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Difference of Curcumin Content in *Curcuma longa* L. (Zingiberaceae) Caused by Hybridization with Other *Curcuma* Species

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ABSTRACT

Curcumin, which is traditionally known to have effects on various types of diseases in humans, is found in Curcuma longa L. Previous reports have indicated that the curcumin content varies between the different lines of this species. To clarify the differences in the amounts of curcumin between the lines, we investigated the outcomes of cultivation experiments with the hybridization or introgression between C. longa and other Curcuma species using the matK gene of chloroplast DNA (cpDNA) and the external transcribed spacer (ETS) of nuclear DNA (nrDNA). The results show that there is heterogeneity of the ETS and incongruence between the matK and the ETS phylogenetic trees, suggesting that hybridization and introgression had taken place in the diversification of the various lines of C. longa. Moreover, although all of the lines had the same cpDNA haplotype of C. longa, the lines of homogeneous C. longa had a high content of curcumin, whereas the lines created by hybridization and introgression with other Curcuma species had a medium or low level. These results suggest that the difference of curcumin content among the various lines of C. longa was caused by hybridization and introgression with other Curcuma species.

Keywords*: *Curcuma longa*, *Curcumin*, *Hybridization*, *Introgression*, *Molecular Analysis*, *Nuclear DNA

1. Introduction

Many chemicals in plants are potential drugs for humans and natural products from plants are found in many therapeutic formulations. Moreover, conscious efforts to search for desirable traits in plants have been underway for the past century, and in recent decades species with desirable traits have come to be regarded as important biological resources in need of conservation [1].

Curcuma longa L., which belongs to the ginger family, Zingiberaceae, is a perennial widely used as a spice, a colorant and also as a major ingredient of curry powder [2]. This species has a long history of use as a traditional medicine in China and India [3], reflecting it's diverse and beneficial health effects. In addition, the *curcuma* species contains phenolic compounds found in the plant's rhizomes. Traditionally, curcumin is well-known to have therapeutic effects on a variety of human diseases, and the cancer preventive activity of curcumin is being intensively studied all over the world. Experiments in animal models indicate that it is a preventive agent against various types of cancer [4]. Specifically, curcumin inhibits the cell growth of various cancer cell lines, induces apoptosis of cancer cells [5-7], and was effective on the cell-cycle regulation of cancer cells [8].

According to previous study there is a difference in the curcumin content among individuals of *C. longa* [9,10], however, it remains unclear why the curcumin content is different. To identify the lines with high curcumin content, Hayakawa *et al.* [10,11] developed a molecular marker. Recent molecular phylogenetic study using chloroplast DNA (cpDNA) sequences indicated that *C. longa* has some closely related species. Also, a large number of previous studies of species within the genus *Drosophila* had illuminated the relationship between genetic distance and reproductive isolation [12,13]. One possibility is that the difference in curcumin content within *C. longa* was caused by including hybrids between *C. longa* and other *Curcuma* species. It is very difficult to detect polymorphisms of morphology of the rhizomes and cpDNA sequences within this species. Al-

though this hypothesis could be clarified by comparing nuclear DNA (nrDNA) sequences, unfortunately, such study has not been done so far.

The number of phylogenetic studies based on molecular data has grown enormously in recent years, and most of the recent studies are concerned with closely related species or variation within species. In particular, the use of molecular markers has considerably improved our knowledge about how past events shape the genetic diversity within a species [14-16]. Recently, various molecular markers have been widely analyzed to assess the genetic variability of wild plants [17]. Among them, nuclear markers are mostly neutral with relatively high mutation rates, and, in association with the history, provide information to estimate the putative parents involved in hybridization and introgression [18,19]. The polymorphisms of the external transcribed spacer (ETS) region in nuclear DNA (nrDNA) are good tools for clarifying the relationship of closely related taxa in many plant groups [20-24]. Here, to test our hypothesis of the hybridization of *C. longa*, we describe the DNA polymorphisms of the ETS region in *C. longa* and its allied species and discuss the possible reasons for the differences in curcumin content of this species.

2. Materials and Methods

2.1. Plant Materials

For the plant materials, we used 1 *Curcuma alismatifolia* Gagnep. cultivar 'Sawang Chiang Mai', 3 *C. aromatica* Salib., 12 *C. longa* L. and 1 *C. zedoaria* Rosc. for molecular analysis in this study (**Table 1**). Of these, 2 *C. aromatica*, 5 to 12 *C. longa* and 1 *C. zedoaria* were cultivated in the field of the Faculty of Agriculture, Kochi University, Japan for 2006-2009 (See detail Hayakawa *et al.* [10]). Rhizomes were transplanted in late May for four years. For fertilizer dressing, a total of 1.5 kg/a of N, 0.6 kg/a of P_2O_5, and 1.4 kg/a of K_2O was applied over

Table 1. List of sample of *Curcuma* species used in this study.

