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RELATIONSHIPS EVALUATION ON SIX HERBAL SPECIES (*CURCUMA*) BY DNA BARCODING

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Abstract

Three chloroplast regions, *rbc*L, *psb*A-*trn*H and *pet*A-*psb*J were applied to assess the genetic relationships among six *Curcuma* medicinal species, which are difficult to distinguish from morphology. The Maximum Parsimony tree was conducted by Kimura 2-parameter model with MEGA 4. The genetic relationships were linked with geographical distributions among these six species; *Curcuma sichuanensis* is a mutation species of *Curcuma longa*, *Curcuma sichuanensis* couldn't be defined as a single species, and *Curcuma chuanhuangjiang* is an individual species.

Key words: *Curcuma*, DNA barcoding, Genetic *rbc*L, *psb*A-*trn*H, *pet*A-*psb*J.

Introduction

Curcuma L. (Zingiberaceae) is a geographically widespread group, comprising approximately 70 species (Jan *et al*., 2011-2012). About 10 *Curcuma* species are distributed in China (Xiao *et al*., 1997; Li *et al*., 2001; Ye *et al*., 2008), of which 6 species are treated as vital Chinese folk herbal medicine in Traditional Chinese Medicine (TCM), and an extract of rhizomes exhibits activity of anti-inflammatory, anticancer and HIV-1 protease inhibitory (Moussavi *et al*., 2006). Four of the six species, *Curcuma longa*, *C. phaeocaulis*, *C. wenyujin*, and *C. kwangsiensis*, are officially recorded as herbal species in the Anon., (2010). However, 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 in folk therapeutic uses (Chen, 1981; Zhu, 1992).

In TCM, the same medicine can be made from these six *Curcuma* species, of which one can be handled as different medicine with its different tissues. Moreover, the morphological characters are very similar within and inter-species of *Curcuma*, and the florescence varies from April to October, and even the color always shows diversity within intra-species, which makes it generally confused to distinguish these species at both vegetative and reproductive stage. It is necessary to adopt various methods to identify these six herbal species (Sasaki *et al*., 2002; Cao & Katsuko, 2003). The DNA barcode has shown some advantages in phylogenetic analysis, identification of related species, even providing a good potential method on the identification and evaluation quality for the genus of medicinal plants (Kress *et al*., 2005, Kress & Erickson, 2007; Xiao *et al*., 2000; Newmaster *et al*., 2006; Taberlet *et al*., 2007; Valentini *et al*., 2009; Chen *et al*., 2010; Shinwari & Shinwari, 2010). Zheng & Xia (2010) studied Zingiberaceae tribe on ITS and *mat*K, and confirmed ambiguity in the two sequences used in the phylogeny of tribe Zingiberaceae. Clearly, more DNA barcodes should be made available for the identification of *Curcuma* species. Beside Zingiberaceae *mat*K and *rbc*L have been applied to several angiosperm families (Shinwari *et al*., 2014; Jamil *et al*., 2014).

The objectives of this paper, based on the study of *rbc*L, *psb*A-*trn*H, *pet*A-*psb*J barcodes, are to distinguish the six related *Curcuma* species; to explore the taxonomic status of *C. sichuanensis* and *C. chuanhuangjiang* species, the relationship between *C. sichuanensis* and *C. wenyujin*; thus to provide helpful information for TCM.

Materials and Methods

The total 32 specimens were used in this study (Table 1). Among them, 26 specimens were collected from different localities in Sichuan Province, and the remaining 6 specimens were collected from Guangxi Medicinal Botanical Garden. Sichuan and Guangxi are the original regions of these six species in China, and Sichuan is the geo-herbalism habitat of *C. longa*, *C. sichuanensis*, *C. phaeocaulis* and *C. chuanhuangjiang* (Hu, 1998).

PCR and sequencing: Total DNA isolation was carried out on fresh leaves by CTAB method (Doyle & Doyle, 1987). The PCR reactions were conducted in a final volume of 25 µL containing 10 µL 2×Taq MasterMiX (CWBIO), 0.5 µL DNA, 14 µL ddH_2O, 0.5 µL primers on a GeneAmp PCR System 9700 thermocycler. Primers and reaction conditions were used in the present study according to Lledo´ *et al*. (1998) and Techaprasan *et al*. (2006). The nucleotide sequence data generated are available in GenBank accession numbers JF719546-JF719577 (*rbc*L), JF73022-JF730252 (*psb*A-*trn*H), and JF730258-JF730288 (*pet*A-*psb*J).

