Comparative Genomic Analysis Uncovers the Chloroplast Genome Variation and Phylogenetic Relationships of Camellia Species

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Abstract: Camellia is the largest genus in the family Theaceae. Due to phenotypic diversity, frequent hybridization, and polyploidization, an understanding of the phylogenetic relationships between Camellia species remains challenging. Comparative chloroplast (cp) genomics provides an informative resource for phylogenetic analyses of Camellia. In this study, 12 chloroplast genome sequences from nine Camellia species were determined using Illumina sequencing technology via de novo assembly. The cp genome sizes ranged from 156,545 to 157,021 bp and were organized into quadripartite regions with the typical angiosperm cp genomes. Each genome harbored 87 protein-coding, 37 transfer RNA, and 8 ribosomal RNA genes in the same order and orientation. Differences in long and short sequence repeats, SNPs, and InDels were detected across the 12 cp genomes. Combining with the complete cp sequences of seven other species in the genus Camellia, a total of nine intergenic sequence divergent hotspots and 14 protein-coding genes with high sequence polymorphism were identified. These hotspots, especially the InDel (~400 bp) located in atpH-atpI region, had sufficient potential to be used as barcode markers for further phylogenetic analysis and species identification. Principal component and phylogenetic analysis suggested that regional constraints, rather than functional constraints, strongly affected the sequence evolution of the cp genomes in this study. These cp genomes could facilitate the development of new molecular markers, accurate species identification, and investigations of the phylogenomic relationships of the genus Camellia.

Keywords: Camellia; chloroplast genome; genetic relationship; marker; phylogenetic analysis

1. Introduction

Camellia, comprising more than 200 species [1], is the largest genus in the family Theaceae, with remarkable economical and phylogenetic value [2]. Camellia plants offer abundant species diversity and include several economically important members. For instance, species in the section (sect.) Oleifera, including C. oleifera and C. meiocarpa, have a long history of cultivation and utilization in China for the acquisition of high-quality edible oil. Species belonging to the sect. Thea, including C. sinensis and C. assamica, provide the world’s oldest and most popular caffeine-containing tea beverage [3]. Moreover, species in the sect. Camellia have great ornamental value, particularly C. japonica. Some other Camellia species, such as C. chekiangoleosa, C. semiserrata, etc., have the additional benefit of bearing beautiful flowers. Because of such a variety of uses, Camellia plants are grown all over the world [2].

Due to phenotypic diversity, frequent hybridization, and polyploidization, a full understanding of the phylogenetic relationships of the genus Camellia is quite problematic [4–6]. Phenotypes are often affected by environmental factors, complicating morphology-based
traditional phylogenetic analysis. Nuclear genome sequencing has frequently been used to analyze phylogenetic relationships, genetic diversity, and evolutionary pathways in the genus *Camellia* to gain insights into the phylogeny of these species [7–10].

The angiosperm chloroplast (cp) genomes that are maternally transmitted with relatively stable structure [6,11] could provide an informative and valuable resource for phylogenetic analyses at family/genus/species levels [12–14]; they are also suitable for the resolution of complex evolutionary relationships [15–17] and trace source populations [18–20]. In the past decades, cp genomes have proven to be particularly powerful in revealing phylogenetic relationships of *Camellia* plants, which have large nuclear genomes [3,21,22]. Cp-derived markers were successfully employed to study the evolutionary relationships of plants in sects. *Thea, Camellia, Theopsis, Stereocarpus, Chrysantha*, and *Corallina*, etc., providing valuable genetic information for accurate identification of species and reconstruction of the phylogeny of the genus *Camellia* [6,11–14,23–27]. However, the lack of suitable polymorphic genetic markers has obstructed the phylogenetic analysis of sect. *Oleifera*, leaving its taxonomic classification rather controversial. For example, *C. vietnamensis*, a species defined in Zhang et al.’s taxonomic classification [5], was classified into a taxonomic variety of *C. oleifera* in Min et al. classification [1]. In turn, a better understanding and utilization of the diversification and evolution of the sect. *Oleifera* is missing. Recent advances in next-generation sequencing techniques and bioinformatics tools have made it convenient to obtain cp genome sequences at relatively low costs.

In this study, we present the complete cp genome sequences of 12 individuals from nine species of *Camellia* using the Illumina sequencing platform. The sequenced individuals covered six species from the sect. *Oleifera*, two species from the sect. *Camellia*, and one species from the sect. *Paracamellia*. Combining with the complete cp sequences of seven other species (Table S1) in the genus *Camellia*, we performed a comparative analysis of cp genomes and identified highly variable regions across species. Combining with 20 reported complete cp genomes in the family Theaceae (Table S1), the new phylogenetic trees were constructed. This study aims to examine variable regions in the *Camellia* cp genomes, especially in the sect. *Oleifera*, to establish a molecular basis for the development of novel DNA markers and reconstruction of phylogenetic relationships among the representative species. Our results provide a robust genomic framework for the phylogenomic characterization of *Camellia* species. It also gives a foundation to develop DNA markers that allow the identification of individual taxa cost-effectively.

2. Materials and Methods
2.1. Plant Materials
Tender leaves of Camellia plants, including *C. oleifera* var. 40, 53, GR3, MY6, *C. meiocarpa*, *C. nanyongensis*, *C. sasanqua*, *C. vietnamensis*, *C. gauchowensis*, *C. chekiangoleosa* var. *Baihua*, *C. semiserrata*, and *C. grijsii*, used in this study were harvested from Research Institute of Subtropical Forestry, Chinese Academy of Forestry (Hangzhou, Zhejiang, China), International Camellia Species Garden (Jinhua, Zhejiang, China), and Dongfanghong Forest Farm of Zhejiang Province (Jinhua, Zhejiang, China) in April 2020 (Table 1). The collected plant materials were classified by Sealy’s taxonomic treatment [5] (Table 1). All the samples were collected according to local, national, or international guidelines and legislation. We complied with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora during the sample collection.
| Taxon                        | Genus      | Section | Collection Sites | Geographical Origin                     | Morphological characters                                                                 |
|-----------------------------|------------|---------|------------------|-----------------------------------------|------------------------------------------------------------------------------------------|
| *Camellia oleifera* var. 40| Camellia   | Oleifera| DFFZP            | Zhejiang province, China                 | Shrub or dungarunga; elliptical or ovate leaves; white flower, petals 5–7                  |
| *Camellia oleifera* var. 53| Camellia   | Oleifera| DFFZP            | Zhejiang province, China                 | Shrub; elliptical or ovate leaves; white flower, petals 5–7                               |
| *Camellia oleifera* var. GR3| Camellia   | Oleifera| DFFZP            | Guangxi zhuang autonomous region, China  | Shrub; elliptical or ovate leaves; white flower, petals 5–7                               |
| *Camellia oleifera* var. MY6| Camellia   | Oleifera| DFFZP            | Fujian province, China                   | Shrub; elliptical or ovate leaves; white flower, petals 5–7                               |
| *Camellia meiocarpa*       | Camellia   | Oleifera| DFFZP            | Jiangxi province, China                  | Shrub; elliptical or ovate leaves; white flower, petals 5–7                               |
| *Camellia nanyongensis*    | Camellia   | Oleifera| ICSG             | Guangxi zhuang autonomous region, China  | Shrub; lanceolate leaves; white flower, petals 6                                          |
| *Camellia sasanqua*        | Camellia   | Oleifera| RISF             | Zhejiang province, China                 | Dungarunga; elliptical leaves; red flower, petals 6–7                                     |
| *Camellia vietnamensis*    | Camellia   | Oleifera| RISF             | Guangdong province, China                | Shrub or dungarunga; elliptical or ovate leaves; white flower, petals 5–7                |
| *Camellia gauchowensis*    | Camellia   | Oleifera| RISF             | Guangdong province, China                | Shrub or dungarunga; elliptical leaves; white flower, petals 7–8                           |
| *Camellia chekiangolocosa* var. Baihua | Camellia   | Camellia| DFFZP            | Zhejiang province, China                 | Dungarunga; elliptical leaves; white flower, petals 7                                     |
| *Camellia semiserrata*     | Camellia   | Camellia| RISF             | Guangxi zhuang autonomous region, China  | Dungarunga; elliptical leaves; red flower, petals 6–7                                     |
| *Camellia grijsii*         | Camellia   | Paracamellia | RISF            | Hunan province, China                    | Shrub or dungarunga; oblong leaves; white flower, petals 5–6                               |

