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Comparison and Phylogenetic Analyses of Nine Complete Chloroplast Genomes of Zingibereae

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Abstract: Zingibereae is a large tribe in the family Zingiberaceae, which contains plants with important medicinal, edible, and ornamental values. Although tribes of Zingiberaceae are well circumscribed, the circumscription of many genera within Zingibereae and the relationships among them remain elusive, especially for the genera of *Boesenbergia*, *Curcuma*, *Kaempferia* and *Pyrgophyllum*. In this study, we investigated the plastome variation in nine species representing five genera of Zingibereae. All plastomes showed a typical quadripartite structure with lengths ranging from 162,042 bp to 163,539 bp and contained 132–134 genes, consisting of 86–88 coding genes, 38 transfer RNA genes and eight ribosomal RNA genes. Moreover, the characteristics of the long repeats sequences and simple sequence repeats (SSRs) were detected. In addition, we conducted phylogenomic analyses of the Zingibereae and related taxa with plastomes data from additional 32 species from Genbank. Our results confirmed that *Stahlianthus* is closely related to *Curcuma*, supporting the idea of merging it into *Curcuma*. *Kaempferia*, *Boesenbergia* and *Zingiber* were confirmed as close relatives and grouped together as the *Kaempferia* group. *Pyrgophyllum* is not allied with the *Curcuma* clade but instead is embedded within the *Hedychium* clade. Our results demonstrate the power of plastid phylogenomics in improving the phylogenetic relationships within Zingibereae and provide a new insight into plastome evolution in Zingibereceae.

Keywords: *Curcuma*; *Pyrgophyllum*; chloroplast genome; phylogenetic analysis

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1. Introduction

Zingiberaceae, commonly known as the ginger family, is the largest family of the order Zingiberales. It comprises over 50 genera and consists of more than 1300 species [1], widely distributed throughout tropical Africa, Asia, and the Americas, with species abundant in South and Southeast Asia [2]. Many species of the ginger family are important ornamental, spice, or medicinal plants [3–5]. The first comprehensive phylogenetic analysis based on nuclear ITS region and plastid *matK* region confirmed the long-suspected complexity of generic concepts in Zingiberaceae and divided the Zingiberaceae family into six tribes and four subfamilies: Zingiberoideae (Zingibereae and Globbeae), Tamijioideae (Tamijieae), Siphonochiloideae (Siphonochileae) and Alpinioideae (Alpinieae and Riedelieae).

The tribe Zingibereae is a large subclade within the family Zingiberaceae and includes ca. 670 species in some 25 genera (Plants of the World Online: http://plantsoftheworldonline.org, IPNI: https://www.ipni.org, accessed on 21 January 2021) [1,6]. Members of Zingibereae are mainly distributed throughout tropical and warm-temperate Asia, with a few species extending to Pacific islands and Australia [1] (Plants of the World Online: http://plantsoftheworldonline.org, accessed on 15 January 2021). Members of Zingibereae are easily distinguished from other gingers by the plane of leaf distichy parallel to the direction of rhizome growth, large and petaloid lateral staminodes, trilocular ovary with axial, basal or free columnar placentation, and labellum usually not connate to the filament [1].

Although tribes of Zingiberaceae are well circumscribed [1], attaining high resolution of the phylogenetic relationships within Zingibereae is still problematic [1,6]. For example, the delimitation of the genus *Curcuma* has been a matter of dispute since its establishment by Linnaeus [7]. Several small or monotypic genera closely related to *Curcuma*, e.g., *Hitchenia* Wall., *Laosanthus* K.Larsen & Jenjitt., *Paracautleya* R.M.Sm., *Smithatris* W.J.Kress & K.Larsen and *Stahlianthus* Kuntze, were recognized based on morphology in the past [1,2,6]. Recently, the study based on the nuclear ITS region and three plastid regions (*trnL-F*, *psbA-trnH*, *matK*) supported a broad generic boundary for *Curcuma*, with the inclusion of *Laosanthus*, *Paracautleya*, *Stahlianthus*, *Smithatris* and some species of *Kaempferia* L. and *Hitchenia* [8]; thus, they were all transferred to *Curcuma* later [9]. However, no character has been found that is both exclusive to *Curcuma* s.l. and present in all of *Curcuma* species. *Pyrgophyllum* (Gagnep.) T.L.Wu & Z.Y.Chen was firstly considered as a subgenus of *Kaempferia* [10], then was transferred to be a section of *Camptandra* Ridl. [11], later to be a section of *Caoulokaempferia* K.Larsen [12]. It was recognized as a separate genus closely related to *Camptandra* [13], whereas its phylogenetic position is unclear in later studies [6,14,15]. Many species of *Kaempferia* were transferred to *Boesenbergia* Kuntze and *Curcuma* recently [9,16,17]. *Boesenbergia* was demonstrated to be polyphyletic [1], while the phylogenetic trees based on *petA-psbJ* spacer recognized the monophyly of *Boesenbergia* [18]. This taxonomic complex is yet another example of the problems that exist in defining clear generic boundaries in Zingibereae.

Chloroplast as an essential organelle is directly and indirectly involved in various metabolic pathways and plays an essential role [19]. Since the first chloroplast genome of tobacco was sequenced and published in 1986 [20], the number of complete chloroplast genomes sequences has increased significantly. Chloroplast genomes vary in length from 120–220 kb and have a quadripartite structure consisting of a large single-copy region (LSC), a small single-copy region (SSC) and a couple of reverse complementary inverted repeats (IRs) which separates the regions of LSC and SSC [21]. Variation in the genome size is due to the loss, contraction and expansion of the IRs region [22–24], such as in some leguminous plants and algae that have completely lost the IRs region [25,26]. The chloroplast genomes are relatively small, highly conserved and have slow mutation rates and are useful in resolving phylogenetic issues brought about by historical diversity, rapid radiation and frequent hybridization [27,28]. Therefore, the chloroplast genome is believed to be a perfect model for phylogenetic and phylogeographic studies [29]. However, only a few chloroplast genomes of Zingibereae species have been published until now.

In order to gain new insights into the evolution of plastomes, and to improve the delineation of the phylogenetic affinities among genera within Zingibereae, we sequenced nine complete chloroplast genomes and compared the previously reported chloroplast genomes of 21 Zingibereae species, nine other Zingiberaceae species and two species from closely related families. Our specific goals are to (1) investigate the genome structure, gene order, and gene content of the whole chloroplast genome of nine Zingibereae species; (2) test whether chloroplast genome data yielded sufficient variation to construct a well-supported phylogeny of Zingibereae, particularly the phylogenetic relationships of *Boesenbergia*, *Curcuma*, *Kaempferia* and *Pyrgophyllum*.

2. Materials and Methods

2.1. Plant Materials and DNA Sequencing

Nine taxa of five genera belonging to Zingibereae (*Boesenbergia kingii* Mood & L.M.Prince, *Curcuma aff. plicata*, *C. aff. singularis*, *C. kwangsiensis* S.K.Lee & C.F.Liang, *C. ruiliensis* N.H.Xia & Juan Chen, *C. wenyujin* Y.H.Chen & C.Ling, *Kaempferia rotunda* L., *Pyrgophyllum yunnanense* (Gagnep.) T.L.Wu & Z.Y.Chen and *Stahlianthus involucratus* (King ex Baker) Craib) were sequenced and analyzed. Voucher specimens are deposited at IBSC (Table 1).

Table 1. List of nine Zingibereae species sampled together with their voucher specimen numbers and GenBank accession numbers.

Number	Species	Voucher Number	Locality	Accession Number
1	*Boesenbergia kingii*	17081502 (IBSC)	Yunnan, China	MW326451
2	*Curcuma ruiliensis*	17082304 (IBSC)	Yunnan, China	MW326454
3	*Curcuma aff. singularis*	1722 (IBSC)	Chiangmai, Thailand	MW326455
4	*Curcuma aff. plicata*	17081107 (IBSC)	Yunnan, China	MW326452
5	*Curcuma kwangsiensis*	17083001 (IBSC)	Guangxi, China	MW326453
6	*Curcuma wenyujin*	201544 (IBSC)	Guangdong, China	MW326456
7	*Kaempferia rotunda*	17081102 (IBSC)	Yunnan, China	MW326457
8	*Pyrgophyllum yunnanense*	2014106 (IBSC)	Sichuan, China	MW326458
9	*Stahlianthusinvolucratus*	19031203 (IBSC)	Laos	MW326459

The fresh leaves were obtained from the nursery of the South China Botanical Garden in Guangzhou, China. The total genomic DNA was extracted by a modified CTAB protocol [30]. The libraries were sequenced on Illumina HiSeq Xten platform (Illumina, Inc., San Diego, CA, USA) at Sangon Biotech Co. Ltd. (Shanghai, China).