No. Sepcies	Locality	Curcumin content[1]				nrDNA[3] Type
		2006	2007	2008	2009	
1 *Curcuma alismatifolia* 'Sawang Chiang Mai'	Chiang Mai. Thailand	–[2]	–	–	–	
2 *C. aromatica* (Kochi)	Kochi Perfecture. Japan	38^{bcd}	71^{b}	47^{b}	126^{c}	
3 *C. aromatica* (Tanegashima)	Tanegashima Island. Kagoshima Perfecture. Japan	41^{abcd}	78^{bc}	36^{b}	122^{c}	
4 *C. aromatica* (Okinawa)	Okinawa Perfecture. Japan	–	–	–	–	
5 *C. longa* (Kochi)	Kochi Perfecture. Japan	327^{ab}	398^{ab}	358^{ab}	392^{c}	Hybrid
6 *C. longa* (Tanegashima)	Tanegashima Island. Kagoshima Perfecture. Japan	301^{abc}	382^{ab}	392^{ab}	361^{c}	Hybrid
7 *C. longa* (Wakayama A)	Wakayama Perfecture. Japan	179^{abcd}	403^{ab}	388^{ab}	374^{c}	Hybrid
8 *C. longa* (Wakayama B)	Wakayama Perfecture. Japan	1^{d}	2^{c}	1^{b}	1^{c}	Introgression
9 *C. longa* (Wakayama C)	Wakayama Perfecture. Japan	–	404^{abc}	396^{ab}	390^{c}	Hybrid
10 *C. longa* (Okinawa A)	Okinawa Perfecture. Japan	–	–	364^{ab}	347^{c}	Hybrid
11 *C. longa* (Okinawa B)	Okinawa Perfecture. Japan	–	–	373^{ab}	305^{c}	Hybrid
12 *C. longa* (Indonesia A)	Bogol. West Java. Indonesia	2849^{a}	2777^{a}	2678^{a}	3059^{a}	Pure line
13 *C. longa* (Indonesia B)	Bogol. West Java. Indonesia	–	229^{abc}	337^{ab}	310^{c}	Hybrid
14 *C. longa* (Indonesia C)	Bogol. West Java. Indonesia	–	–	–	2315^{b}	Pure line
15 *C. longa* (Vietnam A)	Vietnam	–	–	–	2977^{a}	Pure line
16 *C. longa* (Vietnam B)	Vietnam	–	–	–	3198^{a}	Pure line
17 *C. zedoaria*	Unkonwn	1^{cd}	2^{c}	1^{b}	1^{c}	

[1]Curcumin content in primaly branch rhizomes (mg/100g).[2]Not examind. [3]Type of nrDNA in *Curcuna longa*. Values followed by the same latter in a column of each year are not signigicantly at 5% level by one-way ANOVA.

four years. In addition, 200 kg/a of compost fertilizer, 15 kg/a of magnesia lime, and 30 kg/a of chicken droppings were applied. The experimental plots were arranged in a randomized complete design with two replicates, which formed three rows. Due to a lack of seed rhizomes, some lines of *C. longa* were examined with/without replicate using two or three rows. Samples were harvested in early December for four years. The total curcumin content (mg/100g) of primary branch rhizomes was measured by using High Performance Liquid Chromatography (HPLC), according to the method described by Sato *et al*. [25].

2.2. Molecular Analyses

For the molecular analyses, total DNA was isolated from fresh root using a Plant Genomic DNA Mini Kit (VIOGENE, Sunnyvale, USA), according to the manufacturer's protocol. We amplified the *maturase K* (*matK*) gene from cpDNA and the external transcribed spacer (ETS) region of 18S-26S rDNA from nrDNA with primers designed by Johnson and Soltis [26] and Starr *et al.* [27], respectively. The isolated DNA was amplified by PCR in a 50 μl reaction solution containing approximately 50 ng total DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1.25 units *Taq* DNA polymerase (TaKaRa) and 0.5 μM of each primer pair. We used the following thermal cycle profile for amplification by the PCR Thermal Cycler Dice (TaKaRa): 1 min at 94˚C, 2 min at 48˚C, and 2 min at 72˚C for 45 cycles, followed by 15 min of final extension at 72˚C. After amplification, the PCR products of the *matK* and ETS region were subjected to electrophoresis in 1% low-melting-temperature agarose gels to remove by-products and purify amplified products. We sequenced the purified PCR products using a BigDye Terminator ver. 3.1 (Applied BioSystems) and ABI Prism 3100 Genetic Analyzer (Applied BioSystems) according to the manufacturer's instructions. For sequencing, we used the same primers as those used for amplification.

To construct a phylogenetic tree based on the *matK* sequences of *Curcuma* and its allied species and the ETS sequences of *Curcuma* species, the amplified regions were aligned using ClustalW [28] and were improved manually using MEGA 4 [29]. Phylogenetic relationships were analyzed using the neighbor-joining (NJ) method with PAUP* 4.08b [30]. The NJ analyses were performed using MEGA 4 with Kimura's two-parameter model. For the NJ analyses, bootstrapping with 1000 pseudo-replicates was chosen to examine the robustness of the clades and their phylogenetic relationships. The *matK* sequences were collected from DDBJ/EMBL/GenBank International DNA databases (**Table 2**).

For the ETS region, because *C. longa* with a medium curcumin content could not determine its sequence caused by putative heterozygosity, we carried out PCR-RFLP analysis after checking the sequencing results and alignments. The result of the alignments indicated that an autapomorphic character of the nrDNA was the restriction site *Hinf* I. After designating the restriction sites, the amplified products were digested by *Hinf* I at 37˚C for more than an hour. The digested DNAs were separated on 1.0% agarose gel and the size of each band was determined.