RISE, Research Institute of Subtropical Forestry, Chinese Academy of Forestry (Hangzhou, China); ICSG, International *Camellia* Species Garden (Jinhua, China); DFFZP, Dongfanghong Forest Farm of Zhejiang Province (Jinhua, China).
2.2. DNA Sequencing and Chloroplast Genome Assembly

About 20 g of tender leaves from each taxon were used for total genomic DNA extraction using the TaKaRa MiniBEST Plant Genomic DNA Extraction Kit (TaKaRa, Dalian, China) according to the user manual. High-quality genomic DNA was used to construct 350 bp Illumina HiSeq libraries following the manufacturer’s protocol. Raw data were filtered using FASTp v.0.20.0 [28] to obtain clean data by trimming adapter sequences and removing low-quality reads with a Phred quality threshold $Q < 20$. We assembled the cp genome in the following steps: First, the clean reads were assembled into contigs using SPAdes v3.11.1 [29] with a range of iterative $k$-mers sizes of 55, 87, and 121. If SPAdes was unsuccessful at assembling complete cp genomes, we used the following steps to assemble it. Second, the contigs were aligned to the reference cp genome of *C. crapnelliana* (NCBI accession number NC_024541.1) using BLAST [30], and the aligned contigs were extracted, ordered, and the reads of nuclear origins were excluded. The extracted contigs were further assembled to scaffolds using SSPACE v2.0 [31]. Third, clean data were again mapped to the assembled draft cp genomes to verify the assembly results. The majority of gaps were filled by Gapfiller v2.1.1 [32,33].

2.3. Genome Annotation and Visualization

Assembled cp genomes from all taxa were annotated. Prodigal v2.6.3 [34] and Hmmer v3.1b2 [35] were applied to annotate protein-coding genes and rRNAs, respectively. tRNAs were predicted via ARAGORN v1.2.38 [36]. The annotation results were manually corrected for the codon positions and intron/exon boundaries by comparing to the homologous genes from *Camellia* with known cp genomes. The circular maps of the cp genome were drawn using the OGDRAW tool [37]. The exact boundaries of IR/ LSC and IR/ SSC were confirmed by aligning them with the homologous sequences from other *Camellia* species and visualized by a customized Perl script (https://github.com/xul962464/perl-IRscope (accessed on 12 June 2021)), which was similar to IRscope.

2.4. Sequence Divergence Analysis

Forward, reverse, palindromic, and complement LSRs were found and analyzed using vmatch v2.3.0 [38], with the parameters’ minimum length = 30 bp and Hamming distance = 3 in the cp genomes. Reverse and complement repeats were further checked by a customized Perl script. SSRs were detected by MISA v1.0 [39] with the parameters set at $\geq 8$ repeats for mononucleotides, $\geq 5$ repeats for dinucleotides, and $\geq 3$ repeats for trinucleotides, tetrancleotides, pentanucleotides, and hexa-nucleotides. Whole-genome alignments were conducted to evaluate rearrangements and substantial sequence divergence using Mauve [40] and MAFFT v7.0 [41] with default parameters. Based on the whole-genome alignments by MAFFT v7.0, the SNPs and InDels calling was performed using DnaSP 5.0 [42]. Furthermore, to identify the divergent hotspots, the nucleotide diversity ($\Pi$) values for each gene in the cp genomes were evaluated using DnaSP 5.0.

2.5. Selection Pressure Analysis

To evaluate the role of selection on the protein-coding gene regions in the cp genomes of *Camellia* species, we calculated the nonsynonymous mutation rate ($Ka$), synonymous mutation rate ($Ks$), and the $Ka/Ks$ of each gene from the 12 *Camellia* taxa in this study and the seven related *Camellia* species downloaded from NCBI GenBank (Table S1) using the KaKs_Calculator v2.0 [43].

2.6. Principal Component Analysis (PCA) and Phylogenetic Analysis

SNP data of the 12 cp genomes sequenced in this study were used to perform PCA using GCTA v1.25.2 [44], and the first two components were plotted. The complete cp genome sequences of 26 *Camellia* taxa were used for phylogenetic analyses, including 12 cp genomes reported in this study and 14 previously sequenced cp genomes downloaded
from the NCBI GenBank. Another six cp genomes from the genera *Tutcheria*, *Apterosperma*, *Stewartia*, *Anneslea*, *Adinandra*, and *Pyrenaria* in Theaceae were selected as outgroup. The whole-genome alignments of 32 species or their variations were performed using the MAFFT v7.0 program [41]. The aligned results were trimmed by trimAl v1.4. The phylogenetic analyses were implemented using maximum likelihood (ML) and Bayesian analysis methods based on the complete cp genome data, coding region data and noncoding region data, respectively. ML analyses were implemented in RAXML v.8.2.10 [45] and Bayesian analysis was conducted in MrBayes v.3.2.6 [46]. RAXML and MrBayes searches relied on the general time reversible (GTR) model of nucleotide substitution with the gamma model of rate heterogeneity. RAXML was run for the ML trees with 1000 bootstrap replicates. The Markov Chain Monte Carlo (MCMC) algorithm was calculated for 1,000,000 generations with a sampling tree every 1000 generations in Bayesian analysis. The first 25% of generations was discarded as burn-in. Stationary was reached when the average standard deviation of split frequencies was <0.01 and a consensus tree was constructed using the remaining trees.