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

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

Data analysis The DNA sequences were minimally edited and manually aligned in Geneious 4.7.4 (Drummond *et al*., 2006). The incongruence length difference and the partition homogeneity of sequences were implemented in PAUP*4.0b10 (Farris *et al*., 1995; Swofford, 2003), which was conducted to determine whether the three partitions were congruent to be combined into a total molecular evidence analysis. The individual DNA regions and combined data (*rbc*L, *psb*A-*trn*H and *pet*A-*psb*J) were conducted by Maximum Parsimony to assess topology and clade support. The results indicated that single-gene data revealed a general lack of clade support at the basal nodes of *Curcuma* (the nodes of particular interest on *C. sichuanensis* and *C. chuanhuangjiang* in the current study), therefore, single-gene analysis are not shown here.

Results and Discussion

The sequence length and variation were shown in Table 2. The sequence maximum length variation was presented in *C. longa* species, due to missed *pet*A-*psb*J. The combined data was conducted by MEGA version 4 (Tamura *et al*., 2007). Tajima's Neutrality Test for 32 sequences were s = 1 305, p_s = 1.000000, Θ = 0.248309, π = 0.662874, D = 6.472629 (S = Number of segregating sites, p_s = S/m, $\Theta = p_s/a1$, and π = nucleotide diversity).

The pairwise difference was tested, shown in Table 3. There were a total of 1 305 positions in the final dataset. The pairwise differences varied from 0.00 to 0.075. The diversity was coincident with Tajima's Neutrality Test. Because of the limited diversities, some related species (especially *C. longa* and *C. sichuanensis*), could not be distinguished clearly.

The Maximum Parsimony (MP) was carried out with the following options: Parsimony informative characters were unordered and equally weighted, gaps were treated as missing data with 1 000 random stepwise addition replicates with the full bootstrap option (1 000 replicates, seed = 80 332). The MP tree (Fig. 1) was obtained by using the Close-Neighbor-Interchange algorithm in which the initial trees were obtained with the random addition of sequences (100 replicates).

From the dendrogram, the specimens were clustered into three groups totally. All the specimens of *C. longa* and *C. sichuanensis* formed Group 1, Group 2 was composed of *C. chuanhuangjiang* and *C. kwangsiensis*, and Group 3 included all the specimens of *C. wenyujin* and *C. phaeocaulis*. Except for Group 2 and Group 3, Group 1 showed limited diversity between the species. The relationship between *C. longa* and *C. sichuanensis* was closer, compared with other four species. In Group 1, there are two adjacent sister subclades (Clade I and Clade II). Instead of being united, the total seven uncultivated *C. longa* species sparsely distributed in Clade I and Clade II. Three *C. longa* specimens (NO. 17 - 19) collected from Guangxi were included in Clade II, which showed that the genetic relationships had linkage with geographical origin.

C. wenyujin and *C. sichuanensis*, Chen *et al*. (1999) integrated *C. sichuanensis* into *C. wenyujin* on the basis of RAPD markers analysis. The differences were studied by *trn*K sequences between *C. wenyujin* and *C. sichuanensis* (Sasaki *et al*., 2002). Xiao *et al*. (2001) had suspicion Chen's view, for the research excluded *C. longa*. Xia *et al*. (2005) studied curdione/curcumol contents of the five species (*C. kwangsiensis*, *C. wenyujin*, *C. phaeocaulis*, *C. longa*, *C. sichuanensis*) and 5sRNA sequence analysis, inferred that *C. longa* was on close terms with *C. sichuanensis*. Based on the dendrogram, *C. wenyujin* and *C. sichuanensis* were fallen into different clades, and showed great diversities between these two species.