2.2. The Genomes of Plastome Assembly, Annotation and Structure

The raw reads of nine Zingibereae species were trimmed and filtered by NGSQC Toolkit version 2.3.3 [31]. The reads were de novo assembled using SPAdes v3.6.0 (54) and finished using PRICE (Paired-Read Iterative Contig Extension) [32]. The BWA was used to check the de novo assembly in default parameter and the reads were aligned against the assembled genome [33]. The automatic annotator software Unix Program Plann was used to annotate the genome [34]. The annotated genome was matched with open reading frames (ORFs), then the remaining lacking protein evidence ORFs were disregarded [35]. The genes were considered potential pseudogenes which contained one or more frame shift mutations or premature stop codons. In addition, the DRAW tool was used to generate and edited the circular map of the chloroplast genomes [36].

2.3. The Analysis of Codon Usage

The relative synonymous codon usage (RSCU) is used to represent the ratio of the specific and the expected codon frequency. RSCU > 1.00 indicates that a codon is used more frequently than expected, and vice versa. DAMBE5 is used to calculate the RSCU [37].

2.4. Complete Chloroplast Genome Comparison and Molecular Marker Identification

We used the mVISTA with the annotated sequence of *Curcuma kwangsiensis* as a reference to compare similarities and detect any rearrangement or inversion among nine newly sequenced Zingibereae species which make pairwise alignments in the LAGAN model [38]. The rates of nonsynonymous (Ka) and synonymous substitutions (Ks) were calculated in DnaSP 6.0 based on 80 protein coding regions [39]. In DnaSP 6.0, the sequence polymorphism and nucleotide diversity (Pi) values were evaluated.

2.5. The Analysis of Long Repetitive Sequences and Simple Sequence Repeats (SSRs)

The long repeats (forward, reverse, palindromic and complementary) among the complete chloroplast genome of nine newly sequenced Zingibereae species based on the size and location of the long repeats in REPuter were calculated [40]. The detection parameter settings were a minimum repeat size of 30 bp, and the Hamming distance of 3. MISA software (http://pgrc.ipk-gatersleben.de/misa/, accessed on 24 January 2021) was used to detect SSRs. The parameters were set as follows: ≥ten for mono-; ≥five for di-; ≥four for tri-, ≥three for tetra-, ≥three for penta- and ≥three for hexa-. The interruptions (max difference between 2 SSRs) less than 9 bp were termed "complex".

2.6. Phylogenetic Analysis

In this study, 30 accessions of eight genera (one *Boesenbergia* species, one *Cautleya* (Royle ex Benth.) Hook.f. species, 14 *Curcuma* species, two *Hedychium* J.Koenig. species, three *Kaempferia* species, one *Pyrgophyllum* species, three *Roscoea* Sm. species, two *Stahlianthus* species, three *Zingiber* Mill. species) belonging to Zingibereae were analyzed. Nine outgroup species included four *Alpinia* Roxb. species, two *Amomum* Roxb. species, one *Lanxangia* M.F.Newman & Škorničk. species and two *Wurfbainia* Giseke species. Another two species from the closely related family (*Costus viridis* S.Q. Tong and *Musella lasiocarpa* (Franch.) C.Y. Wu) were used to root the trees. Except for nine newly sequenced species, the remaining 32 published chloroplast genomes were downloaded from NCBI. A list of GenBank accessions is provided in Supplementary Table S1.

In order to make a more reasonable utilization of the relationships based on phylogenetic trees, we used a complete chloroplast genome, CDS, LSC and intron sequences for phylogenetic analysis. The software MAFFT version 7.0 was used to align the multiple sequences before inferring the phylogenetic trees [41]. Maximum likelihood (ML) methods in the program PAUP * Version 4.0 were used to construct the phylogenetic trees [42].

3. Results

3.1. The Genome Structure and Content of Nine Zingibereae Species

Chloroplast genomes of nine Zingibereae species (six newly reported) were sequenced and assembled with lengths ranging from 162,042 bp (*Pyrgophyllum yunnanense*) to 163,539 bp (*Curcuma aff. singularis*) (Table 2). All cp genomes had a typical quadripartite circular structure with a pair of IR regions that separated the LSC and SSC regions, and the gene map of the *B. kingii* chloroplast genomes was presented in Figure 1 as representative. The LSC region ranged from 86,943 bp (*C. aff. plicata*) to 88,251 bp (*C. aff. singularis*), accounting for 33.78%–34.11% of the total length. The SSC region ranged from 15,568 bp (*Stahlianthus involucratus* to 16,023 bp (*P. yunnanense*), accounting for 29.14%–29.66% of the total length. The IR regions ranged from 29,379 bp (*P. yunnanense*) to 30,117 bp (*S. involucratus*), accounting for 40.89%–41.30% of the total length.

The complete cp genomes of nine Zingibereae species contain 132–134 genes (113 unique genes), including 86–88 coding genes, 38 transfer RNA genes (tRNA) and eight ribosomal RNA genes (rRNA) (Table 1 and Table S2). Among the 113 unique genes, 18 intron-containing genes were detected, including 14 genes (*atpF*, *clpP*, *ndhA*, *petB*, *petD*, *rpl16*, *rpoC1*, *rps12*, *rps16*, *trnG-GCC*, *trnK-UUU*, *trnL-UAA*, *trnV-UAC* and *ycf3*) in LSC regions and four genes (*ndhB*, *rpl2*, *trnA-UGC* and *trnI-GAU*) in IR regions, and only one gene (*ndhA*) in SSC region (Table S3). Among these 18 genes, only two genes (*ycf3* and *clpP*) contained two introns while the other 16 genes contained one intron, including nine coding genes (*rps16*, *rpoC1*, *rpl2*, *rpl16*, *petD*, *petB*, *ndhB*, *ndhA* and *atpF*) and six tRNA (*trnV-UAC*, *trnL-UAA*, *trnK-UUU*, *trnI-GAU*, *trnG-GCC* and *trnA-UGC*). The *rps12* gene was a special trans-spliced gene with two duplicated 3′ end exons in IR regions and 5′ end exon in LSC region.

Table 2. Complete chloroplast genomes of nine Zingibereae species.

Species	Size	PCGs	tRNAs	rRNAs	Genes	GC%	Length (LSC)	Length (SSC)	Length (IR)	GC% (LSC)	GC% (SSC)	GC% (IR)
B. kingii	163,198	88	38	8	134	36.16	88,009	15,701	29,744	33.95	29.66	41.15
C. aff. plicata	162,169	87	38	8	133	36.20	86,943	15,742	29,742	34.01	29.62	41.14
C. ruiliensis	162,242	87	38	8	133	36.19	87,022	15,740	29,740	33.99	29.56	41.16
C. aff. singularis	163,539	87	38	8	133	36.07	88,251	15,830	29,729	33.83	29.51	41.15
C. kwangsiensis	162,179	87	38	8	133	36.18	87,014	15,665	29,750	33.97	29.65	41.13
C. wenyujin	162,165	87	38	8	133	36.19	87,000	15,665	29,750	33.98	29.66	41.13
K. rotunda	162,391	87	38	8	133	36.25	87,018	15,753	29,810	34.11	29.66	41.12
P. yunnanense	162,042	86	38	8	132	36.06	87,261	16,023	29,379	33.81	29.14	41.30
S. involucratus	163,298	88	38	8	134	36.00	87,496	15,568	30,117	33.78	29.60	40.89

GC guanine–cytosine, LSC large single-copy region, SSC short single-copy region, IRs inverted repeats.