3. Results
3.1. Chloroplast Genome Sequencing and Assembly

Using the Illumina sequencing platform, we sequenced cp genomes of nine *Camellia* species, including six species from the sect. *Oleifera*, two species from the sect. *Camellia*, and one from the sect. *Paracamellia* (Table 1). Illumina paired-end (2 × 150 bp) sequencing produced large data sets for individual samples. We randomly picked 25,000,000 paired-end reads to assemble the cp genomes. In total, 12 cp genome sequences were obtained through de novo genome assembly. Overall, 57,181~439,221 reads with an average insert size of 296~379 bp were assembled into the 12 cp genomes, reaching 112~884 × average coverage (Table 2).

3.2. Characterization of Chloroplast Genomes of Selected Camellia Species

All 12 complete *Camellia* cp genomes exhibited a typical quadripartite structure of most angiosperms with conserved genome arrangement and structure. Among these cp genomes, genome size ranged from 156,545 bp (*C. sasanqua*) to 157,021 bp (*C. nanyongensis*). The sequenced genomes included a pair of inverted repeat regions (IRA and IRB) separated by a large single copy region (LSC) and a small single copy region (SSC) (Table 2 and Figure 1). The length varied from 86,257 bp (*C. sasanqua* and *C. chekiangoleosa* var. *Baihua*) to 86,659 bp (*C. oleifera* var. 40, 53, and MY6) in the LSC. It varied from 18,269 bp (*C. semiserrata*) to 18,415 bp (*C. chekiangoleosa* var. *Baihua*) in the SSC. The length varied from 25,943 bp (*C. meiocarpa*) to 26,060 bp (*C. nanyongensis*) in the IR (Table 2). These cp genome sequences were deposited into the GenBank under accession numbers OL689014~OL689025 (Table S1).

Overall, the cp genomes of all 12 *Camellia* individuals encoded an identical set of 132 genes, including 87 protein-coding, 37 transfer RNA (tRNA), and eight ribosomal RNA (rRNA) genes (Table 2). Due to the uniform gene number, order, and names, the annotated cp genomes of these 12 *Camellia* taxa are represented in one circular map (Figure 1). In all cp genomes, eight protein-coding (*rps12, rps7, ndhB, ycf15, ycf2, rpl23, rpl2, and rps19*), seven tRNA (*trnN-GUU, trnR-ACG, trnA-UGC, trnL-GAU, trnV-GAC, trnL-CAA, and trnI-CAU*), and four rRNA (*rrn16, rrn23, rrn4.5, and rrn5*) genes were duplicated in the IR. The LSC harbored 61 protein-coding and 22 tRNA genes, while the SSC had 11 protein-coding and one tRNA gene. The *rps19* is a uniquely structured gene with the 3'-end exon located in the LSC, while two copies of the 5'-end exon are located in the IRA and IRB, respectively; this arrangement generates an *rps19* pseudogene in the IRA. The *ycf1* is located in the boundary region between the IRA and SSC, leading to an incomplete duplication of the gene within IRs and the generation of a pseudogene in the IRB.
Table 2. A summary of the statistics for the chloroplast genomic assemblies of 12 *Camellia* species and varieties.

| Genome Features                  | C. oleifera var. 40 | C. oleifera var. 53 | C. oleifera var. GR3 | C. oleifera var. MY6 | C. meiocarpa | C. nanongensis | C. sasanqua | C. vietnamensis | C. gauchowensis var. Baliana | C. cheksiangeleosa var. Baihua | C. semiserrata | C. grijsii |
|----------------------------------|---------------------|---------------------|----------------------|---------------------|--------------|----------------|-------------|----------------|---------------------------|-----------------------------|----------------|-----------|
| Genome size (bp)                 | 156,975             | 156,975             | 156,975              | 156,975             | 156,550      | 156,910        | 157,003     | 156,606         | 156,833                    | 157,004                     |               |           |
| LSC size (bp)                    | 86,659              | 86,659              | 86,606               | 86,659              | 86,264       | 86,619         | 86,257      | 86,656          | 86,257                    | 86,656                     | 86,462        | 86,657    |
| SSC size (bp)                    | 18,408              | 18,408              | 18,290               | 18,408              | 18,400       | 18,282         | 18,402      | 18,300          | 18,297                    | 18,415                     | 18,269        | 18,297    |
| IR size (bp)                     | 25,954              | 25,954              | 26,041               | 25,954              | 25,943       | 26,006         | 25,943      | 26,025          | 26,025                    | 25,967                     | 26,051        | 26,025    |
| Number of genes                  | 134                 | 134                 | 134                  | 134                 | 134          | 134            | 134         | 134             | 134                       | 134                        | 134           | 134       |
| Protein coding genes (unique)    | 87 (80)             | 87 (80)             | 87 (80)              | 87 (80)             | 87 (80)      | 87 (80)        | 87 (80)     | 87 (80)         | 87 (80)                   | 87 (80)                     | 87 (80)        | 87 (80)   |
| trRNA genes (unique)             | 37 (29)             | 37 (29)             | 37 (29)              | 37 (29)             | 37 (29)      | 37 (29)        | 37 (29)     | 37 (29)         | 37 (29)                   | 37 (29)                     | 37 (29)        | 37 (29)   |
| rRNA genes (unique)              | 8 (4)               | 8 (4)               | 8 (4)                | 8 (4)               | 8 (4)        | 8 (4)          | 8 (4)       | 8 (4)           | 8 (4)                     | 8 (4)                       | 8 (4)         | 8 (4)     |
| Duplicated genes in IR (%)       | 34                  | 34                  | 34                   | 34                  | 34           | 34             | 34          | 34              | 34                        | 34                          | 34             | 34        |
| GC content (%)                   | 37.29               | 37.29               | 37.29                | 37.29               | 37.29        | 37.29          | 37.29       | 37.29           | 37.29                     | 37.29                       | 37.29          | 37.29     |
| GC content in LSC (%)            | 35.29               | 35.29               | 35.29                | 35.29               | 35.29        | 35.29          | 35.29       | 35.29           | 35.29                     | 35.29                       | 35.29          | 35.29     |
| GC content in SSC (%)            | 30.53               | 30.53               | 30.53                | 30.53               | 30.53        | 30.53          | 30.53       | 30.53           | 30.53                     | 30.53                       | 30.53          | 30.53     |
| GC content in IR (%)             | 43.03               | 43.03               | 42.99                | 43.03               | 42.99        | 43.03          | 42.99       | 43.03           | 42.99                     | 43.03                       | 42.98          | 42.98     |
| Total reads                      | 25,000,000          | 25,000,000          | 25,000,000           | 25,000,000          | 25,000,000   | 25,000,000     | 25,000,000  | 25,000,000      | 25,000,000                | 25,000,000                  | 25,000,000    | 25,000,000|
| Assembled reads                  | 371,694             | 356,834             | 133,442              | 202,707             | 57,181       | 206,798        | 197,214     | 152,500         | 439,221                   | 105,258                     | 95,232         |           |
| Average coverage                 | 860                 | 738                 | 334                  | 495                 | 415          | 395            | 306         | 275             | 884                       | 205                         | 208            |           |
| Average insert size (bp)         | 373                 | 336                 | 379                  | 377                 | 313          | 319            | 321         | 310             | 333                       | 296                         | 356            |           |
12 introns exhibited length polymorphism with 1~26 bp across the 12 Camellia ndhA while only the Baihua var. (Table 4). The variance in the length of introns may be one of the reasons for the different sizes of these cp genomes. C. chekiangoleosa regarded as a unique feature of Camellia containing genes, 12 genes were located in the LSC, five genes were distributed in the IRs, one intron and two genes (trnV-UAC) genes were found, including 16 genes (trnK-UGU, rps16, trnG-UCC, atpF, rpoC1, trnL-UAA, trnV-UAC, rps12, petB, petD, rpl16, rpl2, ndhB, trnL-GAU, trnA-UGC, and ndhA) containing one intron and two genes (ycf3 and clpP) containing two introns (Table 3). Of the 18 intron-containing genes, 12 genes were located in the LSC, five genes were distributed in the IRs, while only the ndhA gene was in the SSC. Notably, the trnG-UCC gene of C. chekiangoleosa var. Baihua contained a longer Exon I (34 bp) and a shorter Exon II (43 bp) as compared to the other 11 Camellia individuals (23 and 48 bp, respectively); this observation could be regarded as a unique feature of C. chekiangoleosa var. Baihua. Of all the 20 introns identified, 12 introns exhibited length polymorphism with 1~26 bp across the 12 Camellia cp genomes (Table 4). The variance in the length of introns may be one of the reasons for the different sizes of these cp genomes.

