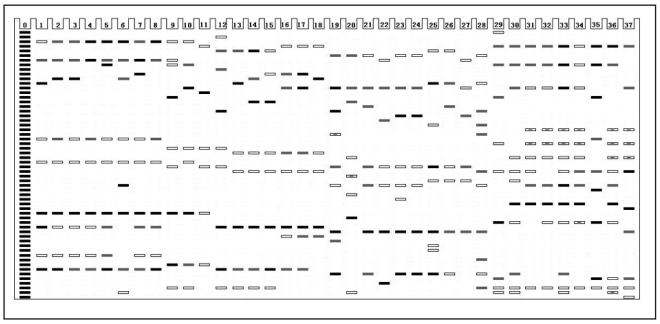


Fig. 3. Isozyme patterns of COD enzyme to identify the 37 specimens of six *Curcuma* species. (Numbers from 1 to 37 refer to the materials listed in Table 1).
Fig. 3. Modelo de distribución de las isoenzimas de la enzima COD para identificar los 37 especímenes de las seis especies de *Curcuma*. (Los números 1 a 37 se refieren a los materiales listados en la Tabla 1).

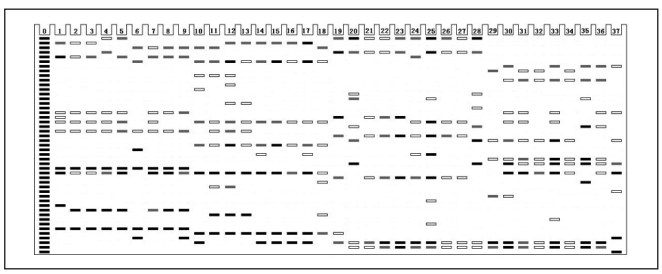


Fig. 4. Isozyme patterns of SOD enzyme to identify the 37 specimens of six *Curcuma* species. (Numbers from 1 to 37 refer to the materials listed in Table 1).
Fig. 4. Modelo de distribución de las isoenzimas de la enzima SOD para identificar los 37 especímenes de las seis especies de *Curcuma*. (Los números 1 a 37 se refieren a los materiales listados en la Tabla 1).

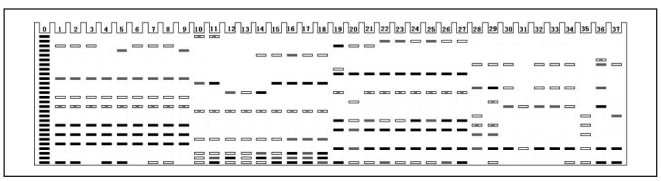


Fig. 5. Isozyme patterns of MDH enzyme to identify the 37 specimens of six *Curcuma* species. (Numbers from 1 to 37 refer to the materials listed in Table 1).
Fig. 5. Modelo de distribución de las isoenzimas de la enzima MDH para identificar los 37 especímenes de las seis especies de *Curcuma*. (Los números 1 a 37 se refieren a los materiales listados en la Tabla 1).

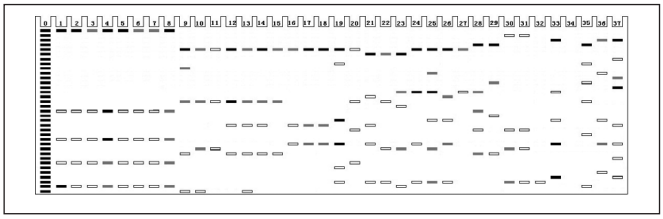
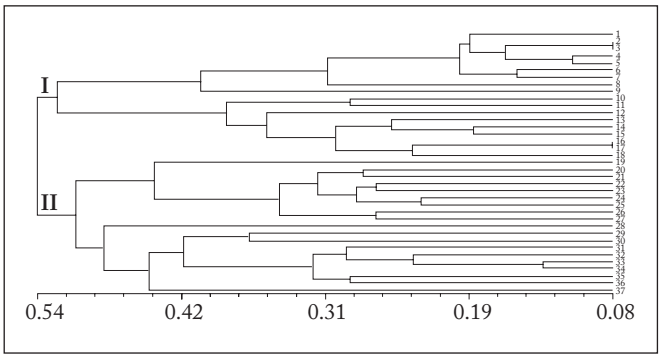


Fig. 6. Dendrogram based on UPGMA analysis of genetic similarity obtained from isozymes data. (Numbers from 1 to 37 refer to the materials listed in Table 1).
Fig. 6. Dendrograma basado en análisis UPGMA de similaridad genética obtenido de los datos de las isoenzimas.
(Los números 1 a 37 se refieren a los materiales listados en la Tabla 1).



C. longa, *C. sichuanensis*). Their results showed that *C. longa* was on intimate relationship with *C. sichuanensis*. Tang et al. (2008) recognized that *C. sichuanensis* was the cultivated mutation species of *C. longa* by isozyme patterns of POD and EST. In the present study, three cultivated (numbers 23, 24 and 27) and two wild specimens (numbers 25 and 26) of *C. sichuanensis* were clustered together with four specimens of *C. longa* at first; two other cultivated specimens (numbers 28 and 29) of *C. sichuanensis* were gathered with *C. chuanhuangjiang*, *C. phaeocaulis*, *C. kwangsiensis* and *C. wenyujin*. No doubt that the taxonomic status of *C. sichuanensis* needs further study.

Although a detailed Flora Sichuanica by Zhu (1992), *C. chuanhuangjiang* is not mentioned in the Flora of China. Liu & Wu (1999) merged *C. chuanhuangjiang* into *C. kwangsiensis*. Xiao et al. (2004a, 2004b, 2004c) thought that *C. chuanhuangjiang* was the cultivated mutation of *C. longa*. In our study, the isozymes patterns of *C. chuanhuangjiang* were dissimilar to the other 36 specimens. Taking into account the previous analysis of Cao & Katsuko (2003) and Tang et al (2008), we believed that it is much more reasonable to retain *C. chuanhuangjiang* as an individual species.

In conclusion, the four isozymes successfully supported the taxonomical classification of the six *Curcuma* species. From the dendrogram, 3/4 of the wild specimens of *C. longa* (numbers 11-18), and the two wild species of *C. sichuanensis* (numbers 25 and 26), clustered together first in groups I and II, and then gathered with other cultivated specimens; it is shown that the protein differentiation already occurred between cultivated and wild species. We strongly suggest paying attention to the distinction between cultivated and wild specimens when making classification, as well as on the clinic of TCM.

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