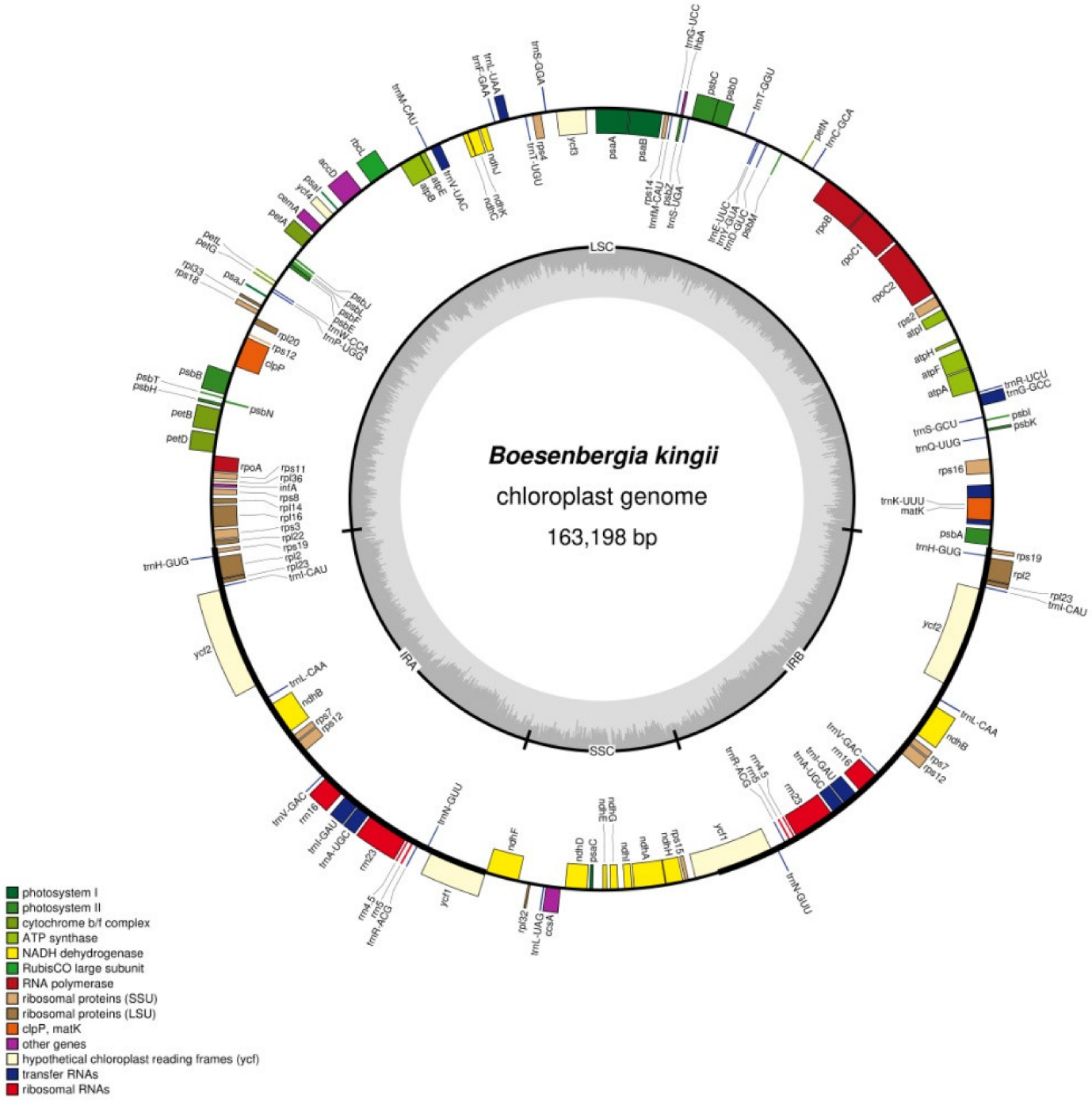

Figure 1. Circular representation of *Boesenbergia kingii* genomes. Genes of different functional groups, small single copy (SSC), large single copy (LSC), and inverted repeats (IRa, IRb), are separated by color. Genes drawn inside the circle are transcribed clock.

3.2. Condon Usage Bias

A total of 67 coding genes were used to estimate the codon usage frequency based on the relative synonymous codon usage (RSCU) value (Table S4). All genes were encoded by 27,705 (*P. yunnanense*) to 27,904 (*S. involucratus*) codons. UAA, UGA and UAG were considered to be the termination codons. For nine Zingibereae species, the serine encoded by AGC had the lowest RSCU value (0.31), while methionine encoded by AUG had the highest one (2.65). The AUU, AAA and GAA encoded isoleucine, lysine and glutamic acid, respectively, had higher frequencies of occurrence than others (more than 1100). In

addition, A or T had a higher nucleotide frequency than G or C in the third codon position, which was relatively common in most angiosperm, and the richness of A or T in the IR regions was the principal reason [43] (Figure 2 and Table S4).

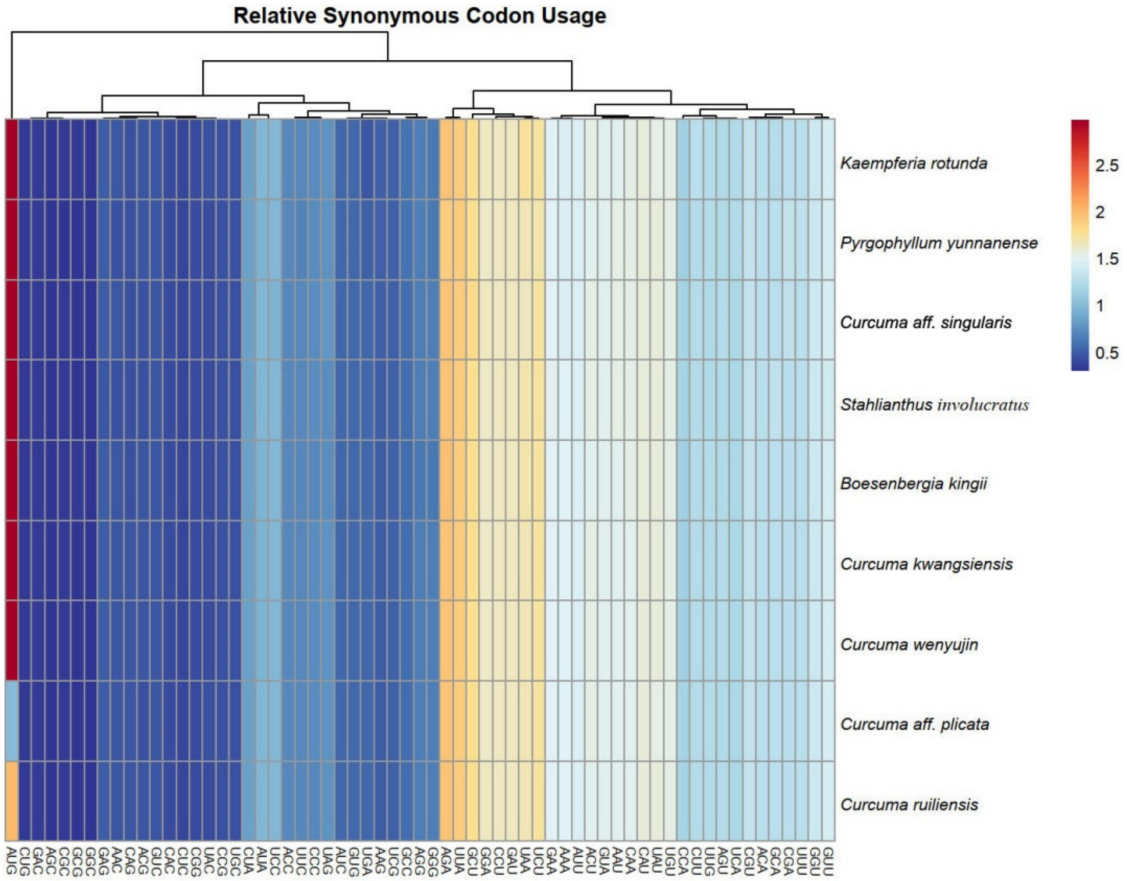

Figure 2. Relative synonymous codon usage (RSCU) index for each amino acid codon and stop codon for nine Zingibereae species chloroplast genomes. The value of RSCU is separated by bar color.

3.3. Comparative Genomic Analysis

A high degree of synteny and gene order conservation indicated a high evolutionary conservatism at plastome level (Figure 3). It is clear that the *Curcuma* species can be separated from the other Zingibereae species by many genes, such as *atpF*, *rpl16* and *atpH-atpI*. However, the divergence among five *Curcuma* species was very low. Notably, the regions of LSC and SSC had more variation than the regions of IRs, and the non-coding regions had a greater differentiation than that of coding regions. Some regions had more variation, such as *matK*, *rps16*, *atpF*, *ndhH*, *clpP* among the coding regions, *ycf1* intron, and *atpH-atpI*, *petN-psbM*, *trnA-psbD* and *rpl*32-*trn*L in the intergenic regions.