Figure 1. A gene map of the Camellia chloroplast genomes. The inner circle indicates the GC content, while the outer circle indicates the genome structure and gene arrangement. Genes shown outside the outer circle were transcribed clockwise and those shown inside were transcribed counterclockwise. Functional categories of genes are presented in the left margin.
## Table 3. List of annotated genes in the sequenced *Camellia* chloroplast genomes.

| Category                        | Group of Genes                  | Name of Genes                                                                 |
|--------------------------------|---------------------------------|-------------------------------------------------------------------------------|
| Photosynthesis                 | Subunits of photosystem I       | psaA, psaB, psaC, psaI, psaJ                                                  |
|                                | Subunits of photosystem II      | psbA, psbB, psbC, psbD, psbE, psbF, psbH, psbI, psbJ, psbK, psbL, psbM, psbN, psbT, psbZ |
|                                | Subunits of NADH dehydrogenase  | ndhA *, ndhB *(2), ndhC, ndhD, ndhE, ndhF, ndhG, ndhH, ndhI, ndhJ, ndhK       |
|                                | Subunits of cytochrome b/f complex | petA, petB *, petD *, petG, petL, petN                                           |
|                                | Subunits of ATP synthase        | atpA, atpB, atpE, atpF *, atpH, atpl                                           |
|                                | Large subunit of rubisco         | rbcL                                                                         |
|                                | Proteins of large ribosomal subunit | rpl14, rpl16 *, rpl2 *(2), rpl20, rpl22, rpl23(2), rpl32, rpl33, rpl36        |
|                                | Proteins of small ribosomal subunit | #rps19, rps11, rps12 **(2), rps14, rps15, rps16 *, rps18, rps19, rps2, rps3, rps4, rps7(2), rps8 |
|                                | Subunits of RNA polymerase       | rpoA, rpoB, rpoC1 *, rpoC2                                                   |
|                                | Ribosomal RNAs                  | rrn16(2), rrn23(2), rrn34.5(2), rrn5(2)                                       |
|                                | Transfer RNAs                   | trnA-UGC *(2), trnC-GCA, trnD-GUC, trnE-UUIC, trnF-GAA, trnG-UCC, trnG-UCC *, trnH-GUG, trnL-CAU(2), trnL-GAU *(2), trnK-UUU, trnL-CAU *, trnL-LUA *, trnL-UAG, trnM-CAL, trnN-GUU(2), trnP-UGG, trnQ-UGU, trnR-ACG(2), trnR-UCU, trnS-GCU, trnS-GGA, trnS-LGA, trnT-GGU, trnT-UAG, trnV-GAC(2), trnV-UAC *, trnW-CCA, trnY-GUA, trnF-M-CAL |
|                                | Other genes                     | matK                                                                         |
|                                | Maturase                        | matK                                                                         |
|                                | Protease                        | clpP **                                                                       |
|                                | Envelope membrane protein        | cemA                                                                         |
|                                | Acetyl-CoA carboxylase           | accD                                                                         |
|                                | c-type cytochrome synthesis gene | ccsA                                                                         |
|                                | Translation initiation factor    | infA                                                                         |
|                                | Unknown function                | Conserved hypothetical chloroplast ORF # ycf1, ycf1, ycf15(2), ycf2(2), ycf3 **, ycf4 |

Notes: Gene *: Genes with one intron; Gene **: Genes with two introns; # Gene: Pseudo-gene; Gene (2): Two gene copies in the IRs.

## Table 4. Information of intron-containing genes in the chloroplast genome of the *Camellia* species.

| Gene  | Location | Exon I (bp) | Exon II (bp) | Exon III (bp) | Intron I (bp) | Intron II (bp) | Intron III (bp) |
|-------|----------|-------------|--------------|---------------|---------------|----------------|-----------------|
| trnK-UUU | LSC      | 37          | 2488-2502    | 35            |               |               |                 |
| rps16  | LSC      | 39          | 851-875      | 225           |               |               |                 |
| trnG-UCC | LSC      | 34 or 23    | 690 or 695   | 43 or 48      |               |               |                 |
| atpF   | LSC      | 159         | 704          | 408           |               |               |                 |
| rpoC1  | LSC      | 485         | 732          | 1626          |               |               |                 |
| ycf3   | LSC      | 126         | 737 or 747   | 228           |               |               |                 |
| trnL-LUA | LSC     | 37          | 523 or 519   | 50            |               |               |                 |
| trnV-LUAC | LSC     | 39          | 586          | 37            |               |               |                 |
| rps12  | IRa      | 346         | 538          | 26            |               |               |                 |
| clpP   | LSC      | 69          | 539-542      | 291           |               |               |                 |
| petB   | LSC      | 6           | 756-762      | 657           |               |               |                 |
| petD   | LSC      | 9           | 696          | 525           |               |               |                 |
| rpl16  | LSC      | 9           | 1014-1025    | 402           |               |               |                 |
| rpl2   | IRb      | 393         | 667          | 435           |               |               |                 |
| ndhB   | IRb      | 777         | 679          | 756           |               |               |                 |
| trnL-GAU | IRb     | 42          | 947 or 948   | 35            |               |               |                 |
| trnA-UUG | IRb     | 38          | 812          | 35            |               |               |                 |
| ndhA   | SSC      | 552         | 1084-1110    | 540           |               |               |                 |
3.3. Expansion and Contraction of the Border Regions

