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Inferring the Phylogeny and Divergence of Chinese *Curcuma* (Zingiberaceae) in the Hengduan Mountains of the Qinghai–Tibet Plateau by Reduced Representation Sequencing

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Abstract: Clarifying the genetic relationship and divergence among *Curcuma* L. (Zingiberaceae) species around the world is intractable, especially among the species located in China. In this study, Reduced Representation Sequencing (RRS), as one of the next generation sequences, has been applied to infer large scale genotyping of major Chinese *Curcuma* species which present little differentiation of morphological characteristics and genetic traits. The 1295 high-quality SNPs (reduced-filtered SNPs) were chosen from 997,988 SNPs of which were detected from the cleaned 437,061 loci by RRS to investigate the phylogeny and divergence among eight major *Curcuma* species locate in the Hengduan Mountains of the Qinghai–Tibet Plateau (QTP) in China. The results showed that all the population individuals were clustered together within species, and species were obviously separated; the clustering results were recovered in PCA (Principal Component Analysis); the phylogeny was ((((((*C. Phaeocaulis*, *C. yunnanensis*), *C. kwangsiensis*), (*C. amarissima*, *C. sichuanensis*)), *C. longa*), (*C. wenyujin*, *C. aromatica*)); *Curcuma* in China originated around ~7.45 Mya (Million years ago) in the Miocene, and interspecific divergence appeared at ca. 4–2 Mya, which might be sped up rapidly along with the third intense uplift of QTP.

Keywords: RRS; ipyrad; phylogeny; divergence; *Curcuma*

1. Introduction

Curcuma, commonly known as turmeric, has been an important flowering plant genus with medicinal, edible, and horticultural utilizations in the Orient from ancient times. Usually *Curcuma* are distributed in the subtropical and tropical area of Asia [1–3], and there are about 10 species in China (*C. longa*, *C. sichuanensis*, *C. amarissima*, *C. yunnanensis*, *C. phaeocaulis*, *C. kwangsiensis*, *C. aromatica*, *C. wenyujin*, *C. flaviflora*, *C. exigua*, and *C. viridiflora*) [4]. Among them, *C. viridiflora* and *C. exigua* may be extinct [5], *C. yunnanensis* and *C. flaviflora* are scarce and difficult to collect [4]. The worldwide *Curcuma* represent a paraphyletic group involving *Hitchenia*, *Stahlianthus*, and *Smithatris* in Zingibereae according to *mat*K and ITS [6], and it has been classified into four lineages (*Hitcheniopsis*, *Pierreana*, *Curcuma*, and *Ecomata*) by using ITS and three chloroplast regions (*trn*L-*trn*F, *psb*A-*trn*H, *mat*K) [2]. Recently, partial lineages in *Curcuma* have been clarified, though the phylogeny

and divergence among some species are still intractable, especially species in China due to the intense uplift of the Qinghai–Tibet Plateau (QTP) [7,8]. Chinese *Curcuma* species are distributed to increase ranging from Tibet (only herbarium specimens represent this species now) to South China, most growing in the Hengduan Mountains Areas of QTP, which play an important role on species distribution, evolution, and divergence in eastern Asia [9–13].

The internal micro-anatomy of the rhizomes and leaves of Chinese *Curcuma* show few differences, and different anatomy traits can support different classification. The Chinese *Curcuma* species were divided into three groups according to the traits of oil cells, vascular bundles, and the number and diameter of xylem vessels (Group I: *C. longa* and *C. sichuanensis*, Group II: *C. kwangsiensis* and *C. exigua*, Group III: *C. wenyujin*, *C. aromatica*, *C. phaeocaulis C. zedoaria*, and *C. yunnanensis*) [14,15]. The hair distribution, stoma density and size, epidermic cell shape and size in leaf, were also suggested to be unique features among Chinese *Curcuma* species (Group I: *C. longa*, *C. wenyujin,* and *C. sichuanensis*; Group II: *C. kwangsiensis* and *C. exigua*; Group III: *C. aromatica*, *C. chuanyujin*, *C. zedoaria*, *C. phaeocaulis,* and *C. yunnanensis*). The study on pollen morphology showed that Chinese *Curcuma* species can be divided into two groups, Group I (pollen < 3 μm) including *C. amarrisima*, *C. flaviflora*, *C. phaecaulis*, *C. sichuanensis*, and *C. wenyujin*, and Group II (pollen > 3 μm) including *C. aromatica*, *C. yunnanensis,* and *C. longa* [16]. Therefore, the phylogeny of Chinese *Curcuma* are always controversial and ambiguous for poor diagnostic characters of morphology (aerial part, underground part, floral morphology, etc.) [17]. Cytological studies supported that some species in this genus were polyploid [18–21]. Chromosome number in Chinese *Curcuma* were studied to find out that *C. kwangsiensis* is 2n = 4x = 84 and *C. flaviflora* is 2n = 2x = 42, and the left of *C. longa*, *C. aromatica*, *C. sichuanensis*, *C. elata*, *C. wenyujin*, and *C. phaeocaulis* are triploid (2n = 3x = 63) [19]. Polyploidy is commonly distributed in *Curcuma,* e.g., *C. longa* 2n = 2x/3x/4x = 32/63/64 [19,22–24], *C. kwangsiensis* 2n = 4x = 64/84 [19,23], and *C. aromatica* 2n = 2x/3x = 42/86 [25,26], making it hard to distinguish them hardly based on ploidy and chromosome number [18,25,26].