A non-synonymous/synonymous mutation (Ka/Ks) ratio was used to assess the significant differences in evolutionary rates (Figure 4 and Table S5). The Ka/Ks ratio of most genes was less than 0.5 (91.25%). The Ka/Ks ratio of three genes were higher than 1, viz. *ccsA*, *ycf1* and *ycf4*, and they may be under positive selection. Most of the genes associated with photosynthesis had the lowest rates of evolution. In addition, the Ka/Ks ratio of 42 genes were 0, including Ka = 0 (*atpA*, *atpI*, *infA*, *lhbA*, *ndhC*, *ndhJ*, *ndhK*, *psaA*, *psaB*, *psbB*, *psbK*, *psbM*, *psbT*, *rps18*, *rps19* and *ycf3*), Ks = 0 (*psbH*, *rpl16*, *rpl32*, *rps12*, *rps14*, *rps15* and *rps16*). The Ka/Ks of *atpH*, *petG*, *petL*, *petN*, *psaC*, *psaI*, *psaJ*, *psbE*, *psbF*, *psbI*, *psbJ*, *psbL*, *psbN*, *psbZ*, *rpl2*, *rpl23*, *rpl33*, *rpl36* and *rps7* were 0, and therefore it indicated that there was no nonsynonymous and synonymous substitution.

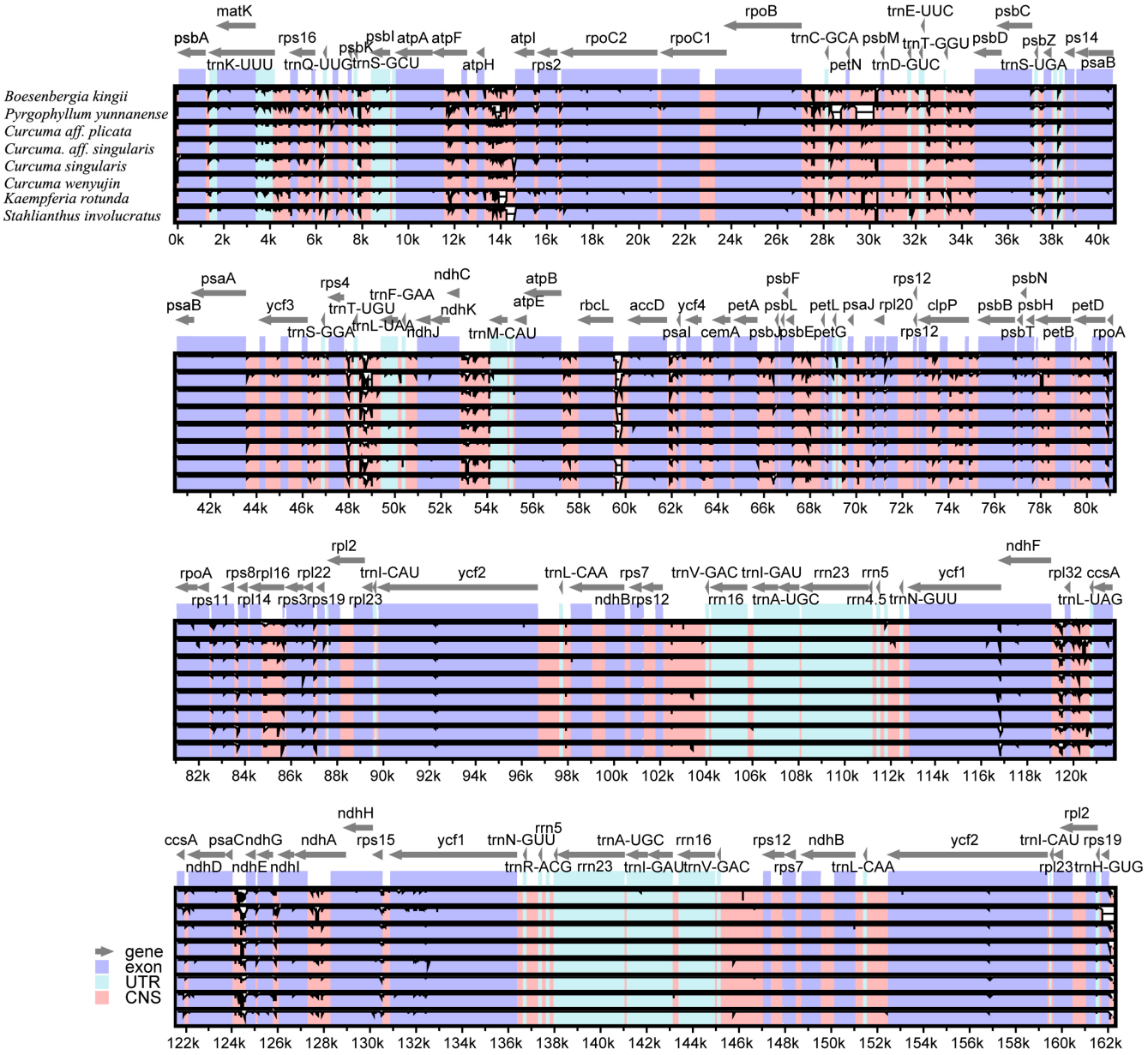

Figure 3. Sequence alignment of nine Zingibereae chloroplast genomes with *Curcuma kwangsiensis* as a reference by using mVISTA. The Y-scale represents the percentage of identity ranging from 50% to 100%.

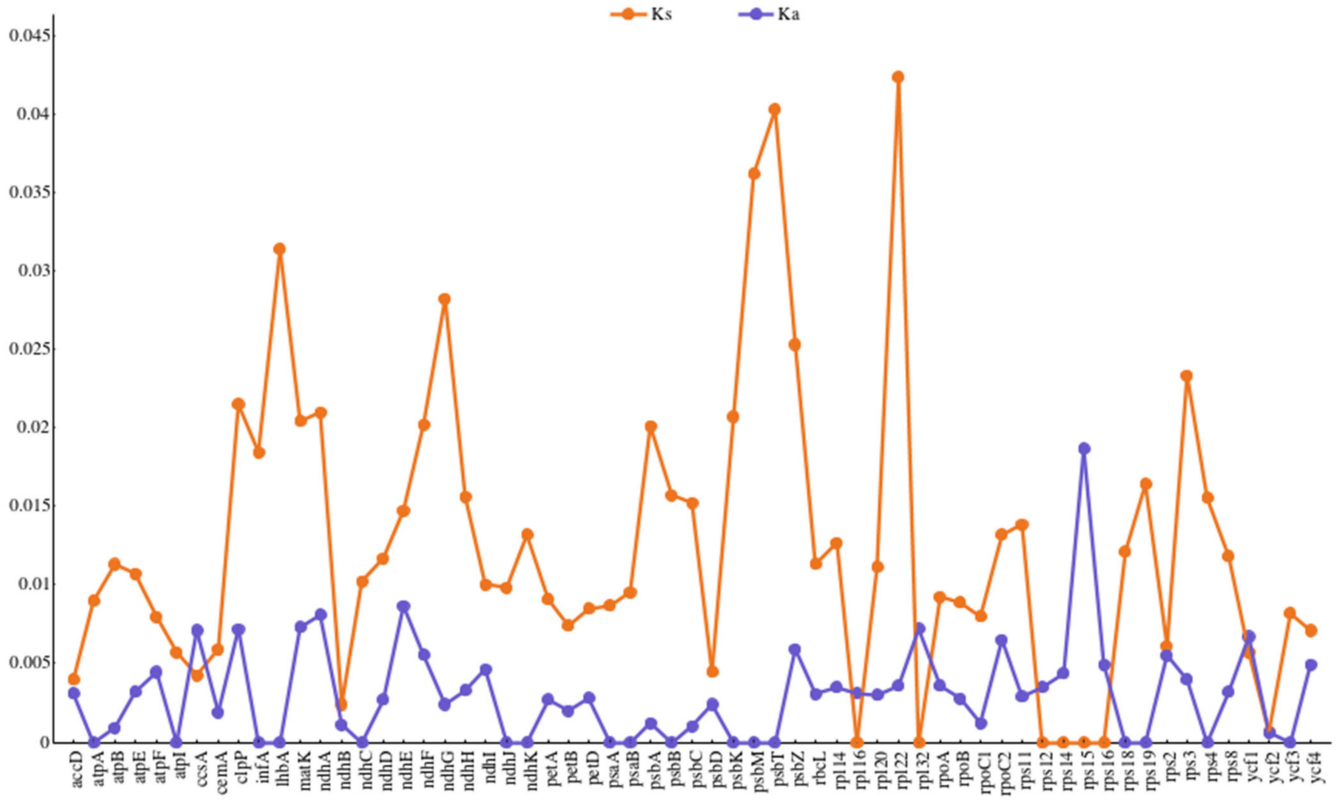

Figure 4. The rates of Ka and Ks in the chloroplast genomes of nine Zingibereae species.