Although overall genomic structure, including gene number and order, is well-conserved, the expansion and contraction of the IRs are common in cp genomes, which is the main reason behind the different sizes of cp genomes [47]. The border regions and adjacent genes of the 12 genomes were compared to analyze the variation in expansion and contraction variation in the junction region (Figure 2). Results suggested that the junction positions of IR/LSC were conserved in these 12 genomes. For example, the rps19 gene, which was located at the boundary of the LSC/IRB in the cp genomes, showed the same length of 279 bp, including 233 bp in the LSC and 46 bp in the IRB, leading to incomplete duplication of the gene and the formation of a pseudo-rps19 (46 bp) within IRA. There were slight differences in the junction positions of IR/SSC in these 12 genomes. The distances from ndhF to the junction of IRB/SSC ranged from 5–68 bp. Furthermore, the distances differed among the four C. oleifera varieties, i.e., 65 bp in C. oleifera var. GR3, rather than 68 bp in other three C. oleifera varieties. The distance was 68 bp in C. meiocarpa and C. sasanqua; 56 bp in C. nanyongensis, C. vietnamensis, C. gauchowiensis, and C. grijsii; 62 bp in C. chekianggoleosa var. Baihua; and only 5 bp in C. semiserrata (Figure 2). ycf1 gene was located on the junction of IRA/SSC with a length of 5616 bp in C. meiocarpa, C. sasanqua, and C. chekianggoleosa var. Baihua, rather than 5622 bp in the other nine cp genomes (Figure 2). The gene ycf1 extended into the IRA with 963 bp in six cp genomes, 1069 bp in five cp genomes, and 1043 bp in C. semiserrata, leading to an incomplete duplication and formation of a pseudo-ycf1 in the IRB with the corresponding length. Meanwhile, this pseudo-ycf1 also extended into the SSC with 2 bp, 16 bp, or 30 bp, respectively (Figure 2). Notably, the length of ycf1 in the IRA of C. oleifera var. GR3 (1069 bp) was different from that in the other three C. oleifera varieties (963 bp). The variations at the IR/SSC boundary regions across the 12 Camellia cp genomes may lead to differences of the lengths of the IR and SSC and the whole-genome sequences.

3.4. Repeat Sequences and Microsatellite Analyses

In this study, we detected forward, palindromic, reverse, and complement repeats (long sequence repeats, LSRs) in all 12 Camellia cp genomes. Overall, 29–39 LSRs with lengths ≥30 bp were identified in each cp genome. Among these, 11–16 were forward repeats, 15–22 were palindromic repeats, and 0–2 were reverse repeats that were separately detected (Figure 3A and Table S2). Only one complement repeat was screened in C. chekianggoleosa var. Baihua. The lengths of repeats in the 12 Camellia cp genomes ranged from 30 to 64 bp. The repeated lengths with 30–39 bp were the most common (48.58%), while those with 50–59 bp (10.43%) and over 60 bp (10.43%) were relatively rare (Figure 3B–D). Most of these LSRs were located in the exon region (57–71%), including some for protein-coding genes such as ycf2, psbN, psaA, and some tRNA genes. LSRs were also located in intergenic sequences (IGSs), while the least number of LSRs was present in introns (Figure 3E,F, and Table S2). The LSRs were primarily shared over the cp genomes of eight species, where C. nanyongensis cp genome has the most unique LSRs (Figure 3G,H). It was found that C. oleifera var. 40, 53, and MY6 shared a set of common LSRs, and C. gauchowiensis, C. sasanqua, and C. vietnamensis shared another set of common LSRs (Figure 3H).

SSRs were also detected in the 12 cp genomes with repeat times of 3–20, and repeated lengths of 1–6 bp (Figure 4 and Table S3). A total of 232–242 SSRs were detected in each genome. The majority of the SSRs were mononucleotide SSRs (especially for A/T) that varied from 142 in C. meiocarpa to 150 in C. vietnamensis. Trinucleotide SSRs (especially for the three-time repeat) was the second most predominant, ranging from 66 in C. semiserrata to 69 in C. meiocarpa, C. nanyongensis, and C. sasanqua. Furthermore, 10–11 tetra-, 4–5 di-, and 6–8 complex-nucleotide SSRs were identified in each cp genome. Additionally, two hexa-nucleotide SSRs were found in C. oleifera, C. nanyongensis, C. chekianggoleosa, and C. semiserrata, while the remaining five cp genomes did not contain any hexa-nucleotide SSRs (Figure 4, Tables 5 and S3). These SSRs were mainly located in the IGS, exons of some protein-coding genes, and tRNA genes, while only 13.56–14.66% of them were located in...
the introns. In the four structural regions, SSRs were distributed unevenly across the cp genomes, with the majority of SSRs were located in the LSC, followed by the SSC and IR (Table 5).

Table 5. Summary of SSRs in the 12 representative *Camellia* cp genomes.

| Species                          | SSR Loci (N) | Region | Location | Styles | P1 a Loci (N) | P2 b Loci (N) | P3 c Loci (N) | P4 d Loci (N) | P5 e Loci (N) | P6 f Loci (N) |
|----------------------------------|--------------|--------|----------|--------|---------------|---------------|---------------|---------------|---------------|---------------|
| *C. oleifera* var. 40            | 237          | LSC    | 139      | 50     | 48            | 88            | 116           | 145           | 5             | 68            | 10            | 2             | 8             |
| *C. oleifera* var. 53            | 237          | LSC    | 139      | 50     | 48            | 88            | 116           | 144           | 5             | 68            | 10            | 2             | 8             |
| *C. oleifera* var. GR3           | 238          | LSC    | 139      | 51     | 48            | 89            | 116           | 147           | 4             | 68            | 10            | 2             | 7             |
| *C. oleifera* var. MY6           | 237          | LSC    | 139      | 50     | 48            | 88            | 116           | 145           | 5             | 67            | 11            | 2             | 7             |
| *C. nanyongensis*                | 242          | LSC    | 142      | 52     | 48            | 88            | 116           | 142           | 4             | 69            | 10            | 0             | 7             |
| *C. sasanqua*                    | 233          | LSC    | 137      | 50     | 46            | 88            | 111           | 143           | 4             | 69            | 10            | 2             | 7             |
| *C. vietnamensis*                | 239          | LSC    | 142      | 51     | 46            | 89            | 116           | 150           | 4             | 68            | 10            | 0             | 7             |
| *C. gauchowensis*                | 237          | LSC    | 140      | 51     | 46            | 90            | 114           | 144           | 4             | 68            | 10            | 0             | 6             |
| *C. chekiangoleosa var. Baihua*  | 235          | LSC    | 137      | 50     | 48            | 88            | 114           | 143           | 4             | 67            | 10            | 2             | 9             |
| *C. semiserrata*                 | 236          | LSC    | 140      | 51     | 46            | 90            | 114           | 147           | 4             | 66            | 10            | 2             | 7             |
| *C. grijsii*                     | 237          | LSC    | 140      | 51     | 46            | 90            | 114           | 148           | 4             | 68            | 10            | 0             | 7             |