The relationship is complex between *C. longa* and *C. sichuanen*sis (Xia *et al*., 1999; Xiao *et al*., 2001). Xiao *et al*. (2000) inferred that *C. sichuanensis* was the cultivated variety of *C. longa* by RAPD marker, and study of histological and morphological on leaves and rhizomes, as well as numerical taxonomy analysis on these species (Xiao *et al*., 2004a, b, c) indicated that both *C. sichuanensis* and *C. chuanhuangjiang* were the cultivated varieties of *C. longa*. Such Xiao's above results about the relationships among *C. wenyujin*, *C. sichuanensis* and *C. longa*, were contradicted: on the study of leaves, *C. wenyujin* and *C. sichuanensis* clustered together, *C. longa* was far from *C. sichuanensis*; while on the study of rhizomes, *C. longa* and *C. sichuanensis* clustered together firstly. Tang *et al*. (2008) and Deng *et al*. (2011) studied POD (Peroxidase), EST (Esterase) and SOD (superoxide dismutase), PPO (polyphenol oxidase), MDH (malate dehydrogenase) and COD (cytochrome oxidase) isozyme patterns relatively, and showed *C. sichuanensis* was the cultivated mutation species of *C. longa*. In this study, most *C. longa* and *C. sichuanensis* specimens mixed together in the phylogenetic tree, and in clade I most *C. longa* specimens (collected from Sichuan province), meanwhile all the *C. sichuanensis* specimens (planting) were included; part of the *C. longa* (three populations from Guangxi Medicinal Botanical Garden) and a single one uncultivated *C. sichuanensis* species (No. 23) gathered into clade II. *C. sichuanensis* and *C. longa* had much more similarities on their morphological characteristics and medicinal ingredients among these six species (Xiao *et al*., 1998; Xie *et al*., 2004), and both the chromosome numbers are 2n = 3x = 63 (Dai, 2008). It is better to infer *C. sichuanensis* as the cultivated mutation of *C. longa*.

Table 2. Sequence length variation for three plastid regions of six *Curcuma* species with 32 specimens.

Species	*rbc*L (bp)	*psb*A-*trn*H (bp)	*pet*A-*psb*J (bp)
Curcuma longa (19)	669-683	676-688	691-747
C. sichuanensis (7)	671-678	687-688	691
C. phaeocaulis (3)	671-685	687	689-691
C. chuanhuangjiang(1)	677	687	691
C. kwangsiensis (1)	678	687	691
C. wenyujin (1)	678	687	691