In the past decades, molecular phylogeny of *Curcuma* around the world has been improved, while the phylogeny and divergence of Chinese *Curcuma* are still unclear [1,2,7]. Two groups, (Group I: *C. chuanhuangjiang*, *C. aromatica*, *C. yunnanensis, C. kwangsiensis,* and *C. phaeocaulis*; Group II: *C. longa*, *C. sichuanensis*, *C. amarissima,* and *C. wenyujin*), were divided by using six mtDNA genes [1]. The *mat*K, *rbc*L, *trn*H-*psb*A, *trn*L-F, and ITS2 were applied to test barcodes within *Curcuma* collected from Myanmar and China, and without enough evidence to identify each species (no barcoding gaps found in four chloroplast regions and little gaps in ITS2) [7]. Based on chloroplast genome, a recent study showed that eight *Curcuma* species in China were divided into two groups (Group I including *C. wenyujin*, *C. phaeocaulis,* and *C. aromatica*, Group II including *C. longa*, *C. yunnanensis*, *C. amarissima*, and *C. sichuanensis*), and *C. flaviflora* is far away from the aforementioned seven species, clustered together with *Zingiber spectablie* (a kind of ginger) and *Z. officinale* [27]. In all, most DNA regions lack enough information on Chinese *Curcuma* species identification, particularly among related species, such as *C. longa* and *C. sichuanensis*, *C. aromatica,* and *C. wenyujin*. Therefore, new technologies need to be introduced to infer the phylogeny and divergence of Chinese *Curcuma* [3,28,29]. High-throughput and next-generation sequencing (NGS) can provide large amounts of genomic data and make great progress on phylogeny, divergence, and biogeography [30–33]. Reduced Representation Sequencing (RRS), one of the NGS approaches, can produce numerous sequence tags, and is successfully used to polymorphism detection in multi-species coalescent-based tree and to deal tricky plant's phylogeny and biogeography [34–40].

The phylogeny and divergence of Chinese *Curcuma* are still under debate due to similar morphological characteristics, unique geographical and climate conditions of the QTP [5,28,41–43]. The rising and climatic fluctuation of Hengduan Mountain Range (presently affected by monsoons from both the Indian and Pacific oceans) and Yunnan–Guizhou Plateau (affected mainly by Pacific monsoons) were induced with rapid uplift

of the Himalayas and the QTP [44,45]. In the Pliocene, the third intense uplift of QTP changed the geographical environment, climate, and species distribution/divergence in China and eastern Asia [9,10,46,47]. *Curcuma* in China are distributed in the QTP generally, such as Sichuan, Yunnan, Zhejiang, Guangdong, and Guangxi, even in Tibet without living individuals collected in recent years (herbarium presented: 02010668, 02010669, 00074714, 00074715, 01376872, 01376873, 01376874, and 01376875 in Herbarium, Institute of Botany, Chinese Academy of Sciences). The uplift of QTP might play a key role in *Curcuma* evolution and divergence in China.

In this study, RRS was utilized (1) to evaluate the phylogeny of *Curcuma* species in China and (2) to explore the impact on the evolution and divergence of Chinese *Curcuma* because of uplift of QTP.

2. Materials and Methods

2.1. Material and Sequencing

A total 60 specimens of eight *Curcuma* species were used in this study, and *Hedychium coronarium* (commonly known as white ginger) was used as outgroup (specimens information in Table 1). All specimens used in this study were identified by Prof. Ruiwu Yang with the Sichuan Agricultural University, and were cultivated at the Sichuan Agricultural University Farmland.

Table 1. Sample of species, with source details and total loci after processing with ipyRAD, of the 61 individuals used in phylogenetic analyses. Each sample has a sample name, species name, collecting information, and loci information.