Figure 2. The comparison of the LSC, IR, and SSC border regions in the 12 *Camellia* chloroplast genomes.
Figure 3. The type and distribution of LSRs in the 12 *Camellia* cp genomes. (A). Number of the four types of repeats; (B–D), Distribution of the forward, palindromic, and reverse repeats by length, respectively; (E,F), Location of repeats in *C. nanyongensis* and *C. meiocarpa*, which harbored the most and least repeats in exon regions in the 12 cp genomes, respectively; (G,H), Summary of shared LSRs among the 12 *Camellia* cp genomes.
3.5. SNP and InDel Variations

Global alignment of the 12 *Camellia* cp genomes for the detection of SNPs and InDels was performed using *C. cheekangoleosa* var. Baihua as a reference. In total, 489 SNPs and 174 InDels were mined (Tables S4 and S5). The largest number of SNPs (40.90%) were distributed in the exon regions, followed by the IGS (33.33%) and intron regions (25.77%) (Table S4). The base substitutions involving C and G were fewer than other types of substitutions, which is in agreement with previous studies [6,48]. The InDels were mainly distributed in the exon regions, followed by the IGS (33.33%) and intron regions (25.77%) (Figure S1). Similar to a previous study [6], single-nucleotide InDels were the most identified SSR motifs in the 12 *Camellia* cp genomes. p3, p4, p6, and c indicate tri-, tetra-, hexa-, and complex nucleotides, respectively.

![Figure 4. The distribution of SSR motifs in the 12 Camellia cp genomes. p3, p4, p6, and c indicate tri-, tetra-, hexa-, and complex nucleotides, respectively.](image-url)

We performed a pairwise comparison of the cp genomes. The results showed that the number of SNPs and InDels ranged from 0 to 203 and 0 to 98, respectively, with the ratio of SNPs to InDels (S/I) ranging from 1.0 to 2.60 (Table 6). Three *C. oleifera* varieties shared an identical cp genome without any SNP and InDel, except for var. GR3. As compared to the intraspecific varieties and other species, var. GR3 possessed similar numbers of SNPs and InDels. The results of the interspecies comparison showed that the cp genome sequences of *C. grijsii* and *C. gauchowensis* were highly similar and contained only two InDels and no SNPs, while the sequences of *C. nanyongensis* were different from the other species with more SNPs and InDels (Table 6).
Table 6. The numbers and ratios of SNPs and InDels in the 12 *Camellia* cp genomes.

|                      | C. oleifera var. 40 | C. oleifera var. 53 | C. oleifera var. GR3 | C. meiocarpa | C. nanyongensis | C. sasanqua | C. vietnamensis | C. gauchovensis | C. grijsii | C. chekiangoleosa var. Baihua | C. semiserrata |
|----------------------|---------------------|---------------------|----------------------|--------------|-----------------|-------------|----------------|----------------|-----------|-------------------------------|---------------|
| C. oleifera var. 40  | -                   | 0                   | 62                   | 0            | 48              | 78          | 42             | 77             | 73        | 74                            | 50            |
| C. oleifera var. 53  | 0                   | -                   | 62                   | 0            | 48              | 78          | 42             | 77             | 73        | 74                            | 50            |
| C. oleifera var. GR3 | 153(2.47)           | 153(2.47)           | -                    | 62           | 68              | 71          | 66             | 64             | 62        | 63                            | 73            |
| C. myocarpa          | 0                   | 0                   | 153(2.47)            | -            | 48              | 78          | 42             | 77             | 73        | 74                            | 50            |
| C. nanyongensis      | 93(2.21)            | 93(2.21)            | 128(1.94)            | 93(2.21)     | 6(1.00)         | 79          | 93             | 87             | 87        | 84                            | 69            |
| C. sasanqua          | 170(2.21)           | 170(2.21)           | 121(1.89)            | 170(2.21)    | 143(2.10)       | 79          | 93             | 87             | 87        | 84                            | 69            |
| C. gauchovensis      | 171(2.34)           | 171(2.34)           | 122(1.97)            | 171(2.34)    | 147(2.10)       | 176(2.23)   | 68             | 70             | 70        | 59                            | 87            |
| C. grijsii           | 171(2.31)           | 171(2.31)           | 123(1.95)            | 171(2.31)    | 146(2.09)       | 198(2.28)   | 150(2.21)      | 62(1.48)       | 77        | 77                            | 98            |
| C. chekiangoleosa var. Baihua | 75(1.5)           | 75(1.5)             | 140(1.92)            | 72(1.22)     | 190(2.26)       | 76(1.43)    | 157(1.96)      | 158(2.05)      | 183(2.01) | -                             |               |
| C. semiserrata       | 194(2.18)           | 194(2.18)           | 173(2.16)            | 173(1.99)    | 162(3.35)       | 177(2.13)   | 170(1.73)      | 173(1.84)      | 173(1.82) | -                             |               |

The upper triangle shows the number of InDels, and the lower triangle indicates the number of total SNPs. The ratios of SNPs to InDels (S/I) are given in brackets.
3.6. Sequence Divergence and Hotspots

To elucidate the level of genome divergence and identify the divergent hotspots, multiple alignments of 19 *Camellia* cp genomes, including 12 genomes sequenced in this work and seven genomes representative *Camellia* species from previous studies (Table S1), were performed using Mauve [40]. With *C. chekiangoleosa* var. *Baihua* as a reference, we plotted the sequence identity (Figure 5). The alignment results revealed high sequence similarity across the 19 *Camellia* cp genomes, suggesting that the genome structure was highly conserved in terms of both gene identity and order (Figure 5). Differences were also detected across the *Camellia* cp genomes. Similar to the findings of a previous study [6], the IR were more conserved than single-copy regions, and coding regions were more conserved than noncoding regions. The most divergent regions were mainly located at positions between 5000–20,000 bp, 30,000–35,000 bp, 45,000–55,000 bp, and 115,000–135,000 bp (The cp genome sequence was coded in counterclockwise order from the interface between the LSC and IRA). Combined with the SNPs and InDels analysis results (Tables S4 and S5), the high variable regions were found that mainly located in *trnH-GUG-psbA, psbK-atpA, atpH-atpI, rpoB-psbM, trnE-UUC-psbD, rps4-ndhJ, ndhC-atpB, ndhF-rpl32*, and *ccsA-ndhD*. There was a marked divergence at the 15,065 bp position (*atpH-atpI* region) of the referent genome, which was in accordance with the existence of InDels with a length of ~400 bp at this locus (Figure 5 and Table S5). These hotpots can be applied to DNA barcode encoding and phylogenetic analysis of *Camellia* genus.

