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Evaluation on genetic relationships among China's endemic *Curcuma* L. herbs by mtDNA

Evaluación de las relaciones genéticas entre hierbas de *Curcuma* L. endémicas de China por mtDNA

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Abstract. Six mitochondrial (mtDNA) markers, cox2, ccmFn, ccb256, cox3, Nad4L-orf25, and atp4, were combined to assess genetic relationships by using Maximum Likelihood (ML) and Maximum parsimony (MP). This was done among nine Chinese *Curcuma* herbal species, included two rare species which are difficult to distinguish from their morphological characters. The relationships are very close among the nine species, and the overall distance was 0.018. In this study, the backbone of such nine species was achieved firstly to date, and being divided into two groups with moderate to strong bootstrap support. Both the ML and MP tree were obtained with similarity topologies of the same group except for partial divergences of deep relationships. The highlights is that *Curcuma* genus was divided into two strong supported groups in the ML tree, Group I ([[C. *kwangsiensis*, C. *phaeocaulis*], [C. *chuanhuangjiang*, C. *aromatica*], C. *yunnanensis*]) with 89 BS (bootstrap) support, Group II ([[[C. *longa*, C. *sichuanensis*], C. *amarissima*], C. *wenyujin*]) with 98 BS support.

Keywords: *Curcuma*; Mitochondrial DNA; Genetic relationship.

Resumen. Seis marcadores mitocondriales (mtDNA), cox2, ccmFn, ccb256, cox3, Nad4L-orf25, y atp4, se combinaron para determinar las relaciones genéticas Probabilidad Máxima (ML) y Máxima Parsimonia (MP). Esto se hizo entre nueve especies de hierba chinas, incluyendo dos especies raras que son difíciles de distinguir a partir de sus caracteres morfológicos. Las relaciones fueron muy cercanas entre las nueve especies, y la distancia total fue 0.018. En este estudio, la columna vertebral de las nueve especies fue alcanzada primero a la fecha, y se dividió en dos grupos apoyo de bootstrap de moderado a fuerte. El árbol para ambas, el ML y el MP fueron obtenidos con topologías de similitud del mismo grupo excepto por divergencias parciales de relaciones profundas. El género *Curcuma* fue dividido en dos grupos fuertemente apoyados en el árbol ML, Grupo I ([[C.*kwangsiensis*, C. *phaeocaulis*], [C. *chuanhuangjiang*, C. *aromatica*], C. *yunnanensis*]) con un apoyo de 89 BS (boostrap), Grupo 2)[[[C. *longa*, C. *sichuanensis*], C. *amarissima*], C. *wenyujin*]) con un apoyo de 98 BS.

Palabras clave: *Curcuma*; DNA mitocondrial; Relación genética.

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INTRODUCTION

Curcuma L. (Zingiberaceae) is native to Southeast Asia, South Asia, and Australia, comprising approximately 70-120 species. Some *Curcuma* species are used as food and herbs in Asia. The curcuminoids, extracted from *Curcuma* rhizomes, have been reported on vital medicinal qualities, including antioxidant, anti-cancer, anti-Alzheimer's, HIV-1 protease inhibitory, and anti-tumor effects (Ramsewak et al., 2000; Anand et al., 2008; Hamaguchi et al., 2010; Pisano et al., 2010; Kunwar et al., 2011) .

Around 10 *Curcuma* species are distributed in China (Xiao et al., 1997), of which four species (*C. longa*, *C. wenyujin*, *C. phaeocaulis*, and *C. kwangsiensis*) are officially recorded in Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2010) as folk herbs named as Radix Curcumae (also named Yujin), Rhizoma Curcumae Longae (also named Jianghuang) and Rhizoma Curcumae (also named Ezhu) in Traditional Chinese Medicine (TCM). The *C. sichuanensis* and *C. chuanhuangjiang* frequently appear as substitutes of the aforementioned four *Curcuma* species in cultivation, herb trading and TCM, and the remaining species are also used as folk therapy at rural areas in China (Chen, 1981; Zhu, 1992).

In TCM, the same medicine can be made from these *Curcuma* species, of which one can be handled as a different medicine because of its different tissues. Moreover, the morphological characters are very similar within and inter-species of *Curcuma*, which makes it generally confusing to distinguish these species at both the vegetative and reproductive stages (Xiao et al., 2004a, 2004b). It is necessary to adopt various methods to study their genetic relationships.