Sample	Species	Source Details	Total of Loci after Ipyrad
SC1	*Curcuma longa*	Muchuan, Sichuan	26,977
SC3	*Curcuma longa*	Fulu, Sichuan	17,898
SC4	*Curcuma longa*	Cuiping, Sichuan	39,772
SC19	*Curcuma longa*	Yibin, Sichuan	30,974
SC18	*Curcuma longa*	Qianwe, Sichuan	26,311
GZ3	*Curcuma longa*	Xingyi, Guizhou	28,912
SC20	*Curcuma longa*	Shuangliu, Sichuan	32,886
YN11	*Curcuma longa*	Menga, Yuannan	20,606
SC5	*Curcuma longa*	Dayi, Sichuan	34,053
SC21	*Curcuma sichuanensis*	Medicinal, Botanical Garden, Guangxi	21,536
SC22	*Curcuma sichuanensis*	Chongzhou, Sichuan	33,411
YN29	*Curcuma sichuanensis*	Yiwu, Yunnan	19,503
SC2	*Curcuma sichuanensis*	Sanjiang, Sichuan	26,973
SC23	*Curcuma sichuanensis*	Cuiping, Sichuan	24,230
SC6	*Curcuma sichuanensis*	Ziyang, Sichuan	27,677
YN10	*Curcuma sichuanensis*	Menga, Yuannan	38,515
SC24	*Curcuma sichuanensis*	Chongzhou, Sichuan	31,072
SC25	*Curcuma sichuanensis*	Qianwei, Sichuan	20,902
SC26	*Curcuma sichuanensis*	Muchuan, Sichuan	21,588
SC11	*Curcuma sichuanensis*	Weiyuan, Sichuan	28,768
SC8	*Curcuma sichuanensis*	GAP Base, Sichuan	30,753
SC9	*Curcuma sichuanensis*	Sanjiang, Sichuan	28,621
SC10	*Curcuma sichuanensis*	Chongzhou, Sichuan	23,043
SC7	*Curcuma sichuanensis*	Yibing, Sichuan	28,068
YN15	*Curcuma sichuanensis*	Menglun, Yunnan	13,089
GX9	*Curcuma sichuanensis*	Medicinal, Botanical Garden, Guangxi	31,039
YN32	*Curcuma amarissima*	Mengkang, Yunnan	16,865
YN7	*Curcuma amarissima*	Menga, Yunnan	17,222
YN8	*Curcuma amarissima*	Menga, Yunnan	17,923
GD4	*Curcuma yunnanensis*	Huaxian, Guangdong	32,976
GD5	*Curcuma yunnanensis*	Huaxian, Guangdong	24,819
GX10	*Curcuma phaeocaulis*	Medicinal, Botanical Garden, Guangxi	23,145
YN27	*Curcuma phaeocaulis*	Yiwu, Yunnan	22,636
SC14	*Curcuma phaeocaulis*	Shuangliu, Sichuan	34,212

Table 1. *Cont.*

Sample	Species	Source Details	Total of Loci after Ipyrad
GX11	*Curcuma phaeocaulis*	Medicinal, Botanical Garden, Guangxi	29,557
SC13	*Curcuma phaeocaulis*	Jianwei, Sichuan	25,208
GX4	*Curcuma phaeocaulis*	Medicinal, Botanical Garden, Guangxi	35,825
GZ2	*Curcuma phaeocaulis*	Anlong, Guizhou	31,112
YN13	*Curcuma phaeocaulis*	Menga, Yunnan	14,425
YN6	*Curcuma phaeocaulis*	Mengxing, Yunnan	21,440
YN31	*Curcuma phaeocaulis*	Mengkang, Yunnan	21,642
SC15	*Curcuma phaeocaulis*	Shuangliu, Sichuan	13,089
YN21	*Curcuma phaeocaulis*	Xishuangbanna, Yunnan	16,744
SC12	*Curcuma phaeocaulis*	Chongzhou, Sichuan	18,289
YN19	*Curcuma phaeocaulis*	Menglun, Yunnan	13,489
YN16	*Curcuma phaeocaulis*	Daluo, Yunnan	26,946
YN17	*Curcuma phaeocaulis*	Xishuangbanna, Yunnan	23,180
YN28	*Curcuma phaeocaulis*	Yiwu, Yunnan	28,065
YN4	*Curcuma kwangsiensis*	Medicinal, Botanical Garden, Guangxi	17,001
GX14	*Curcuma aromatica*	Medicinal, Botanical Garden, Guangxi	26,739
YN5	*Curcuma aromatica*	Mengkang, Yunnan	24,435
SC16	*Curcuma aromatica*	Jianyang, Sichuan	26,817
YN12	*Curcuma aromatica*	Yiwu, Yunnan	18,289
ZJ1	*Curcuma wenyujin*	Taoshan, Zhejiang, cultivated	23,259
ZJ2	*Curcuma wenyujin*	Meiyu, Zhejiang	27,430
GX12	*Curcuma wenyujin*	Medicinal, Botanical Garden, Guangxi	18,273
GD2	*Curcuma wenyujin*	Sanshui, Guangdong	17,080
GX1	*Curcuma wenyujin*	Medicinal, Botanical Garden, Guangxi	22,593
GX3	*Curcuma wenyujin*	Medicinal, Botanical Garden, Guangxi	28,736
GD3	*Curcuma wenyujin*	Huaxian, Guangdong	35,582
JH	*Hedychium coronarium*	Yaan, Sichuan	13,383