Furthermore, the *Pi* values for each gene were calculated separately among the 19 *Camellia* cp genomes (Table S1). The results showed that the distribution patterns of variable nucleotides in the IR/SC were fairly different (Figure 6A and Table S6). The genes in the SSC showed the highest nucleotide diversity with a mean *Pi* value of 0.00116, followed by the LSC (mean *Pi* = 0.00072) and then the IR (0.00049). These findings illustrated that the IRs were more conservative than the single-copy regions. The top ten divergent hotspot genes were identified with *Pi* values. They were *psbK, psbT, psaJ, rpl33, rpl32, ycf1, rpl16, matK*, and *psbl* (Figure 6A and Table S6). Among these divergent genes, eight were located in the LSC and two were located in the SSC. In these ten genes, four genes (*psbK, psbT, psaJ, psbI*) belonged to the ‘Subunits of photosystem’ group, four genes (*rpl16, rpl33, rpl32, rps8*) belonged to the ‘Proteins of ribosomal subunit’ group, and one gene (*matK*) encoded the maturase protein. These ten genes have a higher polymorphism level and can be applied to further phylogenetic analysis and species identification.

3.7. Estimation of the Selection Pressure of the Camellia Cp Genomes

To evaluate the role of selection of the *Camellia* cp genomes, *Ka, Ks*, and *Ka/Ks* values of 87 protein-coding genes were calculated in 19 cp genomes using *C. chekiangoleosa* var. *Baihua* as a reference (Table S7). The results indicated that the selection pressure of these genes was significantly different (Figure 6B). The *Ka/Ks* were not available for some genes because of the low level of divergence, and the *Ka* or *Ks* values were equal to zero. *Ka/Ks* values of most genes in each cp genome were less than 1, suggesting that the purifying selection may have acted on these protein-coding genes. Meanwhile, under the positive selection with *Ka/Ks* values >1, we identified four genes, *ropC2* in *C. gauchowensis, C. vietnamensis, C. grijsii*, and *C. cuspidata*; *petB* in *C.sinensis* and *C. nitidissima*; *ycf2* in *C. nanyongensis*; and *ycf1* in *C. vietnamensis*. *ropC2* and *ycf1* genes showed strong divergence and the *Ka/Ks* values were available for almost all cp genomes in this study (Figure 6B and Table S7). The *ropC2, ycf1, ycf2*, and *petB* had sufficient potential to be used as barcode markers for phylogenetic analysis.
Figure 5. Mauve alignment of the 19 Camellia cp genomes. The C. chekiangoleosa var. Baihua genome is shown at the top as the reference genome. Within each alignment, the pink blocks represent the sequence similarity with the other genomes. The white, green, and red blocks represent protein-coding, tRNA, and rRNA genes, respectively. The regions in the red dotted box are the sequences with high divergence, which include the intergenic regions of atpH-atpI. A, C. chekiangoleosa var. Baihua; B, C. gauchowensis; C, C. meiocarpa; D, C. nanyongensis; E, C. oleifera var. 40; F, C. oleifera var. 53; G, C. oleifera var. GR3; H, C. oleifera var. MY6; I, C. sasanqua; J, C. semiserrata; K, C. vietnamensis; L, C. grijsii; M, C. sinensis (KC143082.1); N, C. japonica (MK353211.1); O, C. amplexicaulis (MT317095.1); P, C. cuspidata (NC022459.1); Q, C. crapnelliana (NC024541.1); R, C. chekiangoleosa (NC_037472.1); S, C. nitidissima (NC_039645.1).
with their common origin in Guangdong and Hunan provinces of China, respectively. Adinandra C. nanyongensis in sect. C. chekiangoleosa C. oleifera showed a large divergence from the other species and formed an independent clade was a sister to the clade Camellia clade, whereas classified into four major clades, of which all the Camellia genomes of 14 species from genus Camellia 3.7. Figure 6. Nucleotide variability in each gene ((A); details in Table S6) and selection pressure of protein-coding genes ((B); details in Table S7) across the 19 Camellia cp genomes.

3.8. PCA and Phylogenetic Analysis of Camellia Species

We performed PCA to evaluate the relationships between the 12 cp genomes using SNPs data (Table S4). Findings suggested that there was a substantial genetic diversity among the genomes (Figure 7). The species of sect. Oleifera were mixed within the other two sections (Figure 7A). Most species with the same geographical origin were grouped (Figure 7B). C. oleifera var. 40, 53, MY6, C. meiocarpa, C. sasanaqua in sect. Oleifera; and C. chekiangoleosa var. Baihua in sect. Camellia were grouped with their geographical origin, i.e., Zhejiang, Jiangxi, and Fujian provinces of China, respectively. C. oleifera var. GR3, C. nanyongensis in sect. Oleifera, and C. semiserrata in sect. Camellia were clustered with the same geographical origin Guangxi Zhuang autonomous region of China. The last group involved C. vietnamensis, C. gauchowensis in sect. Oleifera, and C. gryjsii in sect. Paracamellia with their common origin in Guangdong and Hunan provinces of China, respectively (Table 1 and Figure 7B).

Twelve cp genomes were sequenced in this study, combined with the complete cp genomes of 14 species from genus Camellia and six species from other genera of the Theaceae family published previously, and a total of 32 taxa were used to construct the phylogenetic trees using both the ML and Bayesian analysis methods (Figure 8, and the details of species shown in Table S1). The phylogenetic relationships were evaluated within the genus Camellia and across the members of closely related genera based on the complete cp genome, coding region, and non-coding region data, respectively. The results showed that the six phylogenetic trees constructed were credible and most of their nodes were strongly supported by bootstrap or posterior probability values (≥70%) (Figure 8). The 32 taxa were classified into four major clades, of which all the Camellia species constituted a monophyly; Adinandra and Anneslea located at the basal position showed a close genetic relationship; Stewartia showed a large divergence from the other species and formed an independent clade, whereas Pyrenaria, Tutcheria, and Apterospemra clustered into another clade. This clade was a sister to the clade Camellia and showed a far genetic relationship to the clades formed by Stewartia and by Adinandra and Anneslea.
**Figure 7.** Principal component analysis plots of the 12 *Camellia* individuals. The meanings of each shape are indicated by the legend at the top.