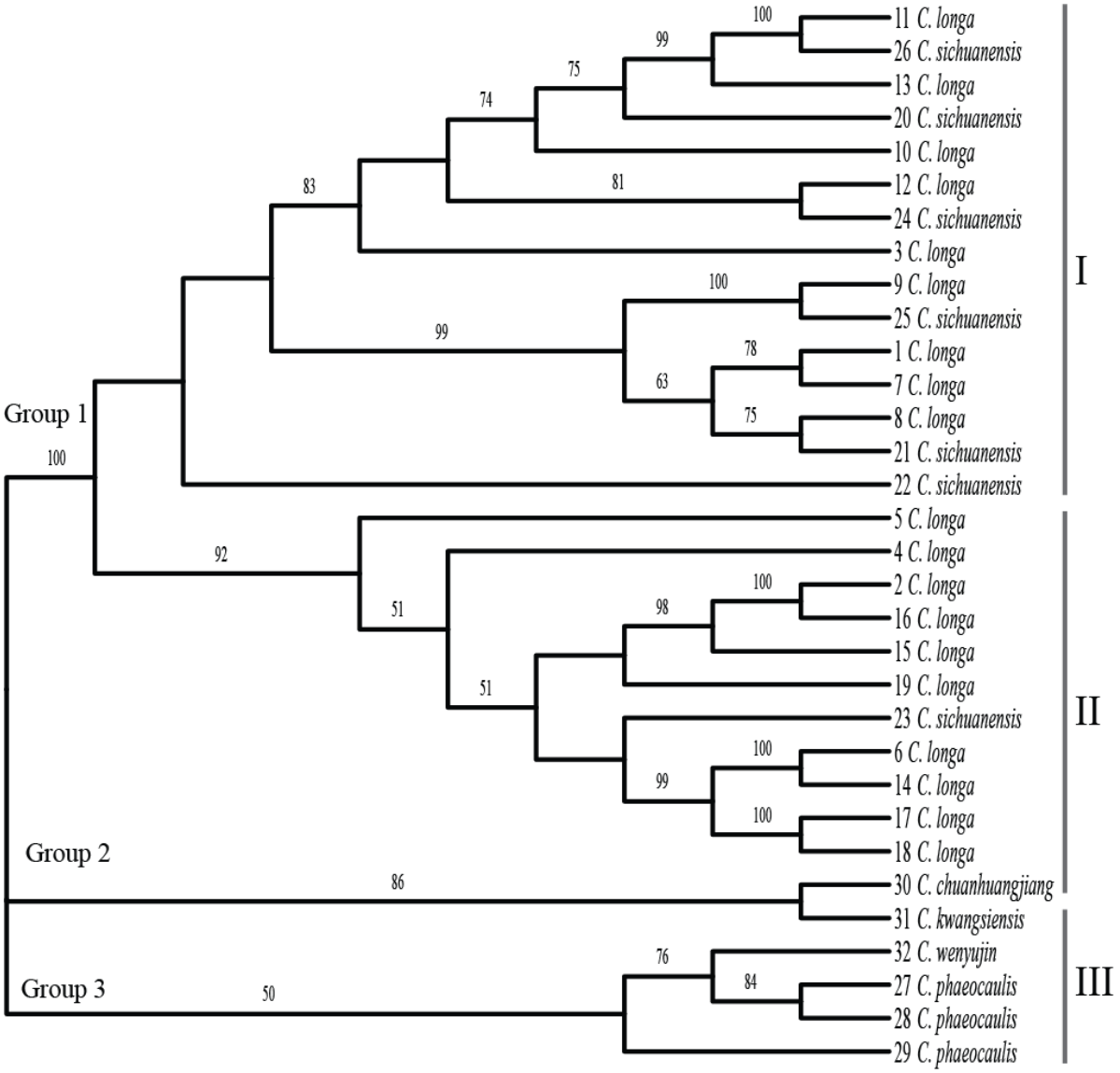

Fig. 1. The Maximum Parsimony tree of combined chloroplast data (*rbc*L, *psb*A-*trn*H and *pet*A-*psb*J) representing the six species of *Curcuma* with 32 specimens. The number at terminal of clades is sample number. Tree length = 11999, CI=0.331361, RI=0.597845, and the composite index is 0.198102.

C. chuanhuangjiang, which originated in Sichuan Province, has special rosin smell among these six species (Zhu, 1992). Liu & Wu (1999) merged *C. chuanhuangjiang* into *C. kwangsiensis* according to morphological analysis; Xiao *et al*. (2004b) confirmed *C. chuanhuangjiang* was the mutant of *C. longa* by morphological characteristics of leaves. From the MP tree, the relationship between *C. chuanhuangjiang* and *C. kwangsiensis* was close with 85 bootstrap supports; and *C. chuanhuangjiang* and *C. longa* clustered into different groups respectively. The chromosome numbers among them are different, 2n = 3x = 63 (*C. chuanhuangjiang*, *C. longa*) (Dai, 2008), 2n = 4x = 84 (*C. kwangsiensis*) (Chen *et al*., 1988). Combining the previous study and our research, we supported *C. chuanhuangjiang* as an *individual* species. Moreover, the medicinal component should be tested to find out whether keeping with the rule of Chinese Pharmacopoeia.

C. phaeocaulis, originated in Sichuan, is a separate species. No. 29 of *C. phaeocaulis*, collected from Medicinal Botanical Garden of Guangxi Autonomous Region, clustered with *C. wenyujin* (Medicinal Botanical Garden of Guangxi Autonomous Region) in MP tree. This may be devoted to the three genes lacking enough information to identify these two species.

This study represents an improvement on the identification of these six *Curcuma* species. We propose to expand the *C. longa* to include *C. sichuanensis*. *C. sichuanensis* is the mutation species of *C. longa*, and *C. chuanhuangjiang* is retained as a single species. The same species from the same localities clustered together in the dendrogram, which reveals that the genetic relationships among these six *Curcuma* species are associated with geographical distribution, and there is no separation of cultivated populations from the uncultivated.