The genomic DNA isolation was carried out on fresh leaves by the CTAB method [48]. The RRS libraries for each sample were prepared using the protocol outline as previously described [49]. We used the restriction enzyme *PstI* (CTGCAG) to digest the extracted genomic DNA from each individual, and then ligated the resulting fragments to a barcode adaptor and a common adaptor with the correct sticky ends. Then, a Qiagen MinElute 96-well PCR purification kit was used in the clean-up step to clean up the products. After PCR, the PicoGreen and a qPCR machine were used to examine the quality of the PCR products. All individuals were pooled into a single RRS library. Sample sequencing was done on Illumina Hiseq PE150 sequencer in Genepioneer Biotechnologies Co. Ltd., Nanjing, China. Raw Reads of the founder lines were deposited in the National Center for Biotechnology Information (NCBI) BioProject ID: PRJNA557061.

2.2. Clustering

The software of pipeline ipyRAD 0.7.29 [50] was used to process the raw data from the Illumina FASTQ files. The pipeline is focusing on preparing RADseq type data for population level analyses [51]. Following seven sequential steps, the ipyRAD pipeline can obtain species or higher variation across clades in clustering and alignment method based on specific parameters in the ipyRAD documentation (https://github.com/dereneaton/ipyrad (accessed on 21 January 2019)). The ipyRAD standard parameter settings were as follows: Nucleotides with Phred scores of <20 were coded as unknown bases (N), and sequences with >5% N's were thrown out. Sequences were clustered within individuals by 90% similarity via the uclust function in USEARCH [52]. Clusters of less than 10 sequences were discarded and the minimum number of individuals per cluster was set to 5. Heterozygous loci among more than two individuals were discarded. The remaining clusters were treated as loci and assembled into a phylogenetic matrix.

2.3. Concatenation-Based Species Tree Inference

The reduced-filtered SNPs were acquired from the filtered SNPs (QC) via quality control by using PLINK 1.9 with standard parameter settings. The standard parameter settings were as follows: Missingness per marker was set to 0.05. Minor allele frequency was set to 0.05. The model of substitution for data was run in MrModeltest [53] by the Akaike Information Criterion (AIC) and obtained the best model of GTR. The maximum likelihood (ML) was implemented under the GTR nucleotide substitution model in RAxML8.2.8 [54]. The maximum parsimony (MP) tree branch with booststrap support was done in the software PAUP * 4.0a134. Optimal MP trees were searched by a heuristic strategy with 1000 random sequence additions and TBR branch swapping. Bootstrap values were calculated using 1000 replicates, 10 random additions per replicate, and TBR branch swapping. Bayesian inference (BI) tree was carried out using MrBayes 3.1.2 [55]. Markov chain Monte Carlo (MCMC) searches were started from a random tree and run for 3,000,000 generations, where the topologies were sampled every 100 generations. Furthermore, 25% of our individuals (which the first 2500 trees) were discarded as burnin. The Bayesian posterior probabilities of the nodal supports were inferred and the 50% majority-rule consensus tree was constructed based on the rest of trees.

2.4. Population Structure and Divergence Time Inference

Principal component analysis (PCA) is a purely mathematical method that reflects the clustering between groups and is based on the degree of SNPs in different individuals by EIGENSOFT version 7.2.1 [56]. Population structure of 60 Curcuma individuals was obtained by ADMIXTURE 1.3.0 [57]. We predefined the ancestral proportions (K) from 3 to 12, and ran the cross-validation error (CV) procedure. The default settings and methods were used for other parameters.

On the basis of the high-quality SNPs, the BEAST version 2.5.0 was used to estimate Chinese Curcuma species divergence time, and the Bayesian tree was dated by setting divergence time between Hedychium coronarium and Curcuma as 42.4 Mya (37.4–47.4 Mya) [58]. The GTR model for nucleotide substitution and the "Bayesian skyline" tree prior model was confirmed with a standard normal prior. Substitution model and site heterogeneity model were used the optimal model based on the Bayesian analysis to select the model "relaxed clock" and MCMC runs 200 million generations, and every 1000 steps in the individuals were to ensure effective sample size (ESS) in each parameter greater than 100. The output file assessed convergence in Tracer 1.5. The phylogenetic tree used TreeAnnotator 1.5.3 to discard 25% as burnin. Finally, the divergence time was analyzed and obtained in FigTree 1.1.2 [59].

3. Results

3.1. Sequences Discovery and Characterization

A total of 53.15 Gb raw data were produced by RRS (Raw Reads in NCBI BioProject ID: PRJNA557061). Then, 437,061 unique RRS loci across all the individuals were revealed by using the denove clustering method in ipyRAD. The reduced-filtered SNPs (1295 bp) were obtained under quality control (QC) in filtered SNPs by using Plink 1.9 to analyze the phylogeny, evolution, and divergence.