The DNA markers have shown some advantages on genetic relationship, identification, and phylogenetic analysis among medicinal plants (Chen et al., 2010; Li et al., 2014; Newmaster et al., 2013; Takamiya et al., 2011). However, the genetic relationships within *Curcuma* were poorly resolved using some classic universal genes, including *rbc*L, *mat*K, and ITS (Cao & Komatsu, 2003; Kress et al., 2005; Techaprasan et al., 2006; Zaveska et al., 2012). Clearly, more DNA markers should be tested on *Curcuma* species for their identification, genetic relationships, and phylogeny. At present, some studies have achieved ideal results on genetic relationships and phylogeny by mitochondrial DNA (mtDNA) within the angiosperm group (Group, 1998; Qiu et al., 1999; Lutzoni et al., 2000; Miya et al., 2003; Schneider et al., 2004).

In this study, six mtDNA markers (cox2, ccmFn, ccb256, cox3, Nad4L-orf25, and atp4), were employed to test genetic relationships and diversities among Chinese *Curcuma* species, and to explore the taxonomic status of *C. sichuanensis*, *C. chuanhuangjiang*, *C. amarissima*, and *C. yunnanensis*. This will provide some helpful information for the phylogeny of *Curcuma*, as well as for species identification for TCM in China.

MATERIALS AND METHODS

Taxon sampling. We sampled 12 taxa (9 ingroup and 3 outgroup) in this study (Table 1). Within the genus, *C. exigua*, was locally extinct at some locations and narrowly distributed in Miyi County, Panzhihua City, Sichuan Province (Liu, 2003); *C. viridiflora* only from Taiwan was not sampled by the present authors (Wu & Larsen, 2000); *C. zanthorrhiza* and *C. flaviflora* are na-

Table 1. The geographic locations of sampled *Curcuma* species, and their data statement. Dataset is available in TreeBase (ID:20808).
Tabla 1. Ubicación geográfica de las especies de *Curcuma* muestreadas, y el informe de sus datos. La serie de datos está disponible en TreeBase (ID:20808).

Taxon	Location	atp4	ccb256	ccmFn	cox2	cox3	Nad4L-orf25
C. chuanhuangjiang Z.Y. Zhu	Jianyang, Sichuan	1	1	1	1	1	1
C. sichuanensis X.X. Chen	Neijiang, Sichuan	1	1	1	1	1	1
C. yunnanensis N. Liu & S. J. Chen	Guangzhou, Guangdong	1	1	1	1	1	1
C. kwangsiensis S. G. Lee & C.F. Liang	Hengxian, Guangxi	1	1	1	1	1	1
C. amarissima Roscoe	Meng'a, Yunnan	1	1	1	1	1	1
C. longa Linnaeus	Leshan, Sichuan	1	1	1	1	1	1
C. phaeocaulis Valeton	Shuangliu, Sichuan	1	1	1	1	1	1
C. wenyujin Y. H. Chen & C. Ling	Medicinal Botanical Garden, Guangxi	1	1	1	1	1	1
C. aromatica Salisbury	Meishan, Sichuan	1	1	1	1	1	1
Zingiber officinale Roscoe	Local market	1	1	1	0	1	1
Hedychium coronarium J. König	Local market	1	0	1	0	0	1
Alpinia japonica (Thunberg) Miquel	Local market	1	0	1	0	0	1

Note: "1" means the data was newly produced by our Lab; "0" represented missing data in this study.
Nota: "1" significa que los datos son nuevos y producidos en nuestro laboratorio; "0" representa datos faltantes en este estudio.

tive to Indonesia, Thailand, etc., which were reported scattered in Yunnan Province before and cannot be sampled in China actually. In this study, we added two rare species of *Curcuma* in China: *C. amarissima* and *C. yunnanensis* to study genetic and phylogenetic relationships among the *Curcuma* species.

DNA extraction, amplification and sequencing. DNA was extracted from dried leaf tissue using the CTAB procedure (Doyle & Doyle, 1987). PCR amplifications were performed using primers and procedures as described in previous studies (Duminil et al., 2002; Zeng et al., 2010). Each 50 μL of polymerase chain reaction (PCR) contained 2 μL of DNA solution (20 ng), 5 μL of PCR reaction buffer, 5 μL dNPT mix (0.2 mM), 2 μL of each primer (10 μmol/L), and 1.5 U *Taq*DNA polymerase (Takara, Japan). Sequencing reactions were performed using the dideoxy chain termination method running on an ABI PRISM 3730 automated sequencer.