3.4. Expansion and Contraction of Inverted Repeats (IRs)

Among nine Zingibereae species, the sizes of the IR regions varied from 29,379 bp (*P. yunnanense*) to 30,117 bp (*S. involucratus*). The *rpl22* genes were located on the boundaries of LSC regions, and the distance between *rpl22* and the boundary of LSC/IRb ranged from 23 bp (*C. aff. singularis*) to 538 bp (*P. yunnanense*). The *rps19* coding gene was located in the IRb region but that gene of *P. yunnanense* was located in the LSC region. The distance between *rps19* and the boundary of LSC-IRb ranged from 3 bp (*P. yunnanense*) to 167 bp (*S. involucratus*). The IRb/SSC and SSC/IRa boundary was crossed by the *ycf1* gene, which was a critical gene. In the IRb/SSC boundary, the *ycf1* gene located in the SSC region was from 4 bp (*S. involucratus*) to 43 bp (*C. ruiliensis*). At the SSC/IRa boundary, the *ycf1* gene located in the SSC region was from 1210 bp (*S. involucratus*) to 1592 bp (*P. yunnanense*). For *S. involucratus*, the *ndhF* gene spanned the IRb region and the SSC region. However, in the other eight Zingibereae species, the *ndhF* gene was located in the SSC region. At the SSC/IRa boundary, the *rps15* gene located in the SSC region was from 1657 bp (*S. involucratus*) to 2037 bp (*P. yunnanense*). The *psbA* gene was located on the right side of IRa/LSC regions with the distance of 103 bp (*C. aff. singularis*)–260 bp (*K. rotunda* L.) (Figure 5).

3.5. Repeat Structure and SSR Analysis

There were a total of 836 long repeats among nine Zingibereae species, including forward repeats, palindromic repeats, reverse repeats and complement repeats (Figure 6 and Table S6). *Curcuma aff. plicata* had the largest number of repeats, including 39 forward, 45 palindromic, 40 reverse and 20 complement repeats, while *Boesenbergia kingii* had the least number of repeats, including 23 forward, 28 palindromic, ten reverse and three complement repeats. *Curcuma aff. singularis* had the least number of complement repeats (having only one). In all, the repeats mostly ranged from 30 to 137 bp. The majority of these repeats showed lengths of 30, 31 and 33 bp.

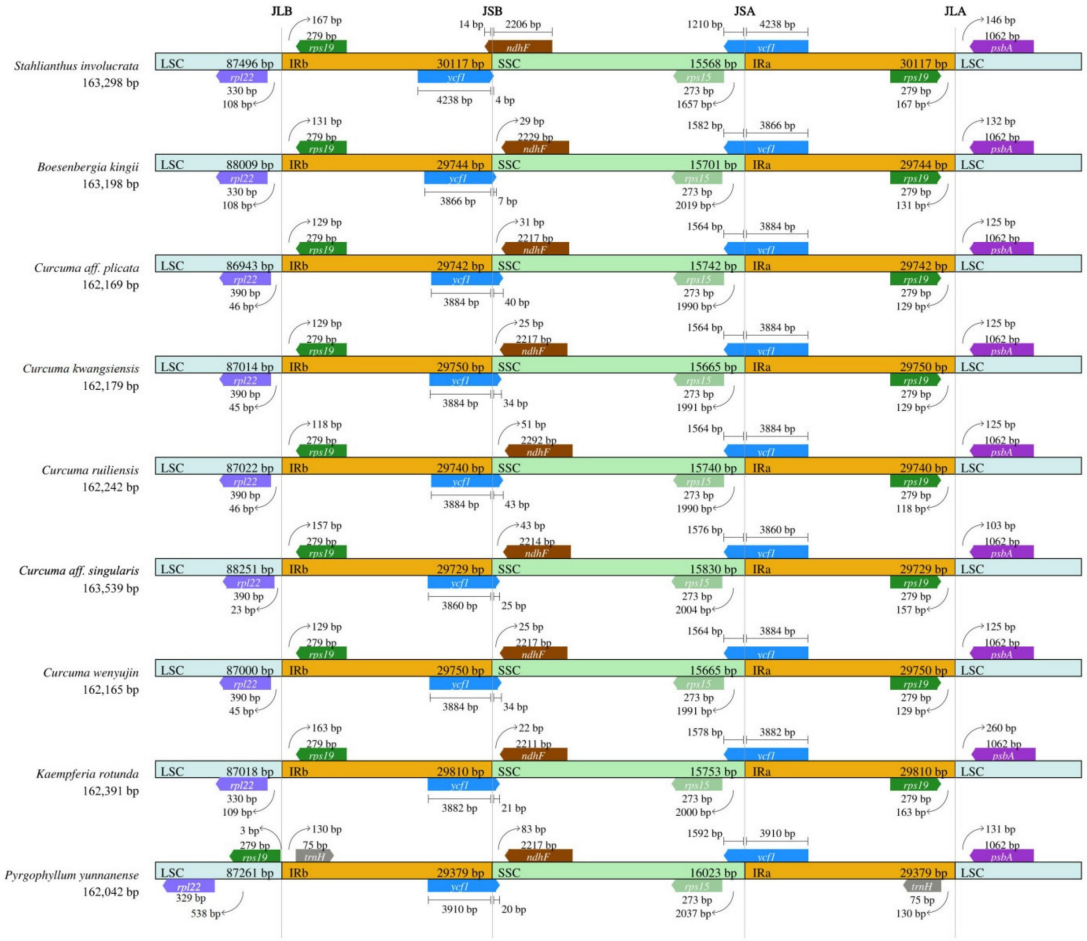

Figure 5. Comparison of the border positions between the LSC, SSC and IR regions among nine Zingibereae chloroplast genomes. The figure is not drawn to scale. Complete genes and portions of genes adjacent to the junctions are depicted by differently colored blocks.

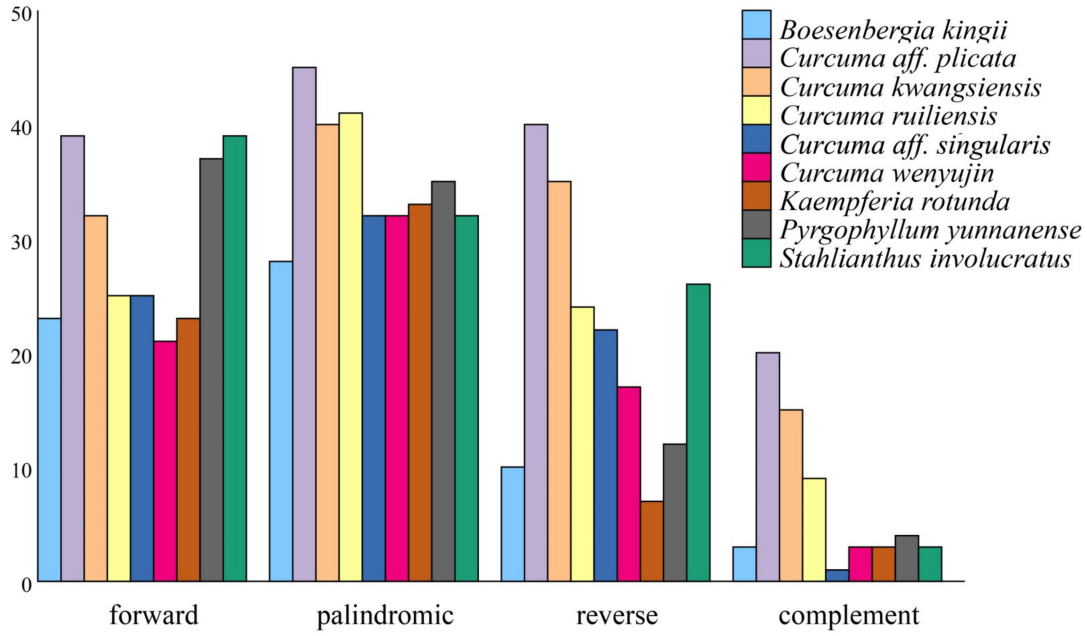

Figure 6. Number of long repetitive repeat types on the complete chloroplast genome sequence of nine Zingibereae species. The species are separated by color.