3. Results

3.1. Curcumin Content

The gradient of curcumin content between species decreased as follows; South Asian *C. longa* > domestic *C. longa* and *C. longa* (Indonesia B) > *C. aromatica* > *C. zedoaria* and *C. longa* (Wakayama B) (**Table 1**) [10] and the level of curcumin content was divided into three groups; high, medium and low.

3.2. Molecular Analyses

To construct the molecular phylogenetic tree of *Curcuma* and its allied species, we determined the sequences of the *matK* gene of *Curcuma* cpDNA and seven outgroup taxa (**Table 2**). The lengths of the *matK* gene of the *Curcuma* species varied from 1539 bp (*C. alismatifolia* 'Sawang Chiang Mai' and *C. longa* (Wakayama B)) to 1554 bp (*C. thorelii*). The result of the phylogenetic analysis of *matK* indicated that *C. longa* had a conserved sequence in this species and was closely related to *C. aromatica* and *C. zedoaria*, whereas *C. alismatifolia* 'Sawang Chiang Mai' was located in the basal position of the phylogenetic tree and the sister to *C. thorelii* with a high boot strap value (**Figure 1**).

In addition, we sequenced the ETS region of nrDNA to detect polymorphisms among *Curcuma* species. The lengths of the ETSs of *Curcuma* species were 514 bp (*C. alismatifolia*) to 517 bp (*C. aromatica*). The sequences have been deposited into the DDBJ/EMBL/GenBank International DNA databases (*C. alismatifolia*, AB588183; *C. aromatica*: AB588181, *C. longa*: AB588182, AB588185 and AB588186, *C. zedoaria*: AB588184). The results of the phylogenetic analyses of the ETSs indicated that *C. longa* and closely related species were divided into two monophyletic groups: clade 1 and clade 2 (**Figure 2**). Clade 1 consisted of all individuals of *C. longa* and clade 2 consisted of *C. longa*, *C. aromatica* and *C. zedoaria*. Although all homogeneous *C. longa* with its high curcumin content appeared in clade 1, *C. longa* (Wakayama B) with its low curcumin content was located in clade 2 (**Figure 2**, **Table 1**). This suggested

Table 2. Accession numbers of *matK* using phylogenetic analysis of *Curcuma* and outgroup taxa.

	matK	
Species	Accession No.	Reference
Curcuma aeruginosa	AF478840	Kress *et al.* (2002)
C. amarissima	AB047751	Cao *et al.* (Unpubl.)
C. alismatifolia 'Sawang Chiang Mai'	AB588187	this study
C. aromatica	AB047731	Cao *et al.* (2001)
C. aromatica (Kochi)	AB551929	Hayakawa *et al.* (2010)
C. aromatica (Tanegashima)	AB551929	Hayakawa *et al.* (2010)
C. aromatica (Okinawa)	AB551929	Hayakawa *et al.* (2010)
C. attenuata	GQ248110	Hollingsworth *et al.* (Unpubl.)
C. bicolor	AF478837	Kress *et al.* (2002)
C. chuanezhu	AB047736	Cao *et al.* (2001)
C. chuanhuangjiang	AB047732	Cao *et al.* (2001)
C. chuanyujin	AB047733	Cao *et al.* (2001)
C. comosa	AF478838	Kress *et al.* (2002)
C. elata	AB047747	Cao *et al.* (Unpubl.)
C. exigua	AB047750	Cao *et al.* (Unpubl.)
C. kwangsiensis A	AB047744	Cao *et al.* (2001)
C. kwangsiensis B	AB047745	Cao *et al.* (Unpubl.)
C. longa (Kochi)	AB551930	Hayakawa *et al.* (2010)
C. longa (Tanegashima)	AB551930	Hayakawa *et al.* (2010)
C. longa (Wakayama A)	AB551930	Hayakawa *et al.* (2010)
C. longa (Wakayama B)	AB551931	Hayakawa *et al.* (2010)
C. longa (Wakayama C)	AB551930	Hayakawa *et al.* (2010)
C. longa (Okinawa A)	AB551930	Hayakawa *et al.* (2010)
C. longa (Okinawa B)	AB551930	Hayakawa *et al.* (2010)
C. longa (Indonesia A)	AB551930	Hayakawa *et al.* (2010)
C. longa (Indonesia B)	AB551930	Hayakawa *et al.* (2010)
C. longa (Indonesia C)	AB551930	Hayakawa *et al.* (2010)
C. longa (Vietnam A)	AB551930	Hayakawa *et al.* (2010)
C. longa (Vietnam B)	AB551930	Hayakawa *et al.* (2010)
C. longa	AB047738	Cao *et al.* (2001)
C. phaeocaulis	AB047735	Cao *et al.* (2001)
C. roscoeana A	AB047741	Cao *et al.* (Unpubl.)
C. roscoeana B	AF478839	Kress *et al.* (2002)
C. sichuanensis A	AB047739	Cao *et al.* (Unpubl.)
C. sichuanensis B	AB047740	Cao *et al.* (Unpubl.)
C. thorelii	AF478841	Kress *et al.* (2002)
C. wenyujin	AB047746	Cao *et al.* (2001)
C. xanthorrhiza	AB047752	Cao *et al.* (Unpubl.)
C. yunnanensis	AB047749	Cao *et al.* (Unpubl.)
C. zedoaria	AB551932	Hayakawa *et al.* (2010)
C. zedoaria A	AB047734	Cao *et al.* (2001)
C. zedoaria B	AB047743	Cao *et al.* (2001)
Outgroup		
Boesenbergia rotunda	AF478827	Kress *et al.* (2002)
Cautleya spicata	AF478834	Kress *et al.* (2002)
Cornukaempferia aurantiflora	AF478835	Kress *et al.* (2002)
Curcumorpha longiflora	AF478842	Kress *et al.* (2002)
Kaempferia marginata	AB232054	Sitthithaworn and Komatsu (Unpubl.)
Scaphochlamys biloba	AF478889	Kress *et al.* (2002)
Zingiber mioga	AB047755	Cao *et al.* (Unpubl.)