Table 3. The pair wise difference analysis involved 32 nucleotide sequences of six *Curcuma* species with 32 specimens.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
2	0.002																														
3	0.001	0.001																													
4	0.002	0.000	0.001																												
5	0.007	0.005	0.006	0.005																											
6	0.001	0.001	0.000	0.001	0.006																										
7	0.007	0.007	0.006	0.007	0.012	0.006																									
8	0.009	0.010	0.009	0.010	0.005	0.009	0.011																								
9	0.004	0.004	0.003	0.004	0.009	0.003	0.009	0.012																							
10	0.006	0.005	0.005	0.005	0.001	0.005	0.011	0.005	0.008																						
11	0.007	0.007	0.006	0.007	0.003	0.006	0.011	0.005	0.009	0.002																					
12	0.005	0.005	0.005	0.005	0.002	0.005	0.011	0.005	0.008	0.001	0.002																				
13	0.002	0.002	0.002	0.002	0.008	0.002	0.008	0.011	0.005	0.007	0.008	0.006																			
14	0.005	0.005	0.004	0.005	0.010	0.004	0.005	0.008	0.007	0.009	0.008	0.008	0.005																		
15	0.002	0.000	0.001	0.000	0.005	0.001	0.007	0.010	0.004	0.005	0.007	0.005	0.002	0.005																	
16	0.001	0.001	0.000	0.001	0.006	0.000	0.006	0.009	0.003	0.005	0.006	0.005	0.002	0.004	0.001																
17	0.005	0.005	0.005	0.005	0.002	0.005	0.011	0.005	0.008	0.001	0.002	0.000	0.006	0.008	0.005	0.005															
18	0.009	0.009	0.008	0.009	0.005	0.008	0.011	0.007	0.011	0.005	0.005	0.004	0.010	0.011	0.009	0.008	0.004														
19	0.006	0.005	0.005	0.005	0.001	0.005	0.011	0.005	0.008	0.000	0.002	0.001	0.007	0.009	0.005	0.005	0.001	0.005													
20	0.007	0.007	0.006	0.007	0.003	0.006	0.011	0.005	0.009	0.002	0.002	0.002	0.008	0.008	0.007	0.006	0.002	0.005	0.002												
21	0.005	0.006	0.005	0.006	0.002	0.005	0.011	0.005	0.008	0.002	0.002	0.001	0.007	0.009	0.006	0.005	0.001	0.005	0.002	0.002											
22	0.070	0.071	0.070	0.071	0.066	0.070	0.075	0.067	0.074	0.067	0.067	0.066	0.072	0.073	0.071	0.070	0.066	0.070	0.067	0.067	0.065										
23	0.008	0.009	0.008	0.009	0.005	0.008	0.013	0.005	0.011	0.005	0.005	0.004	0.010	0.011	0.009	0.008	0.004	0.005	0.005	0.005	0.003	0.067									
24	0.010	0.010	0.009	0.010	0.005	0.009	0.014	0.005	0.012	0.005	0.005	0.005	0.011	0.011	0.010	0.009	0.005	0.007	0.005	0.006	0.005	0.067	0.005								
25	0.006	0.006	0.005	0.006	0.002	0.005	0.010	0.004	0.008	0.002	0.001	0.001	0.007	0.008	0.006	0.005	0.001	0.005	0.002	0.001	0.002	0.067	0.005	0.005							
26	0.008	0.008	0.008	0.008	0.005	0.008	0.012	0.006	0.011	0.004	0.005	0.003	0.009	0.010	0.008	0.008	0.003	0.004	0.004	0.005	0.004	0.069	0.002	0.006	0.004						
27	0.007	0.007	0.006	0.007	0.002	0.006	0.012	0.005	0.009	0.002	0.003	0.002	0.008	0.008	0.007	0.006	0.002	0.005	0.002	0.003	0.002	0.064	0.005	0.005	0.002	0.005					
28	0.007	0.007	0.006	0.007	0.003	0.006	0.011	0.005	0.009	0.002	0.002	0.002	0.008	0.008	0.007	0.006	0.002	0.004	0.002	0.003	0.002	0.067	0.004	0.003	0.002	0.003	0.003				
29	0.006	0.005	0.005	0.005	0.001	0.005	0.011	0.005	0.008	0.000	0.002	0.001	0.007	0.009	0.005	0.005	0.001	0.005	0.000	0.002	0.002	0.067	0.005	0.005	0.002	0.004	0.002	0.002			
30	0.008	0.006	0.007	0.006	0.002	0.007	0.013	0.007	0.010	0.002	0.004	0.002	0.008	0.011	0.006	0.007	0.002	0.006	0.002	0.004	0.003	0.068	0.006	0.007	0.003	0.005	0.004	0.004	0.002		
31	0.006	0.005	0.005	0.005	0.001	0.005	0.011	0.005	0.008	0.000	0.002	0.001	0.007	0.009	0.005	0.005	0.001	0.005	0.000	0.002	0.002	0.067	0.005	0.005	0.002	0.004	0.002	0.002	0.000	0.002	
32	0.006	0.005	0.005	0.005	0.001	0.005	0.011	0.005	0.008	0.000	0.002	0.001	0.007	0.009	0.005	0.005	0.001	0.005	0.000	0.002	0.002	0.067	0.005	0.005	0.002	0.004	0.002	0.002	0.000	0.002	0.000

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