Simple sequence repeat (SSR), also known as tandem repeats or microsatellites, consists of DNA repeat with sizes of 1–6 bp and can be used as important molecular markers for species identifications [44–46]. There were seven kinds of SSRs in nine Zingibereae species: mononucleotide, dinucleotide, trinucleotide, tetranucleotide, pentanucleotide, hexanucleotide and complex. There were 95–118 SSRs in each species (Figure 7a). Among each species, mononucleotide repeats were the most common one, with numbers ranging from 45–57; followed by dinucleotide ranging from 25–36; tetranucleotide SSRs ranging from 13–23; trinucleotide SSRs ranging from 3–10; complex SSRs ranging from 3–10; pentanucleotide SSRs ranging from 1–2; hexanucleotide SSRs ranging from 0–1 (only found in *P. yunnanense*).

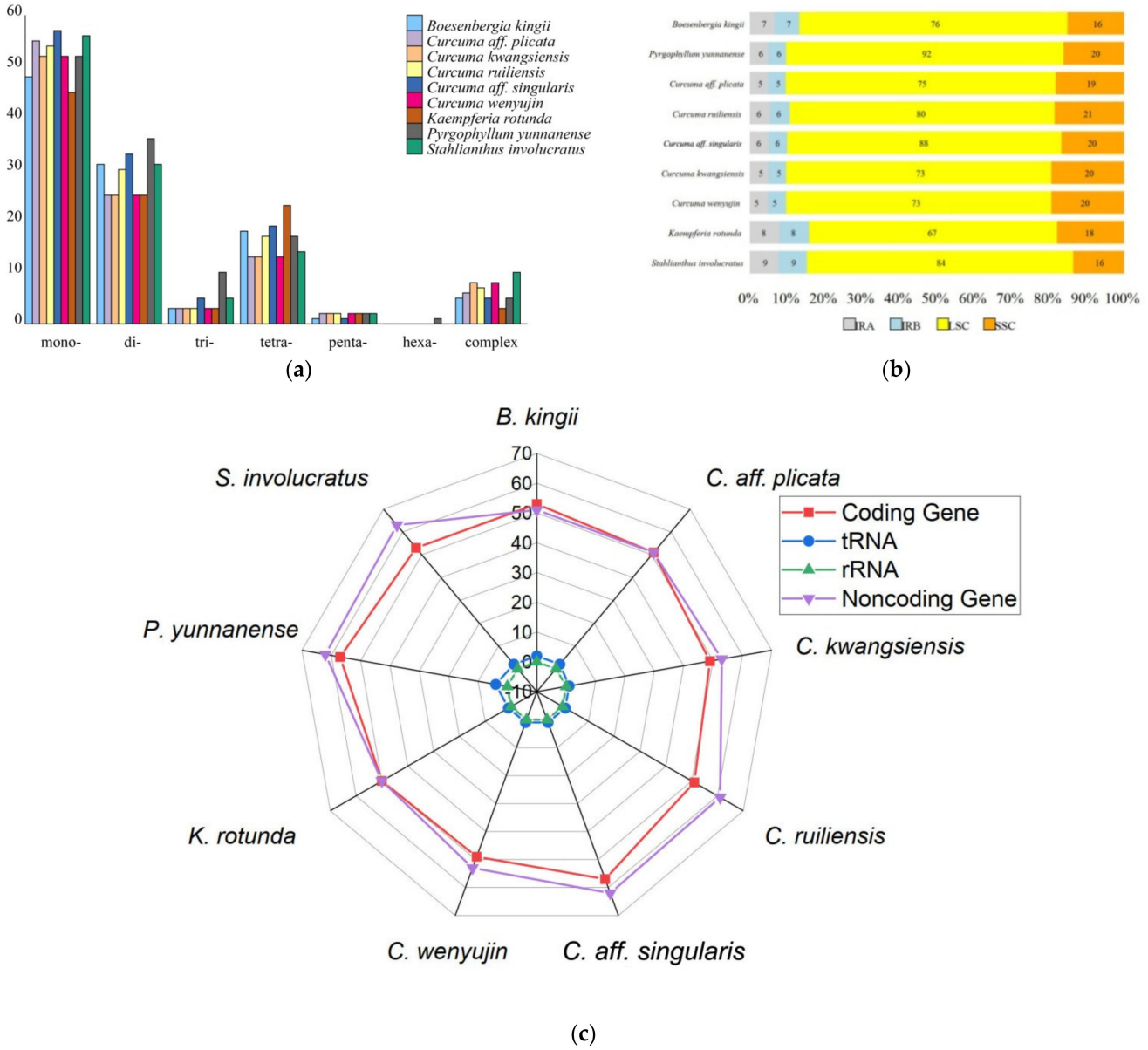

Figure 7. The comparison of SSR distribution in nine Zingibereae chloroplast genomes; (**a**) number of different SSR types; (**b**) frequency of SSRs in different region; (**c**) frequency of SSRs in the intergenic regions, protein-coding genes and introns.

Among nine Zingibereae species, the results showed that the mononucleotide A/T repeats accounted for 43.95% and 54.44%, respectively (Table S7). The mononucleotide C/G repeats accounted for 1.06% and 0.55%, respectively. The number of mononucleotide

A/T repeats in *C. aff. singularis* was 23/34, which was the highest, while *Kaempferia rotunda* had the lowest (17/26).

The number of SSRs located in the LSC regions was much more than that in the SSC and IR regions (Figure 7b). Moreover, the SSRs were more dispersed in the noncoding gene regions (50–63) than in the coding genes (49–57) and in tRNA (1–4) but none in rRNA (Figure 7c). The SSR loci were located in the 13 coding genes (*matK*, *trnK-UUU*, *atpF*, *rpoC2*, *rps14*, *psbF*, *rps18*, *rps12*, *ycf2*, *rps16*, *trnG-GCC*, *atpF*, and *ycf3*) and 62 intergenic regions of the nine Zingibereae species (Table S6).

3.6. Sequence Divergence Hotspots

In the chloroplast genomes, the divergence hotspots can provide useful information and are often applied to assess geographic distribution and phylogeny [28,47–49]. Our results indicated that the Pi values in the coding regions were lower than those in the intergenic regions (Table S8). For the coding regions, the values of the LSC regions ranged from 0.0010–0.0933, followed by the values of the SSC regions ranging from 0.000–0.0753 and the values of the IR regions ranging from 0.0000–0.0175. Three high divergence hotspots, viz. *rps2*, *rpoC2* and *rps15*, were selected as potential molecular markers to identify related species (Figure 8).

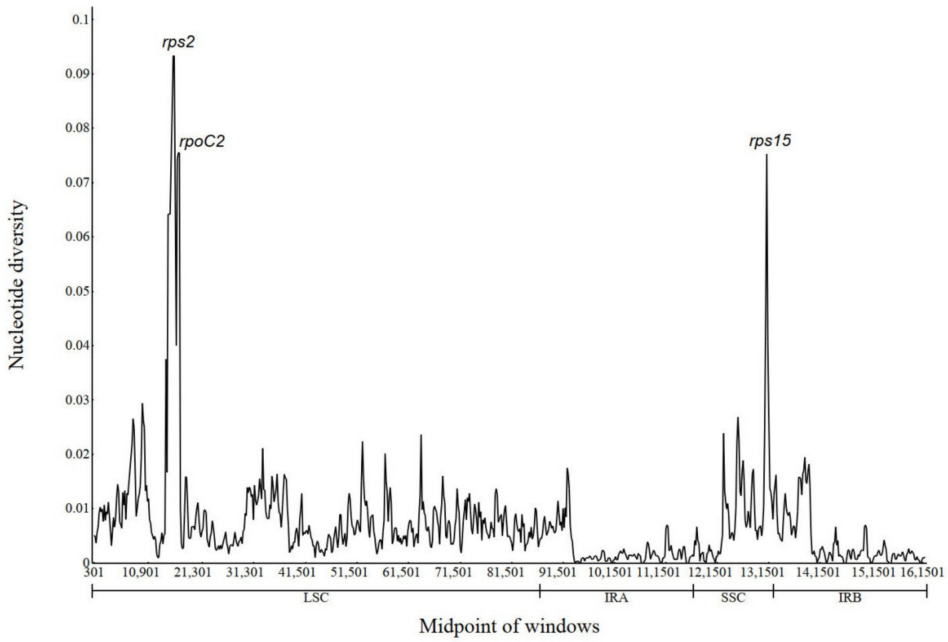

Figure 8. Sliding window analysis of the nucleotide variability (Pi) values of nine Zingibereae chloroplast genomes. *x*-axis: position of the midpoint of a window, *y*-axis: nucleotide diversity of each window.