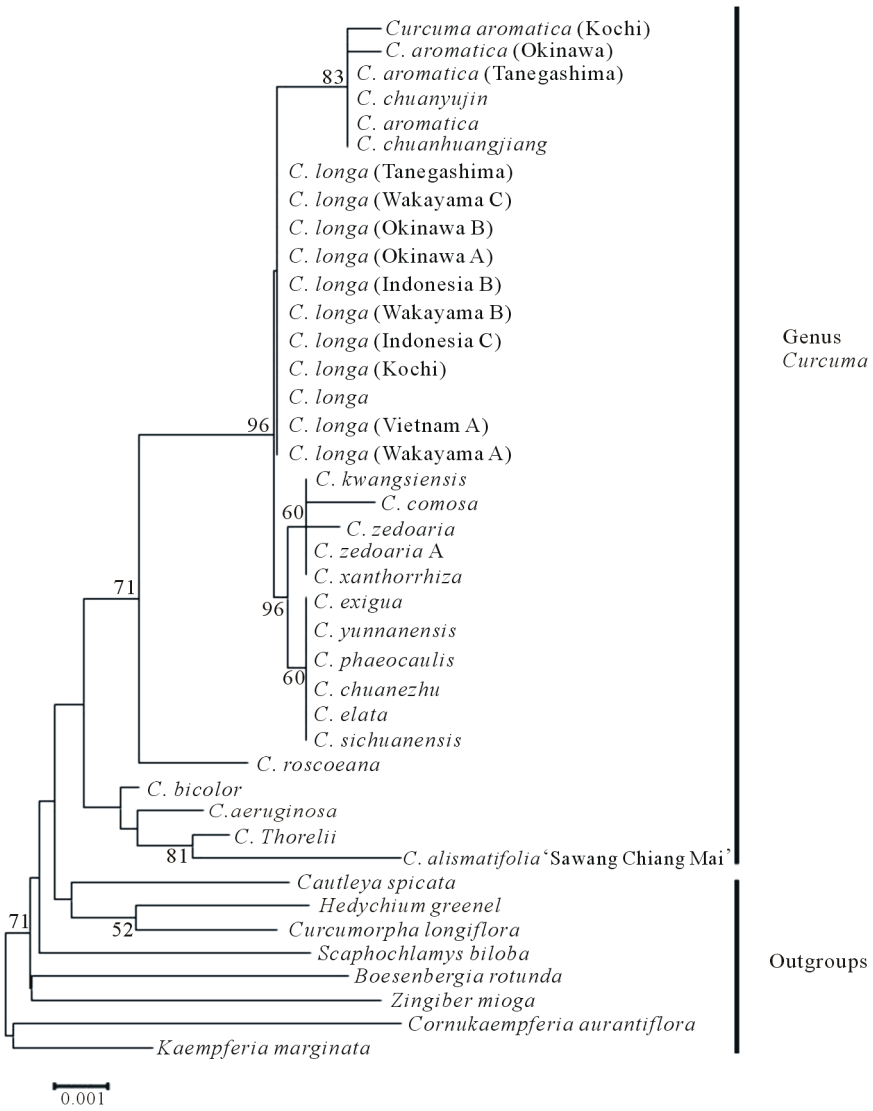

Figure 1. Phylogenetic tree of *Curcuma* and its allied species in the *matK* gene of cpDNA using the neighbor-joining (NJ) method. The numbers below the branches indicate the bootstrap value.