Sequence alignment and analysis. Sequences were aligned using the L-INS-i algorithm in MAFFT (Katoh et al., 2002). The alignments were then manually checked and adjusted in Mesquite 2.75 (Maddison and Maddison, 2011). For Maximum Likelihood (ML), we performed phylogenetic analysis based on six combined mtDNA dataset using as implemented in RaxML 1.3 (Silvestro & Michalak, 2012). The nucleotide substitution was tested with the combined data set in jModelTest (Darriba et al., 2012). We chose GTR+I model in RAxML with the default settings for the optimization of individual per-site substitution rates. The support was evaluated with 1000 bootstrap. For maximum parsimony (MP), MP tree was conducted in PAUP v4.0 (Swofford, 2003) to perform a heuristic search with 1000 random addition sequence replicates and default parameters (TBR branch swapping with one tree held at each step during stepwise addition). MP support was evaluated with 2000 bootstrap replicates, and 50% Majority-rule consensus tree was produced from 10234 trees. The dataset and ML tree with MP bootstrap are available for further/downstream analyses in TreeBase (ID: 20808).

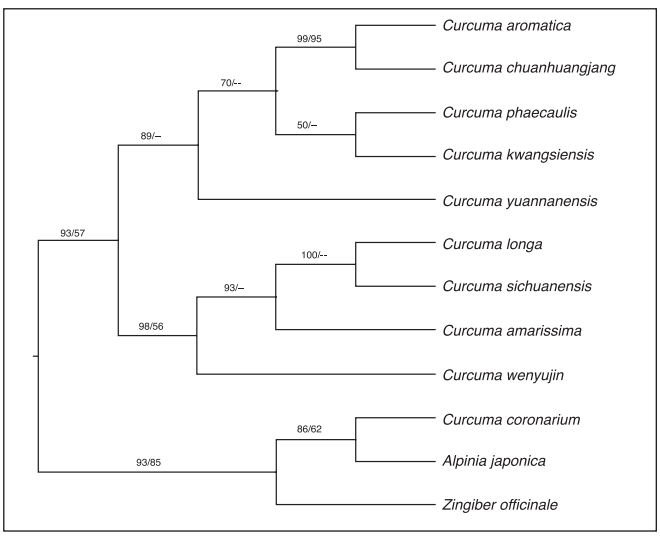

Fig. 1. The Maximum Likelihood (ML) phylogenetic tree of *Curcuma* species. The numbers at the branch are aps of ML and MP (Maximum Parsimony). "-" means the bootstrap absent or < 50.
Fig. 1. Árbol filogenético de máxima probabilidad de las especies de *Curcuma*. Los números sobre las líneas horizontales son el resultado de la técnica no paramétrica de bootstraping de ML y MP (Máxima Parsimonia). "-" los promedios ausentes ó <50 del bootstrapping.

RESULTS

The alignment length of dataset are 505 bp (atp4), 259 bp (ccb256), 506 bp (ccmFn), 353 bp (cox2), 705 bp (cox3), 588 bp (Nad4L-orf25) and 3186 bp (combined mtDNA dataset). Each single locus was difficult to evaluate their relationships among the nine *Curcuma* species because of their low separating capacity of pairwise genetic distances (Table 2) (Tamura et al., 2011). For atp4 has 9 0-distance with distance range of 0-0.069; ccb256 has 10 0-distance with distance range of 0-0.066; ccmFn has 7 0-distance with distance range of 0-0.004; cox2 has 11 0-distance with distance range of 0-0.037; cox3 has 10 0-distance with distance range of 0-0.004; Nad4L-orf25 has 15 0-distance with distance range of 0-0.014, and combined data has 3 0-distance with range of 0-0.027. Then, we concatenated six loci as a combined mtDNA dataset (see Supplementary dataset) for genetic relationships and phylogeny analyses.

The ML and MP tree showed similar topologies of two same group divided (Group I and Group II in Fig. 1) with partial divergence of deep relationships. In both trees, the bootstrap support is moderate to strong at the key nodes or branches (MP/