3.7. Phylogenetic Analysis Based on Chloroplast Genomes

In this study, we utilized 41 complete cp genomes, including nine newly sequenced genomes and 20 previously reported chloroplast genomes of Zingibereae species, nine other Zingiberaceae species and two species from closely related families to infer phylogenetic relationships. The phylogenetic trees were constructed based on a complete cp genome, the coding regions (CDS), LSC region and intron data. The phylogenetic trees using four different datasets had different topologies, but all recognized the monophyly of the Zingibereae (Figure 9, Figures S1–S3). ML phylogeny inferred from CDS was the best resolved, thus is displayed here and discussed below (Figure 9). Two clades are recognized, namely the *Curcuma* clade and the *Hedychium* clade (Figure 9). Only the *Curcuma* clade is strongly supported (Figure 9). Resolution within the *Curcuma* clade is rather high. Three groups, '*Ecomatae*', '*Hitcheniopsis*' and '*Curcuma*', are strongly supported, although the *Ecomatae* group

was only represented by one species here, namely *Curcuma aff. singularis*. The *Hitcheniopsis* group, represented by *C. alismatifolia* and *Stahlianthus involucratus*, was resolved in a sister position to the *Ecomatae* group. The *Curcuma* group was represented by the remaining species. Relationships within the *Curcuma* group were not satisfactorily resolved.

Within the *Hedychium* clade, *Hedychium*, *Kaempferia*, *Roscoea* and *Zingiber* are supported to be monophyletic with a high support value (bootstrap value = 100%) (Figure 9). *Kaempferia*, *Zingiber* and *Boesenbergia* formed a group separate from the rest with strong support (bootstrap value = 100%), while the relationships among the remaining genera are unresolved. *Pyrgophyllum* is nested within the *Hedychium* clade. Similar results were also found in Figures S1–S3.

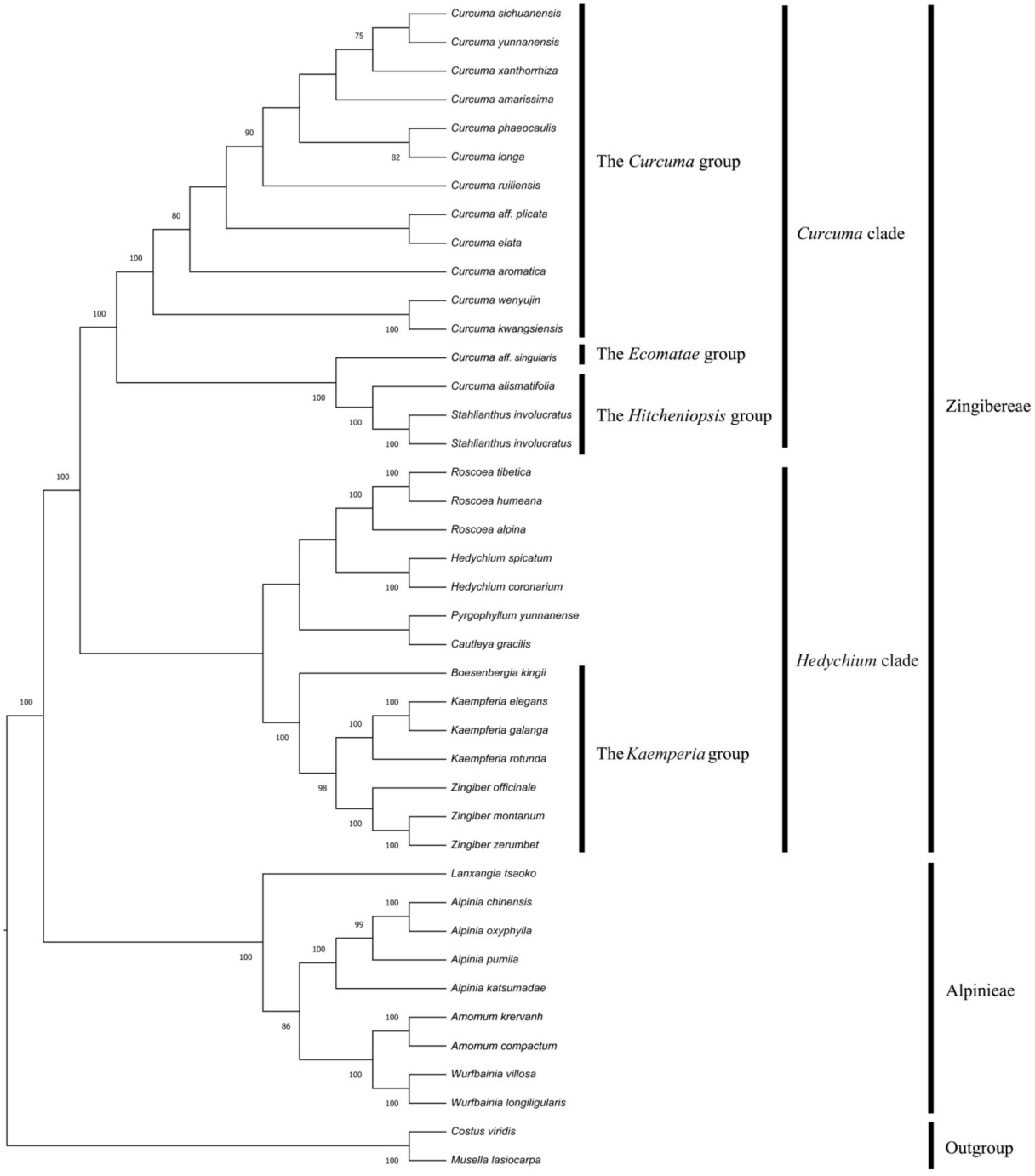

Figure 9. Phylogenetic trees of the Zingibereae species inferred from maximum likelihood (ML) analyses based on the chloroplast genome constructed using coding region data. Support for branches is given by bootstrap values (if values $\geq$ 75%).

4. Discussion

All nine complete cp genomes of Zingibereae species had a typical four-segment structure, including 84–88 coding genes, 38 tRNAs and 8 rRNAs. The genome size of nine Zingibereae species ranged from 162,042 to 163,539 bp with GC content ranging from 36.00% to 36.25%. The size range of these sequenced cp genomes are similar to the sizes of the earlier reported cp genomes of Zingibereae species [24,50–52]. The IRs of earlier reported species were found to be different in length between 28,950 bp and 30,150 bp but the IRs of the nine species reported here varied from 29,379 bp to 30,117 bp. Thus, the expansion and contraction of the size in IR region was the main reason for the genome sizes variation among the Zingibereae species. Additionally, IR expansion or contraction is generally accompanied with the change of gene location. For example, the *rps19* gene as a pseudogene frequently spanned the LSC-IR and SSC-IR boundaries in some angiosperms [29,53]. However, in Zingibereae species, *rps19* coding gene was located in the IRb regions, while it was located in the LSC region in *P. yunnanense*. In nine Zingibereae species, the *rps19* gene was fully duplicated in accordance with the results reported in other Zingiberaceae species [24,51,52,54]. Pseudogene *ψycf1* was also related to the contraction and expansion of the IR region. *ψycf1* was present in Zingibereae species, which was truncated at the IR/SSC boundary. In previous studies, *ycf1* has been used in the phylogeny of some taxa [55,56], while our results showed *ψycf1* had no phylogenetic significance in Zingibereae species. Differences in the location of genes between species provide useful information on evolutionary relationships in genetic research. In this study, it was clear that the organization, genome size and structure of the nine chloroplast genomes were highly conserved. The largest variation of Zingibereae cp genomes was the intergenic areas, which was similar to other chloroplast genomes [19].