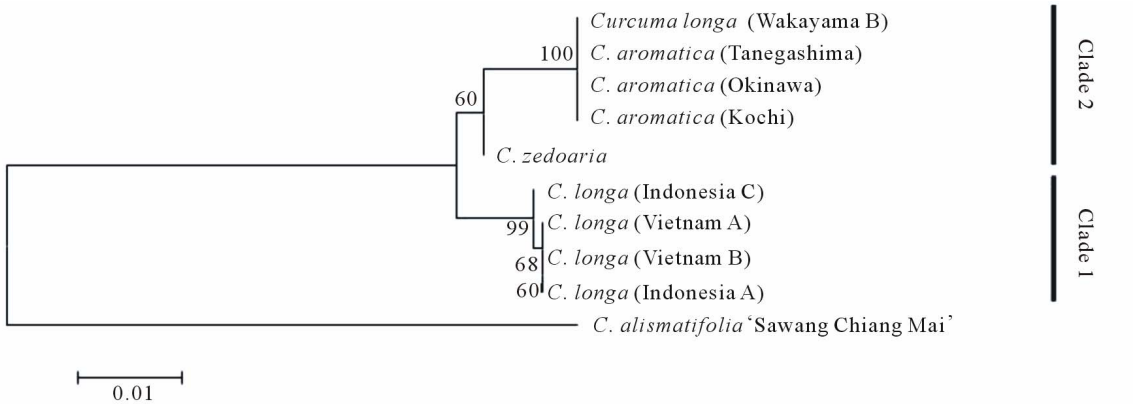

Figure 2. Phylogenetic tree of *Curcuma* species in the ETS region of nrDNA using the NJ method. The numbers below the branches indicate the bootstrap value.

that *C. longa* (Wakayama B) was introgressive with clade 2 because it had the cpDNA haplotype of *C. longa*. However, some individuals could not be sequenced in the ETS region because of the putative heterogeneity of *C. longa* and other *Curcuma* species. To detect their heterogeneity, we conducted a PCR-RFLP analysis because restriction of the site of *Hinf* I to distinguish *C. longa* with other *Curcuma* species was in the ETS region (**Figure 3**). The result was that the digestion pattern of all samples of homogeneous *C. longa* and *C. aromatica* showed the expected patterns, and heterogeneous *C. longa* showed the combined patterns of homogeneous *C. longa* and *C. aromatica* (**Figure 4**). Moreover, *C. longa* (Wakayama B) showed same band pattern as *C. aromatica.* We therefore confirmed that the medium and low curcumin contents of *C. longa* were hybrid and introgressive between *C. longa* and other *Curcuma* species on clade 2 (**Figure 4**, **Table 1**).

4. Discussion

In general, hybrids typically display a mosaic of parental and intermediate morphological characters, although extreme and novel characters appear quite often. A species with morphological characteristics intermediate between two recognized species has always been considered to be a hybrid [31]. However, morphological characteristics alone, such as the rhizomes of the *Curcuma* species, are of limited value when identifying natural hybrids, but molecular studies have greatly enhanced our knowledge in this field [32]. Interspecific hybrids are most commonly identified by the heterogeneity of nrDNA and the incongruences between cpDNA and nrDNA phylogenies that may indicate different parental contributions to the hybrid genome [33,34]. In particular, incongruence between cpDNA and nrDNA phylogenies is very likely the result of interspecific gene flow and subsequent

Figure 3. Expected restriction sites of *Hinf* I for molecular characteristics of ETS regions by PCR-RFLP. H: restriction site.

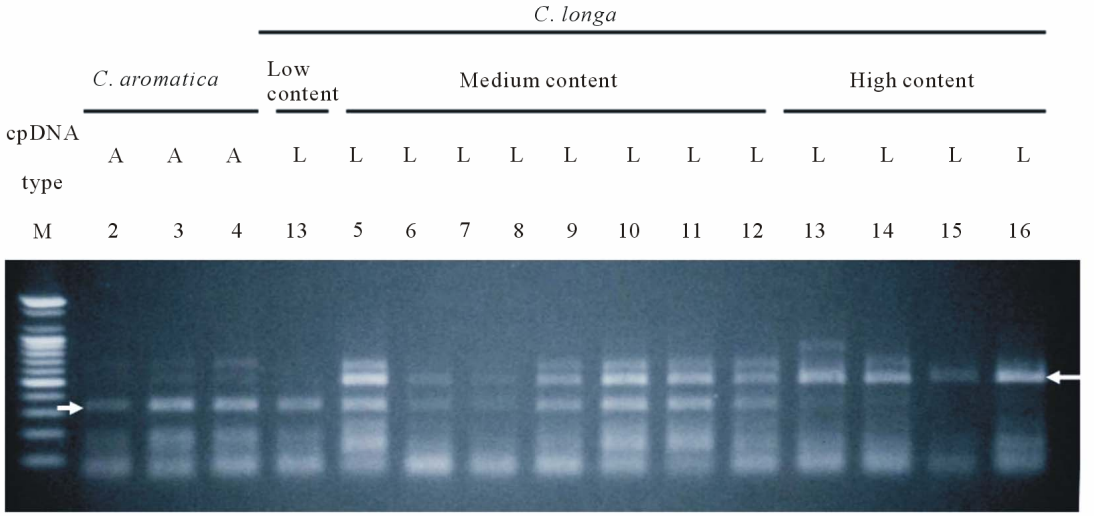

Figure 4. PCR-RFLP profile of various lines of *C. longa* and *C. aromatica*. Arrows indicate expected fragments of both *C. longa* and *C. aromatica*. M: size marker. A: *C. aromatica*. L: *C. longa*. Plant number corresponds to the numbers in Table 1.