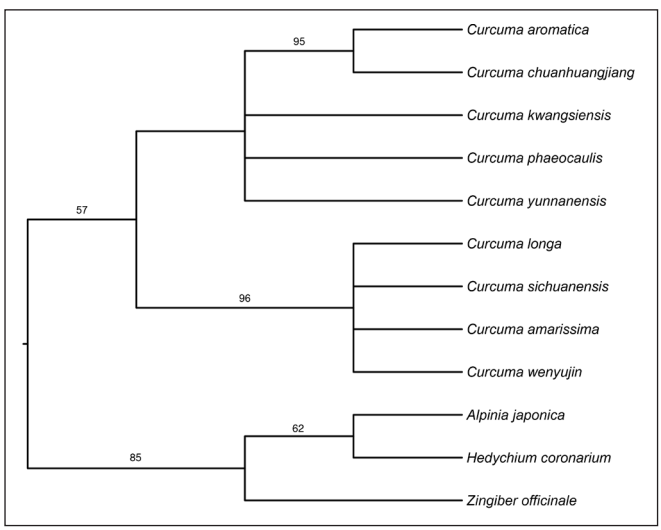

Fig. S1. 50% Majority-rule consensus MP tree of *Curcuma* species. Supplementary dataset. The dataset of combined with six loci.
Fig. S1. Regla del consenso mayoritario del 50% en el árbol MP de las especies de *Curcuma*. Datos suplementarios. Datos de la combinación con seis loci.

Table 2. The pairwise genetic distances between sequences.
Tabla 2. Las distancias genéticas apareadas entre secuencias.

Taxon		Distance						
		atp4	ccb256	ccmFn	cox2	cox3	Nad4L-orf25	Combined
C. yunnanensis	*C. wenyujin*	0.065	0.066	0.004	0.034	0.001	0.007	0.027
C. yunnanensis	*C. kwangsiensis*	0	0.064	0.004	0.031	0.001	0.014	0.018
C. wenyujin	*C. kwangsiensis*	0.065	0.002	0.004	0.003	0.003	0.007	0.013
C. yunnanensis	*C. longa*	0.065	0.064	0.002	0.031	0	0.007	0.026
C. wenyujin	*C. longa*	0	0.002	0.002	0.003	0.001	0	0.001
C. kwangsiensis	*C. longa*	0.065	0	0.002	0	0.001	0.007	0.012
C. yunnanensis	*C. aromatica*	0.002	0.064	0.004	0.031	0.003	0.007	0.017
C. wenyujin	*C. aromatica*	0.067	0.002	0.004	0.003	0.004	0	0.013
C. kwangsiensis	*C. aromatica*	0.002	0	0.004	0	0.004	0.007	0.003
C. longa	*C. aromatica*	0.067	0	0.002	0	0.003	0	0.012
C. yunnanensis	*C. sichuanensis*	0.065	0.064	0.002	0.031	0	0.007	0.026
C. wenyujin	*C. sichuanensis*	0	0.002	0.002	0.003	0.001	0	0.001
C. kwangsiensis	*C. sichuanensis*	0.065	0	0.002	0	0.001	0.007	0.012
C. longa	*C. sichuanensis*	0	0	0	0	0	0	0
C. aromatica	*C. sichuanensis*	0.067	0	0.002	0	0.003	0	0.012
C. yunnanensis	*C. phaeocaulis*	0	0.066	0.002	0.037	0	0.009	0.017
C. wenyujin	*C. phaeocaulis*	0.065	0.004	0.002	0.008	0.001	0.002	0.013
C. kwangsiensis	*C. phaeocaulis*	0	0.002	0.002	0.006	0.001	0.009	0.003
C. longa	*C. phaeocaulis*	0.065	0.002	0	0.006	0	0.002	0.012
C. aromatica	*C. phaeocaulis*	0.002	0.002	0.002	0.006	0.003	0.002	0.003
C. sichuanensis	*C. phaeocaulis*	0.065	0.002	0	0.006	0	0.002	0.012
C. yunnanensis	*C. chuanhuangjiang*	0.004	0.066	0.004	0.034	0.001	0.007	0.018
C. wenyujin	*C. chuanhuangjiang*	0.069	0.004	0.004	0	0.003	0	0.013
C. kwangsiensis	*C. chuanhuangjiang*	0.004	0.002	0.004	0.003	0.003	0.007	0.004
C. longa	*C. chuanhuangjiang*	0.069	0.002	0.002	0.003	0.001	0	0.012
C. aromatica	*C. chuanhuangjiang*	0.002	0.002	0	0.003	0.001	0	0.001
C. sichuanensis	*C. chuanhuangjiang*	0.069	0.002	0.002	0.003	0.001	0	0.012
C. phaeocaulis	*C. chuanhuangjiang*	0.004	0.004	0.002	0.008	0.001	0.002	0.003
C. yunnanensis	*C. amarissima*	0.065	0.064	0.002	0.031	0	0.007	0.026
C. wenyujin	*C. amarissima*	0	0.002	0.002	0.003	0.001	0	0.001
C. kwangsiensis	*C. amarissima*	0.065	0	0.002	0	0.001	0.007	0.012
C. longa	*C. amarissima*	0	0	0	0	0	0	0
C. aromatica	*C. amarissima*	0.067	0	0.002	0	0.003	0	0.012
C. sichuanensis	*C. amarissima*	0	0	0	0	0	0	0
C. phaeocaulis	*C. amarissima*	0.065	0.002	0	0.006	0	0.002	0.012
C. chuanhuangjiang	*C. amarissima*	0.069	0.002	0.002	0.003	0.001	0	0.012
overall	0.038	0.016	0.002	0.009	0.001	0.003	0.011	