3.2. Phylogenetic Analyses

The MP, ML, and BI trees were obtained by reduced-filtered SNPs with strong support values (Figure 1), and the same clustering results were recovered in PCA (Figure 2). The phylogeny shown respectively in MP, ML, and BI tree were consistent in this study.

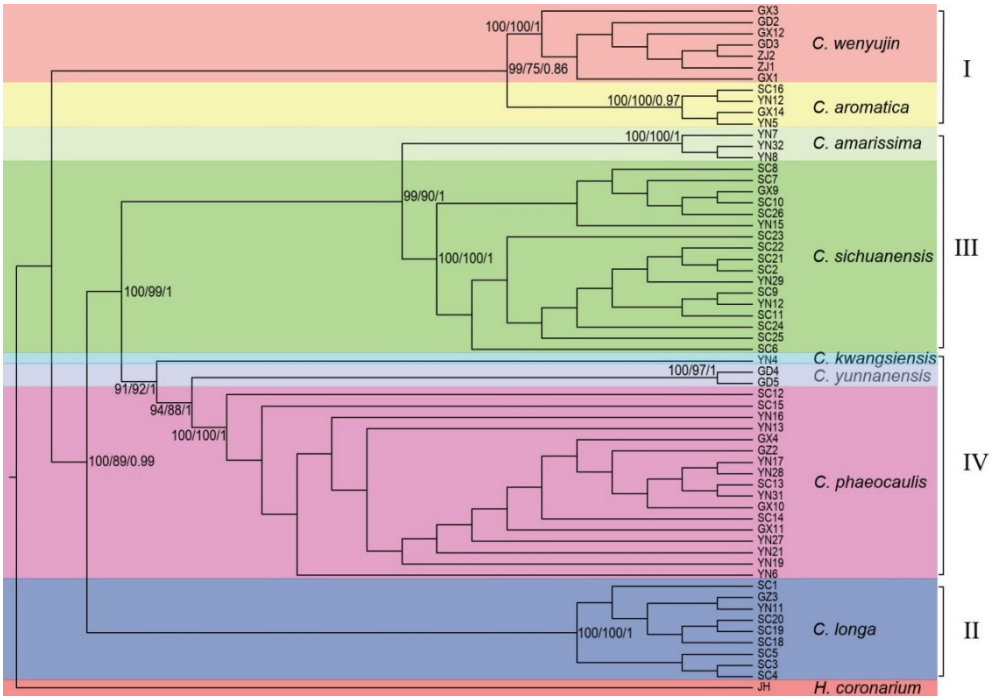

Figure 1. The ML, MP, and BI trees of Chinese *Curcuma*. The values (maximum likelihood bootstrap/maximum parsimony bootstrap/Bayesian support value) were on the species clade. The Group I consisted of *C. wenyujin* and *C. aromatica*; Group II consisted of *C. longa*; The Group III consisted of *C. amrissima* and *C. sichuanensis*; The Group IV consisted of *C. kwangsiensis*, *C. yunnanensis*, and *C. phaeocaulis*.

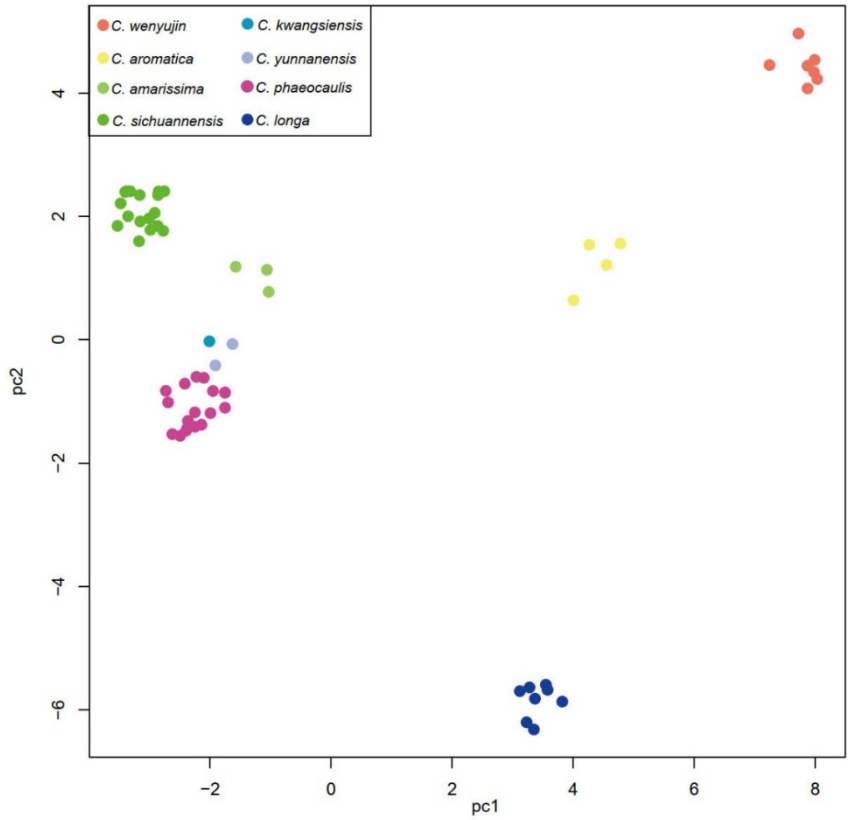

Figure 2. The principle components analysis of Chinese *Curcuma*. Principal component analysis (PCA) of the 60 individuals and different species with different colors.