chloroplast capture. In fact, some studies indicate that introgression and asymmetric capture of the cpDNA are common phenomena in hybridized species [19,35]. Our results indicated that hybrid and introgressive individuals with other *Curcuma* species were included in *C. longa* although hybrid and introgressive have same haplotype of *C. longa* based on *matK* sequences of cpDNA (**Figure 1**). Moreover, it is very interesting that homogeneous *C. longa* has a high curcumin content, and that a heterogeneous hybrid of the *Curcuma* genome has a medium amount of curcumin. Additionally, an introgressive sample with incongruent haplotypes between cpDNA and nrDNA has a low content of curcumin (**Table 1**, **Figure 4**). Therefore, the pattern of decreased curcumin content was congruent with the hybridization or introgression between *C. longa* and other *Curcuma* species, such as *C. aromatica*, which have low curcumin content. These results indicated that hybridization or introgression with other *Curcuma* species could affect the content of curcumin of *C. longa*. In this study, *C. longa* proved to be the seed parent of the hybrid and introgression samples because all of the haplotypes on the *matK* of cpDNA matched this species. In the future, an analysis of the curcumin content of hybrid and introgression samples in which *C. longa* is the pollen parent needs to be conducted. In addition, the recent resurgence in plant development study has been accelerated, in part, by success in elucidating the molecular genetic basis of plant developmental processes, including the isolation and characterization of genes that synthesize curcumin in *C. longa* [36-38]. As a result of these studies, it is considered very important to isolate and analyze the homologous genes of *C. longa* apart from other *Curcuma* species. As for hybrids, Allard [39] claimed that interspecific hybrids are very useful in introducing genetic divergence, and, in fact, hybrids have been used for many crops and ornamental plants. The way it was stated now appears that it is negating the well established knowledge of hybrid vigor and our results could not support this claim because interspecific hybrids contribute to the decrease in the curcumin content of *C. longa*.

Genetic variation is one of the fundamental underpinnings of biological diversity. The genetic structure and history of a given species is an important research focus, because this knowledge is needed to plan species conservation and to understand the evolutionary processes leading to diversity. Our study results suggest that reproductive isolation mechanisms were not acting in the case of the small phylogenetic distances among the *Curcuma* species (**Figure 1**). The evolution of reproductive isolation is one of the defining characteristics of speciation [40], and reproductive isolation contributes to the diversification of species by creating genetically independent lineages and phylogenetic tree branches [41]. Each branching of the tree is a speciation event; however, reproductive isolation alone does not create a new branch. Each branch by itself cannot produce the phenotypic divergence represented by the angular departure of a branch from the ancestral form [41]. Therefore, the diversification in the *Curcuma* species may have other factors involved rather than just reproductive isolation. In the future, it will be necessary to consider the phylogenetic implications in order to understand the detailed evolutionary history of the *Curcuma* species.

In summary, we have provided a hypothesis for the differences of curcumin by analyzing cpDNA and nrDNA

data. Our results, using a molecular approach, were highly effective in revealing the histories of hybridization and introgression of *C. longa*. However, our data was less effective in definitively answering the question concerning the differences of curcumin content. Further studies will be needed to determine whether more comprehensive samplings and additional genetic evidence support the working hypothesis we have developed here.

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REFERENCES

[1] S. D. Tanksley and S. R. McCouch, "Seed Banks and Molecular Maps: Unlocking Genetic Potential from the Wild," *Science*, Vol. 277, No. 5329, 1997, pp. 1063-1066. doi:10.1126/science.277.5329.1063

[2] A. Asai and T. Miyazawa, "Dietary Curcuminoids Prevent High-Fat Diet-Induced Lipid Accumulation in Rat Liver and Epididymal Adipose Tissue," *Journal of Nutrition*, Vol. 131, No. 11, 2001, pp. 2932-2935.

[3] A. C. Beynen, J. J. Visser and J. A. Schouten, "Inhibitory Effect on Lithogenesis by Ingestion of a *Curcuma* Mixture (Temoe Lawak Singer)," *Journal of Food Science Technology*, Vol. 24, 1987, pp. 253-256.

[4] P. Anand, C. Sundaram, S. Jhurani, A. B. Kunnumakkara and B. B. Aggarwal, "Curcumin and Cancer: An 'Old-Age' Disease with an 'Age-Old' Solution," *Cancer Letters*, Vol. 267, No. 1, 2008, pp. 133-164. doi:10.1016/j.canlet.2008.03.025

[5] S. Aggarwal, Y. Takada, S. Singh, J. N. Myers and B. B. Aggarwal, "Inhibition of Growth and Survival of Human Head and Neck Squamous Cell Carcinoma Cells by Curcumin via Modulation of Nuclear Factor-Kb Signaling," *International Journal of Cancer*, Vol. 111, No. 5, 2004, pp. 679-692. doi:10.1002/ijc.20333

[6] Z. Wang, Y. Zhang, S. Banerjee, Y. Li and F. H. Sarkar, "Inhibition of Nuclear Factor Kappab Activity by Genistein is Mediated via Notch-1 Signaling Pathway in Pancreatic Cancer Cells," *International Journal of Cancer*, Vol. 118, No. 8, 2006, pp. 1930-1936. doi:10.1002/ijc.21589

[7] N. M. Weir, K. Selvendiran, V. K. Kutala, L. Tong, S. Vishwanath, M. Rajaram, S. Tridandapani, S. Anant and P. Kuppusamy, "Curcumin Induces G2/M Arrest and Apoptosis in Cisplatin-Resistant Human Ovarian Cancer Cells by Modulating Akt and p38 MAPK," *Cancer Biology and Therapy*, Vol. 6, No. 2, 2007, pp. 178-184. doi:10.4161/cbt.6.2.3577