ML > 50). The ML tree with support values (ML and MP) was shown, and the MP tree was as supplementary (Fig. 1S) in this study. In MP tree, some deep relationship was not solvable for the parsim-informative sites limited. In the ML tree, *Curcuma* genus was divided into two strong supported groups, Group I ([[*C. kwangsiensis*, *C. phaeocaulis*], [*C. chuanhuangjiang*, *C. aromatica*], *C. yunnanensis*]) with 89 BS (bootstrap) support, Group II ([[[*C. longa*, *C. sichuanensis*], *C. amarissima*], *C. wenyujin*]) with 98 BS support, and the third Group with 93 BS included the total three species which were picked as outgroup, then this clade clustered with the two *Curcuma* groups with 93 BS, is suitable as outgroup in this study.

DISCUSSION

Two species, *C. yunnanensis* and *C. amarissima*, not included into relationships analysis within Chinese *Curcuma* before, were enclosed in the present study. They were clustered into two different groups with strong BS support in Fig. 1. *C. yunnanensis* was the basal branch in Group I, and *C. wenyujin* was the first branch in Group II. In Group II, *C. amarissima* was close to [*C. longa*, *C. sichuanensis*].

Curcuma chuanhuangjiang is an endemic species, exclusively located in Jianyang, Sichuan province not included in China's Flora (Wu & Larsen, 2000). It was supposed as an individual species (Zhu, 1992; Tang et al., 2008; Deng et al., 2011), and was confirmed to be a variety of *C. longa* based on the morphological characteristics of its leaves (Xiao et al., 2004b). *C. chuanhuangjiang* was included into Group I in our study, and clustered with *C. aromatica* with 99/95 BS (ML/MP), and *C. longa* was in Group II (98/96 BS ML/MP). Besides, it was still possible to separate *C. chuanhuangjiang* from *C. longa* using alternative methods such as chemical compound (Duan, 2013). The aforementionded studies did not involve *C. aromatica* into their analysis. *C. chuanhuangjiang* was close to *C. aromatica*, and it should be treated as an individual species or a variant of *C. aromatica* according to the ML/MP trees and their similar morphological characters.

Curcuma sichuanensis was identified as a variety of *C. longa* in a previous analysis using RAPD markers (Xiao et al., 1999). This identification was further examined by numerical taxonomy and by the histological and morphological characteristics of leaves and rhizomes (Xiao et al., 2004a, 2004b, 2004c). The findings of the above numerical, taxonomical, histological and morphological characters studies, which included *C. wenyujin*, *C. sichuanensis* and *C. longa*, were contradictory: Based on the leaf morphology, *C. wenyujin* and *C. sichuanensis* clustered together, and *C. longa* was isolated from *C. sichuanensis* in the topology; also, the study of rhizomes showed that *C. longa* and *C. sichuanensis* cluster together. Isozyme analyses suggested that *C. sichuanensis* was a variety of *C. longa* (Tang et al., 2008; Deng et al., 2011). Based on our study, *C. sichuanensis* clustered with *C. longa* (100 BS/ML, BS/MP < 50) in Group II, and we suggested that *C. sichuanensis* is an individual species (or a variety) close to *C. longa* (Xiao et al., 1998; Quan et al., 2005; Tang et al., 2008; Deng et al., 2011a, 2011b, 2015). The previous studies checked the relationships between *C. sichuanensis* and *C. longa*, focusing on *C. sichuanensis* is whether an individual species or a variety of *C. longa* used by morphological characteristics, isozymes, contents of curdione and turmerol, and chloroplast markers. Because the relationships between these two species were very close and with the same chromosome numbers 2n = 3× = 63 (Dai, 2009), more methods should test their relationships in the future (e.g., especially mitochondrial, chloroplast, and nuclear DNA markers).

In this study, we firstly yielded the robust results on the phylogenetic backbone of China's *Curcuma*, and achieved the systematic status of *C. sichuanensis*, *C. chuanhuangjiang*, *C. amarissima*, and *C. yunnanensis* within *Curcuma*.

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