In the three phylogenetic trees, all population specimens from the same species were clustered together firstly and named as a clade in this study, and the eight *Curcuma* species were divided into four groups (including eight clades) with robust support values: Group I (including *C. wenyujin* clade and *C. aromatica* clade) with MP BS = 75, ML BS = 99, and BI PP = 86; Group II (*C. longa* clade) with MP BS = 100, ML BS = 100, and BI PP = 100; Group III (including *C. amarissima* clade and *C. sichuanensis* clade) with MP BS = 90, ML BS = 99, and BI PP = 100; and Group IV (including *C. kwangsiensis* clade, *C. yunnanensis* clade, and *C. phaeocaulis* clade) with MP BS = 92, ML BS = 91, and BI PP = 100. *C. longa* formed a monophyletic branch with MP BS = 89, ML BS = 100, and BI PP = 99; *C. wenyujin* clade (MP BS = 100, ML BS = 100, and BI PP = 100) was sister to *C. aromatica* clade (MP BS = 100, ML BS = 100, and BI PP = 97); *C. amarissima* clade (MP BS = 100, ML BS = 100, and BI PP = 100) clustered with *C. sichuanensis* clade (MP BS = 100, ML BS = 100, and BI PP = 100); *C. kwangsiensis* clade, the first branch in Group IV, was sister to *C. yunnanensis* clade (MP BS = 97, ML BS = 100, and BI PP = 100) and *C. phaeocaulis* clade (MP BS = 100, ML BS = 100, and BI PP = 100).

To test the evolution of Chinese *Curcuma*, a Bayesian clustering algorithm with admixed models was used to estimate the ancestral proportions (*K*) for each specimen (Figure 3). Based on CV error, the *K* = 12 represented the best model for these 60 samples. When *K* = 3, the eight species belong to three gene pools (blue gene pools, green gene pools, and red gene pools). *C. longa* had independent gene pools (blue). The species of *C. amarissima*, *C. sichuanensis*, *C. kwangsiensis*, *C. yunnanensis,* and *C. phaeocaulis* shared green gene pools. The red gene pools were shared by *C. wenyujin* and *C. aromatica*; When *K* = 4 to 12, *C. longa* could be distinguished by constitution of gene pool, and Group I (*C. wenyujin* and *C. aromatica*) is close to Group IV (*C. kwangsiensis*, *C. yunnanensis,* and *C. phaeocaulis*).

3.3. Divergence Time Inference

Curcuma occurred in the Miocene (~7.45 Mya) in China (Figure 4). *C. longa* appeared around 6.43 Mya, the intraspecific of *C. longa* diversified at ~2.45 Mya from the late Pliocence to Quaternary. Group I (*C. amarissima* and *C. sichuanensis*) and II (*C. kwangsiensis*, *C. yunnanensis* and *C. phaeocaulis*) separated at ~4.83 Mya. The divergence in Group I, occurred at ~3.57 Mya in the Pliocence, and the intraspecific diversification of *C. sichuanensis* and *C. amarissima* were at ~1.89 Mya and ~0.67 Mya, respectively. In Group II, *C. kwangsiensis* occurred at ~3.74 Mya, *C. yunnanensis* and *C. phaeocaulis* emerged and separated ca 2.79 Mya, and the diversification within species of *C. yunnanensis* and *C. phaeocaulis* were at ~0.81 Mya and ~1.45 Mya. *C. wenyujin* and *C. aromatica* originated at ~6.08 Mya during the Miocene and Pliocence. The intraspecific diversification within *C. wenyujin* and *C. aromatica* were at ~1.17 Mya and ~0.56 Mya, respectively.

Figure 3. Population genetic structure of Chinese *Curcuma*. When the kinship (*K*) = 3–12, each vertical bar represented a *Curcuma* sample and different color represented different putative ancestral background in putative.