Meanwhile, the low ratios of Ka/Ks and evolutionary rate were assessed among nine Zingibereae species. Most of the genes (Ka/Ks = 0) with the lowest evolutionary rates were photosynthetic genes, e.g., *ndhC*, *ndhJ*, *ndhK*, *petG*, *petL*, *psaC*, *psaI*, *psbE* and *psbF*. The *ycf1*, *ycf4* and *ccsA* genes evolved more quickly and had higher Ka/Ks ($\geq$1). The evolutionary rate of *clpP* was species-specific [57], while the *clpP* gene among nine Zingibereae species experienced negative selection and the ratio of Ka/Ks was 0.3326, which was far less than that of many taxa [58–60]. One previous study showed that the gene had gone through spells of relatively accelerated sequence evolution, and thus led to the intron loss in some plants [57]. In this study, the *clpP* gene contained two introns in nine Zingibereae species, which might be the reason for the low ratio of Ka/Ks. Zingibereae species mostly grow in disturbed habitats, and the environmental conditions of their habitats vary from tropical rainforest (wet–hot) to Qinghai–Tibet Plateau (cold–drought). This promotes gene exchange among colonies of the population in inferior and unfavorable habitats. Genes under positive selection often bring on many repeating amino acid sequence insertions in varying degrees and that may be involve in a recent increase in diversification rate after adapting to a new ecological environment [61]. To understand the ratios of Ka/Ks and the evolutionary rate of genes would provide us valuable information on how Zingibereae species adapt to their environment.

The SSRs are typically mononucleotide tandem repeat DNA sequences that are widely used for species identification and genetic diversity research [62,63]. The SSRs mainly consist of short polyadenine or polythymine repeats and ranged from 95 to 118 among nine Zingibereae species, which were in agreement with previous studies [24,51,52,54]. Due to a lack of genome resources in Zingibereae, the SSRs can be used for species identification and genetic diversity research on Zingibereae species and their relatives.

Chloroplast genome sequences have been valuable in molecular, evolutionary, and phylogenetic studies. Numerous analyses on the basis of cp genome sequence comparison have resolved various phylogenetic problems and improved our understanding of complex evolutionary associations among angiosperms [27,64,65]. Our phylogenomic analyses based on cp genome sequences also revealed that the phylogenetic resolution within Zingibereae has been greatly improved (with high support and the similar topology among

different analyses) in comparison to the most comprehensive previous phylogenetic studies of the Zingibereae based on the nuclear ribosomal ITS region and the plastid *matK* and *trnL-F* regions [1,6]. Our results strongly supported that Zingibereae was separated from Alpinieae, which agreed with the past study [1]. Based on *matK* and ITS combined, a *Kaempferia* clade, including *Boesenbergia*, *Kaempferia*, *Zingiber* is weakly supported [1], but we obtain strong support from cp genome sequences, and which is similar to the conclusion made in other studies by DNA barcodes [66,67]. Based on the combination of *trnL-F* region and ITS, the tribe is divided into two major clades, the *Curcuma* clade and the *Hedychium* clade. Nonetheless, these two studies showed that the relationships within these clades remained uncertain because statistical support was weak. Our phylogenetic trees demonstrated that these two major clades were identified in the Zingibereae; namely, the *Curcuma* clade in the sense of Kress et al. (2002) [1] with strong support and the *Hedychium* clade in the sense of Ngamriabsakul et al. (2004) [6] with weak support.

Within the *Curcuma* clade, *Stahlianthus* is closely related to *Curcuma* at the molecular level, supporting the idea of merging it into *Curcuma* [9]. Our results confirmed the monophyly of *Curcuma* and the infrageneric classification proposed by Záveská et al. (2012) in which *C.* subg. *Curcuma* and *C.* subg. *Hitcheniopsis* (Baker) K.Schum. were retained and a new subgenus, *C.* subg. *Ecomatae* Škorničk. & Šída f. was proposed [8]. The representatives of the *Hitcheniopsis* group resolved here correspond to *Curcuma* subg. *Hitcheniopsis* [11]. The *Curcuma* group includes species traditionally classified in subgenus *Curcuma.* In accordance with previous studies [1,8], *Curcuma* subg. *Ecomatae* represented by *C. aff. singularis* here is more closely related to *C.* subg. *Hitcheniopsis* than *C.* subg. *Curcuma* based on the cpDNA data. However, relationships of species within these clades are complex because polyploidization and hybridization were important for the speciation of *Curcuma* species. More detailed analyses of species relationships within *Curcuma* will be the subject of further studies.

Within the *Hedychium* clade, a *Kaempferia* group in the sense of Kress et al. (2002) [1] consisting of *Boesenbergia*, *Kaempferia* and *Zingiber* was also identified with strong support (bootstrap value = 100%), whereas the relationships of the remaining members were unresolved. According to the complete cp genome, the coding regions (CDS), LSC region and intron data, *Kaempferia* is supported to be monophyletic and is sister to *Zingiber*. Since only one species, *B. kingii*, belonging to *Boesenbergis* was sampled, the relationship within *Boesenbergia* was unable to be further investigated.

In the previous phylogeny study [15], *Pyrgophyllum yunnanense* was very closely related to the genus *Curcuma*. However, *P. yunnanense* is not allied with the *Curcuma* clade but instead is embedded within the *Hedychium* clade. Despite these findings, the systematic relationships of *P. yunnanense* remain uncertain. The natural hybridization and polyploidization were the main cause of inconsistency in Zingibereae. Considering the Zingibereae hybrid origin, the features of maternal inheritance in the chloroplast genome could provide more evidence to clarify their phylogenetic relationships. Further sampling of Zingibereae species may prove their relationships.

5. Conclusions

In this study, complete chloroplast genomes of nine Zingibereae species including *Boesenbergia kingii*, *Curcuma aff. plicata*, *C. aff. singularis*, *C. ruiliensis*, *Kaempferia rotunda*, and *Pyrgophyllum yunnanense* were firstly published. The chloroplast genomes of nine Zingibereae species were similar in structure, composition and gene order, showing that the chloroplast genomes studied here are highly conserved. Moreover, we also identified the SSR sites and three divergence hotspots (*rps2*, *rpoC2* and *rps15*), which could provide powerful markers for phylogenetic and identification analyses within Zingibereae.

Our results shed a new light on the phylogenetic relationships within Zingibereae and demonstrated the continuing power of plastome sequencing to improve phylogenetic resolution among the complicated taxa of Zingiberaceae. The phylogenomic analysis strongly supported the idea that Zingibereae is monophyletic and can be divided into

two clades, namely the *Curcuma* clade and the *Hedychium* clade. The monophyly of the genus *Curcuma* and three subgenera in *Curcuma* are confirmed with high support. Our results also showed that *Hedychium*, *Kaempferia*, *Roscoea* and *Zingiber* are strongly supported to be monophyletic. *Pyrgophyllum yunnanense* is not allied with the *Curcuma* clade but instead is embedded within the *Hedychium* clade. However, the systematic relationships of *Pyrgophyllum* and *Boesenbergia* remain unresolved. Further work based on broader sampling within Zingibereae is needed.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/f12060710/s1, Figure S1: Phylogenetic tree constructed using the complete chloroplast genome data. Figure S2: Phylogenetic tree constructed using intron data. Figure S3: Phylogenetic tree constructed using LSC data. Table S1. The GenBank accession numbers of 32 species using in phylogenetic analysis. Table S2. Genes contained in nine sequenced Zingibereae chloroplast genome. Table S3. The genes with introns in the nine Zingibereae chloroplast genomes. Table S4. Codon usage and codon–anticodon recognition pattern of nine Zingiberaeace species. Table S5. The mean Ka/Ks of 77 genes among nine Zingibereae species. Table S6. The comparison of long repeats among nine Zingibereae species. Table S7. The comparison of SSRs among nine Zingibereae species. Table S8. The nucleotide variability (Pi) value of nine Zingibereae species.

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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 Key Laboratory of Plant Resources Conservation and Sustainable Utilization/Guangdong Provincial Key Laboratory of Digital Botanical Garden, South China Botanical Garden, Chinese Academy of Sciences, China. The GenBank accession numbers are available in NCBI: MW326451, MW326454, MW326455, MW326452, MW326453, MW326456, MW326457, MW326458 and MW326459.

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Abbreviations

CTAB	Cetyltrimethy lammonium Ammonium Bromide
CP	Chloroplast
IRs	Inverted repeats
Ka	Non-synonymous site
Ks	Synonymous site
Ka/Ks	the ratio of non-synonymous site and synonymous site
LSC	Large single-copy region
ML	Maximum likelihood
mono-	Mononucleotides
Pi	Nucleotide diversity values
rRNA	Ribosomal RNAs
RSCU	Relative synonymous codon usage
SSC	Small single-copy region
SSRs	Simple-sequence repeats
tRNA	Transfer RNAs

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