[8] E. Liu, J. Wu, W. Cao, J. Zhang, W. Liu, X. Jiang and X. Zhang, "Curcumin Induces G2/M Cell Cycle Arrest in a p53-Dependent Manner and Upregulates Ing4 Expression in Human Glioma," *Journal of Neuro-Oncology*, Vol. 85, No. 3, 2007, pp. 263-270. doi:10.1007/s11060-007-9421-4

[9] K. Aoi, K. Kaburagi, T. Seki, T. Tobata, M. Sarak and M. Kuroyanagi, "Studies on the Cultivation of Turmeric (*Curcuma longa* L.) I: Varietal Differences in Rhizome Yield and Curcuminoid Content," *Bulletin of National Institute of Hygiene Science*, Vol. 104, 1986, pp. 124-128.

[10] H. Hayakawa, T. Kobayashi, Y. Minamiya, K. Ito, A. Miyazaki, T. Fukuda and Y. Yamamoto, "Development of a Molecular Marker to Identify a Candidate Line of Turmeric (*Curcuma longa* L.) with a High Curcumin Content," *American Journal of Plant Siences*, Vol. 2, No. 1, 2011, pp. 15-26. doi:10.4236/ajps.2011.21002

[11] H. Hayakawa, T. Kobayashi, Y. Minamiya, K. Ito, A. Miyazaki, T. Fukuda and Y. Yamamoto, "Molecular Identification of Turmeric (*Curcuma longa*, Zingiberceae) with a High Curcumin Content," *Journal of Japanese Botany*, Vol. 85, No. 5, 2010, pp. 263-269.

[12] J. A. Coyne and H. A. Orr, "Patterns of Speciation in *Drosophila*," *Evolution*, Vol. 43, No. 2, 1989, pp. 362-381. doi:10.2307/2409213

[13] J. A. Coyne and H. A. Orr, "Patterns of Speciation in *Drosophila* Revisited," *Evolution*, Vol. 51, No. 1, 1997, pp. 295-333. doi:10.2307/2410984

[14] J. C. Avise, J. Arnold, R. M. Ball, E. Bermingham, T. Lamb, J. E. Neigel, C. A. Reeb and N. C. Saunders, "Intraspecific Phylogeography: The Mitochondrial DNA Bridge between Population Genetics and Systematics," *Annual Review of Ecolgy, Evolution, and Systematics*, Vol. 18, No. 1, 1987, pp. 489-552.

[15] J. C. Avise, "Molecular Markers, Natural History, and Evolution," Chapman & Hall, New York, 1994.

[16] J. C. Avise, "Phylogeography: The History and Formation of Species," Harvard University Press, Cambridge, 2000.

[17] J. A. Doyle, "Phylogeny of Vascular Plants," *Annual Review of Ecolgy, Evolution, and Systematics*, Vol. 29, No. 1, 1998, pp. 567-599. doi:10.1146/annurev.ecolsys.29.1.567

[18] L. H. Rieseberg, J. Whitton and C. R. Linder, "Molecular Marker Incongruence in Plant Hybrid Zones and Phylogenetic Trees," *Acta Botanica Neerlandica*, Vol. 45, 1996, pp. 243-262.

[19] J. F. Wendel and J. J. Doyle, "Phylogenetic Incongruence: Window into Genome History and Molecular Evolution," In: P. S. Soltis, D. E. Soltis and J. J. Doyle, Eds., *Molecular Systematics of Plants II*, Kluwer, Dordrecht, 1998, pp. 265-296.

[20] B. G. Baldwin and S. Markos, "Phylogenetic Utility of the External Transcribed Spacer (ETS) of 18S-26S rDNA: Congruence of ETS and ITS Trees of *Calycadenia* (Com-

positae)," *Molecular Phylogenetics and Evoltion*, Vol. 10, No. 3, 1998, pp. 449-463. doi:10.1006/mpev.1998.0545

[21] C. R. Linder, L. R. Goertzen, B. V. Heuvel, J. Francisco-Ortega and R. K. Jansen, "The External Transcribed Spacer of the Rdna Repeat: A New Nuclear Region for Low-Level Taxonomic Analysis of the Asteraceae and Closely Allied Families," *Molecular Phylogenetics and Evoltion*, Vol. 14, 2000, pp. 285-303. doi:10.1006/mpev.1999.0706

[22] S. Markos and B. G. Baldwin, "Higher-Level Relationships and Major Lineages of *Lessingia* (Compositae, Astereae) Based on Nuclear Rdna Internal and External Transcribed Spacers (ITS and ETS) Sequences," *Systematic Botany*, Vol. 26, No. 1, 2001, pp. 168-183.