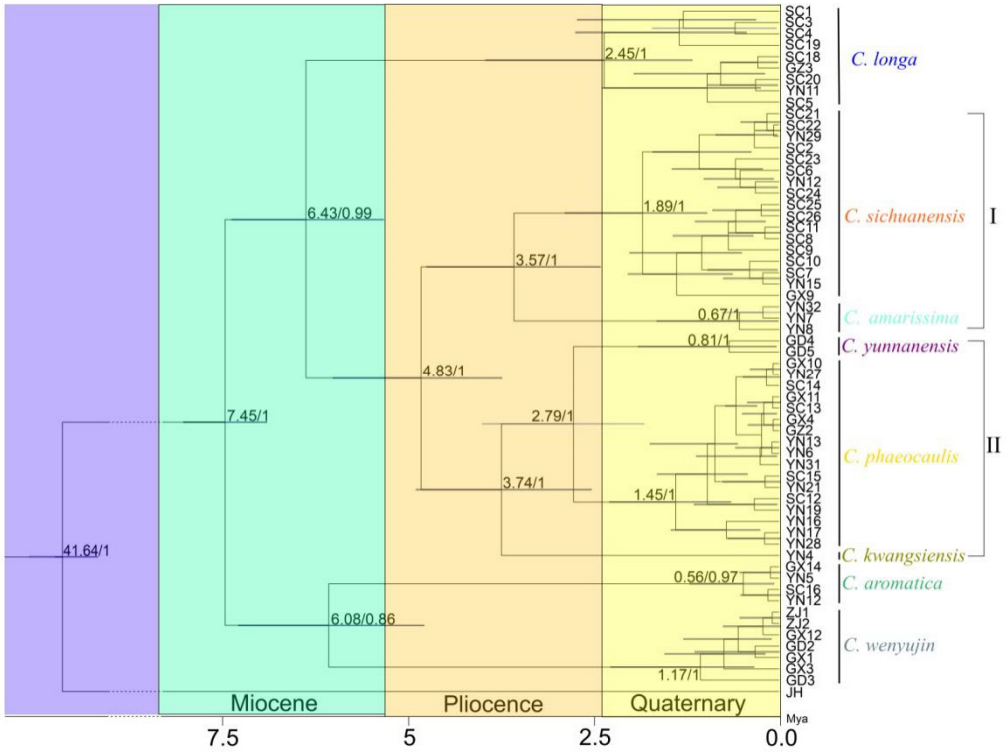

Figure 4. Phylogeny tree resulting from the Bayesian inference and divergency time of Chinese *Curcuma* in BEAST (Divergence time/Bayesian support). Group I consisted of *C. amarissima*, *C. sichuanensis*, *C. kwangsiensis*, *C. yunnanensis*, and *C. phaeocaulis*. Group II was made up of *C. kwangsiensis*, *C. yunnanensis*, and *C. phaeocaulis*.

4. Discussion

4.1. Application of "Next-Generation" Sequencing in Estimating the Phylogeny and Biological Implication of Curcuma

The recent rapid speciation of species might lead to a mistake in inference by using a single phylogenetic tree, and this might be presented a poor fit in the multi-species coalescent supermatrix data and model [60,61]. A large amount of sequence data is ideal for establishing and reconstructing phylogenetic trees [5,28,41,62–64]. In this study, RRS was firstly used to produce big data to analyze the phylogeny and divergence of Chinese *Curcuma*. More loci and variable sites might improve the phylogeny reconstruction of Chinese *Curcuma* [5,41]. Compared to limited individuals and data size introduced in some previous studies, more individuals were involved to produce big data for mining more accurate information to recover a well-resolved phylogeny in this study [31].

4.2. Phylogeny Inference

The phylogeny of *Curcuma* species is difficult to resolve by depending on traditional approaches for their complicated origins (hybridization, introgression, and common appearance in species) [2,8,17]. The genetic relationship among Chinese *Curcuma* species is very close in this study, which is consistent with several previous studies [1,3,7].

Studies based on various morphological evidences reveal different results and lead to unreliable classifications of *Curcuma*: Based on pollen grains, Chen and Xia believed *C. aromatica*, *C. yunnanensis*, and *C. longa* had a close relationship, and the relationships among *C. amarrisima*, *C. elata*, *C. flaviflora*, *C. phaecaulis*, *C. sichuanensis*, and *C. wenyujin* were very close and hard to distinguish [16]. These species in Xiao et al. were delineated as Group I: *C. longa*, *C. xanthorrhiza*, and *C. sichuanensis*, Group II: *C. kwangsiensis* and *C. exigua*, Group III: *C. wenyujin*, *C. aromatica*, *C. phaeocaulis C. zedoaria*, and *C. yunnanensis* by using oil cells and vascular bundles [14]; and the morphology of leaves produced different results (Group I: *C. longa*, *C. xanthorrhiza*, *C. wenyujin*, and *C. sichuanensis*; Group II: *C. kwangsiensis*

and *C. exigua;* and Group III: *C. aromatica*, *C. chuanyujin*, *C. zedoaria*, *C. phaeocaulis,* and *C. yunnanensis*) [15].

On the basis of molecular marker data (isoenzyme and DNA barcode), Deng et al. (2011a and 2011b) and Li et al. believed the relationship between *C. longa* and *C. sichuanensis* was very close, and supported that *C. sichuanensis* originated from the cultivated mutation of *C. longa* [1,3,65]. The genetic relationships among *C. kwangsiensis*, *C. yunnanensis*, *C. aromatica,* and *C. phaeocaulis* were poorly supported, and *C. wenyujin* clustered with *C. longa*, *C. sichuanensis,* and *C. amarissima* [5]. Chloroplast genomes study showed *C. aromatica*, *C. wenyujin,* and *C. phaeocaulis* have a close relationship and supported *C. yunnanensis*, *C. amarissima*, *C. sichuanensis,* and *C. longa* as a group [27]. In this study, the phylogeny of Chinese *Curcuma* is (((((*C. Phaeocaulis*, *C. yunnanensis*) *C. kwangsiensis*), (*C. amarissima*, *C. sichuanensis*)), *C. longa*), (*C. wenyujin, C. aromatica*)). *C. longa* is a single clade (MP BS = 100, ML BS = 100, and BI PP = 100), separated from *C. sichuanensis* clade, was sister to *C. amarissima* clade with strong support (MP BS = 90, ML BS = 99, and BI PP = 100), was inconsistent with the study of Deng et al. (*C. sichuanensis* clustered together with *C. longa*) [3]. The clade of *C. wenyujin* was close to *C. aromatica* clade [5] in Group I with MP BS = 75, ML BS = 99, and BI PP = 86. Based on RAMP and ISSR markers, *C. phaeocaulis* and *C. yunnanensis* had a close relationship [66,67]. In this study, *C. kwangsiensis*, *C. yunnanensis,* and *C. phaeocaulis* clustered together (Group IV) with robust support values (MP BS = 92, ML BS = 91, and BI PP = 100).

4.3. Divergence Time

The estimates of divergence indicated that the earliest appearance of *Curcuma* in China was in the Miocene. In the Miocene, large-scale orogenesis and geological events frequently emerged and influenced the speciation of plants living in QTP area during that times [68]. During the time, the drought climate (the Asian Monsoon) was related to intense uplift of QTP [69,70]. *Curcuma* lies dormant in winter, rhizomes fleshy with tuber-bearing roots, and blooms in the rainy season, which is similar to the drought-resistant plants to satisfy such drought climate [17]. The interspecific divergence of Chinese *Curcuma* occurred in ca. 4–2 Mya, which was coincided with the drought-resistant and deciduous plant sprang up and expanded rapidly in Hengduan Mountains ca. 4–2 Mya [71]. Chinese *Curcuma* expanded rapidly during this period, and the third intense uplift of QTP sped up their interspecific divergence.

In addition, hybridization and polyploidy play an important role on speciation and diversification [72,73]. According to previous studies on *Curcuma*, the distribution range overlapped, and the introgressive hybridization existed extensively [74]. Several Chinese *Curcuma* species were most likely to be of hybrid origin (*C. aromatica* and *C. kwangsiensis*) [8]. The cytomixis and chromosome doubling could promote the production of a large number of new phenotypes in a short time, and polyploids tend to be more adaptive than their parents [75–77]. Hybridization and polyploidization in plants are commonly distributed in the QTP regions, where the species overlap generally and the environment vary frequently, to satisfy the harsh environment of the QTP regions [78,79]. Chinese *Curcuma* species are confirmed to be polyploidy, which might help to adapt the harsh living environment at that time [27,74].

4.4. Future Directions

The phylogeny of major Chinese *Curcuma* species was improved in this study. Chinese *Curcuma* species are only a subset of *Curcuma* around the world. More species with population samples, as well as species from the genera of *Hitchenia*, *Stahlianthus*, and *Smithatris*, and more data should be involved to analyze the phylogeny, evolution, and diversification of *Curcuma* [6].

In addition, *Curcuma* has a complex evolutionary history; hence, more new methods such as ddRRS, ddRad, and transcriptome should be used to ensure the reliability of data

for the phylogeny, evolution, and diversification reconstruction on such recent radiated genera [80].

5. Conclusions

The RRS was firstly involved to improve the phylogeny and divergence of Chinese *Curcuma*. The third intense uplift of QTP might speed up the interspecific divergence of *Curcuma* in China. Overall, this study provides valuable information on the origin of Chinese *Curcuma*.

Author Contributions: H.L. and R.Y. designed the experiments and analyzed the data. H.L. wrote the original manuscript. G.G. and J.D. collected the plant materials. K.X. and R.Y. identified the materials. L.Z., C.D., and H.W. assisted with manuscript preparation. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: These plant materials are required for the collection of plant individuals. The plant materials are maintained in accordance with the institutional guidelines of the College of Life Sciences, Sichuan Agricultural University, China. The Raw Reads are available in NCBI BioProject ID: PRJNA557061.

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