[23] L. E. Urbatsch, R. P. Roberts and V. Karaman, "Phylogenetic Evaluation of *Xylothamia*, *Gundlachia*, and Related Genera (Asteraceae, Astereae) Based on Ets and Its Nrdna Sequence Data," *American Journal of Botany*, Vol. 90, No. 4, 2003, pp. 634-649. doi:10.3732/ajb.90.4.634

[24] O. Hidalgo, N. Garcia-Jacas, T. Garnatje and A. Susanna, "Phylogeny of *Rhaponticum* (Asteraceae, Cardueae-Centaureinae) and Related Genera Inferred from Nuclear and Chloroplast DNA Sequence Data: Taxonomic and Biogeographic Implications," *Annals of Botany*, Vol. 97, No. 5, 2006, pp. 704-714. doi:10.1093/aob/mcl029

[25] M. Sato, K. Shimura and K. Hashizume, "Quality Valuation of Turmeric Powders on Market," *Annual Report of Mie Prefecture Health and Enviromant Research Institute*, Vol. 6, 2004, pp. 52-54.

[26] L. A. Jonson and D. E. Soltis, "*matK* DNA Sequences and Phylogenetic Reconstruction in *Saxifragaceae* s.str.," *Systematic Botany*, Vol. 19, No. 1, 1994, pp. 143-156. doi:10.2307/2419718

[27] J. R. Starr, S. A. Harris and D. A. Simpson, "Potential of the 5' and 3' Ends of the Intergenic Spacer (IGS) of Rdna in the Cyperaceae: New Sequences for Lower-Level Phylogenies in Sedges with an Example from *Uncinia* Pers," *Internat Journal of Plant Sciencese*, Vol. 164, 2003, pp. 213-227. doi:10.1086/346168

[28] J. D. Thompson, D. G. Higgins and T. J. Gibson, "CLUSTAL W: Improving the Sensitivity of Progressive Multiple Sequence Alignment through Sequence Weighting, Positions-Specific Gap Penalties and Weight Matrix Choice," *Nucleic Acids Research*, Vol. 22, No. 22, 1994, pp. 4673-4680. doi:10.1093/nar/22.22.4673

[29] K. Tamura, J. Dudley, M. Nei and S. Kumar, "MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software. Version 4.0," *Molecular Biology and Evolution*, Vol. 24, No. 8, 2007, pp. 1596-1599. doi:10.1093/molbev/msm092

[30] D. L. Swofford, "paup*. Phylogenetic Analysis Using Parsimony (*and Other Methods)," 4th Edition, Sinauer Associates, Sunderland, 2001.

[31] V. Grant, "Plant Speciation," Columbia University Press, New York, 1981.

[32] K. Marhold, J. Lihova, M. Perny, R. Grupeand and B. Neuffer, "Natural Hybridization in *Cardamine* (Brassicaceae) in the Pyrenees: Evidence from Morphological and Molecular Data," *Botanical Journal of Linnean Society*, Vol. 139, No. 3, 2002, pp. 275-294. doi:10.1046/j.1095-8339.2002.00066.x

[33] J. Yokoyama, T. Fukuda, A. Yokoyama and M. Maki, "The Intersectional Hybrid between *Weigela hortensis* and *W. maximowiczii* (Caprifoliaceae)," *Botanical Journal of the Linnean Society*, Vol. 138, No. 3, 2002, pp. 369-380. doi:10.1046/j.1095-8339.2002.00033.x

[34] H. Hayakawa, H. Hamachi, Y. Muramatsu, A. Hirata, Y. Minamiya, K. Matsuyama, K. Ito, J. Yokoyama and T. Fukuda, "Interspesific Hybridization between *Arisaema sikokianum* Franch. Et Savat. and *A. tosaense* Makino (Araceae) Revealed from Nuclear and Chloroplast DNA Comparisons," *Acta Phytotaxonomica et Geobotanica*, Vol. 61, No. 2, 2010, pp. 57-63.

[35] J. C. Avise, "Molecular Markers, Natural History, and Evolution," 2nd Edition, Sinauer Associates, Sunderland, 2004.

[36] Y. Katsuyama, M. Matsuzawa, N. Funa and S. Horiuchi, "*In Vitro* Synthesis of Curcumoids by Type III Polyketide Synthase from *Oryza sativa*," *Journal of Biological Chemistry*, Vol. 282, No. 52, 2007, pp. 37702-37709. doi:10.1074/jbc.M707569200

[37] Y. Katsuyama, T. Kita and S. Horiuchi, "Identification and Characterization of Multiple Curcumin Synthases from the Herb *Curcuma longa*," *FEBS Letter*, Vol. 583, No. 17, 2009, pp. 2799-2803. doi:10.1016/j.febslet.2009.07.029

[38] Y. Katsuyama, T. Kita, N. Funa and S. Horiuchi, "Curcumoid Biosynthesis by Two Type III Polyketide Synthases in the Herb *Curcuma longa*," *Journal of Biological Chemistry*, Vol. 284, No. 17, 2009, pp. 11160-11170. doi:10.1074/jbc.M900070200

[39] R. W. Allard, "Principle of Plant Breeding," Jhon Wiley and Sons, New York, 1960.

[40] M. King, "Species Evolution: The Role of Chromosome Change," Cambridge University Press, Melbourne, 1993.

[41] R. Dowkins, "Mechanism of Evolution," In: N. A. Campbell, J. B. Reece and L. G. Mitchell, Eds., *Biology*, 5th Edition, Addison Wesley Longman, Boston, 1999, pp. 412-487.