**Figure 8.** Cont.
Figure 8. Phylogenetic trees of Camellia species and closely related genera in the family Theaceae. (A,B) Phylogenetic trees constructed using complete cp genome data with maximum likelihood and Bayesian analysis methods, respectively. (C,D) Phylogenetic trees constructed using the coding region data with maximum likelihood and Bayesian analysis methods, respectively. (E,F) Phylogenetic trees constructed using the noncoding region data with maximum likelihood and Bayesian analysis methods, respectively. Seven genera in Theaceae family are highlighted with vertical bars in different colors and eight sections in Camellia are marked with rectangles in different colors on the cladogram. The subclade I and II are marked with red dotted boxes. Numbers next to the branches correspond to maximum likelihood bootstrap support and Bayesian posterior probability values, respectively. The hyphen refers to the bootstrap or posterior probability values of <70%.

In the monophyly of Camellia, the phylogenetic relationships among 26 species and varieties were in general consistent across the six different phylogenetic trees, and the 26 taxa were divided into 3–6 distinct subclades (Figure 8). Subclade I was the largest one and involved 12–14 taxa, followed by subclade II with 7–9 taxa. The species and varieties of sect. Oleifera were predominant in subclade I and comprised a mix of three species (C. chekiangolosa var. Baihua, C. japonica, and C. azalea) from sect. Camellia, C. crapnelliana from sect. Furfuracea, and C. grijsii from sect. Paracamellia. In subclade II, four species from sect. Thea were clustered into a branch and formed a sister group to the branches formed by C. amplexicaulis, C. renshanchiangiae, and C. nanyongensis. In the monophyly of Camellia, the six species from sect. Camellia were divided into several subclades, while most other species from the same sections were grouped (Figure 8). C. semiserrata, C. nanyongensis, and C. semiserrata had a rather distant phylogenetic relationship to the other taxa sequenced in this study, which is consistent with the results of PCA.

4. Discussion

4.1. Materials

As the largest and most important genus in the family Theaceae, the Camellia species have a great deal of economic importance, mainly due to their use in making beverages, ornaments, and edible oils. Regardless, the phylogenetic resolution of species in this genus remains a challenge. It has been suggested that cp genomes can suitably provide information for Camellia phylogenetic analyses [6,11,13,49]. In previous studies, detected taxa mainly focused on sect. Thea, while we performed phylogenetic analyses on plants from the sect. Oleifera by sequencing their cp genomes. We made this choice as these plants are widely grown for the production of high-quality edible seed oil in China. Meanwhile, C. oleifera has the most widely distribution in sect. Oleifera and formed several ecotypes. In this study, we selected four C. oleifera clones with geographical origins of central, southeast, and southwest regions of China, respectively. The other species samples were collected from one tree in the core of their geographical origin.
4.2. Genome Organization

Structural rearrangements and gene loss-and-gain events often occur in angiosperms. The rpl23 gene translocates from the IR to the LSC regions in Poaceae plants [50]. Phalaenopsis and Oncidium lose most of their ndh genes [51,52]. It has often been reported that ycf1, accD, rpl23, rps16, and ycf2 are absent in some plants [53–55]. The Camellia cp genomes show structural characteristics and genetic properties that are typical of the angiosperm plastomes. The genomic organization of Camellia in this study was well-conserved, where the gene order was identical to that in the Diospyros, Artemisia, and the other Camellia species [6,11,20,49,56]. The genome lengths of these taxa were ~157 kb on average. The genomes were organized into quadripartite regions with no structural variation among the taxa. Each genome encoded the same number of genes (87 protein-coding, 37 tRNA, and 8 rRNA genes), which included a pseudo-ycf1 and a pseudo-rps19 (Table 3 and Figure 2).

We did not detect any gene loss-and-gain events in the 12 Camellia cp genomes.

IR expansion and contraction are commonly observed in plant cp genomes and are considered to be the primary reason for their length variations [47]. In Poaceae, for example, ndhH and ndhF, which are situated near the opposite ends of SSC, have been reported to migrate repeatedly into and out of the adjacent IRs [57]. IR expansions in monocot cp genomes lead to the duplication of the trnH-rps19 gene cluster near the IR-LSC junctions [58]. Meanwhile, the rps19 gene cannot be found in the cp genome of Gossypium raimondii because of IR contraction [59]. Similar to previous studies, the IR/SC boundary regions of 12 Camellia cp genomes studied here showed only slight differences (Figure 2) without remarkable IR expansions or contractions [6,11].

4.3. Sequence Divergence

Repeated sequences in plastome play important roles in genome rearrangement and are useful for phylogenetic studies [20,60,61]. Variations in the length and copy numbers of repeated sequences in the cp genome are widely used in plant population genetics, polymorphism investigations, and evolutionary research. This is especially important in well-conserved cp genomes of Camellia. In this study, we detected both the LSRs (≥30 bp) and SSRs. In contrast to the results of Yang et al. [11], the number of repeats in the 12 Camellia cp genomes matched well with that in other angiosperm [20,56,62]. The number and distribution of repeats were rather conserved among the Camellia cp genomes. The number of SSRs was more than LSRs. The majority of SSRs were located within exons (Table S2), while SSRs were mainly located in IGS, followed by exons (Table 5). Most SSRs were mononucleotide repeats, where the A/T content was far greater than the G/C content (Figure 4), which is consistent with previous reports [6,62,63]. These repetitive sequences are important resources for studying differences in cp genes.

Previous research had suggested that SNPs and InDels play another important role in inducing variation [64,65]. We also detected SNPs and InDels in the whole cp genomes of the 12 Camellia taxa. SNPs were the most abundant variations, where 489 SNPs were identified in the 12 Camellia taxa (Table S4). Generally, the number of InDels decreases rapidly with an increase in their length [66,67]. In line with this, we observed that single-nucleotide InDels (1 bp) were the most abundant InDel type. However, 5–6 bp InDels were more common than 2–4 bp InDels (Figure S1). Such 5–6 bp InDels were likely caused by adjacent 5–6 bp motif duplication or loss, making them more abundant than the shorter 2–4 bp InDels [6,59]. Some InDel mutations lead to frame-shift or a change in the codon sequence length in the protein-coding genes of Camellia cp genomes. A 5 bp InDel mutation that caused a frame-shift was identified in rps16 in C. semiserrata. Two hexa-nucleotide deletion mutations made the rpoC2 protein shorter by four amino acids in C. semiserrata. One hexa-nucleotide insertion mutation made the rpoC2 protein longer by two amino acids in C. chekiangoleosa var. Baihua as compared to rpoC2 protein in other Camellia taxa (Table S5).

S/I values of taxa pairs ranged from 1.0 to 2.60 (Table 6), which is significantly lower than that for other species pairs from the Camellia genus [6]. This is consistent with the
fact that the S/I values increase with divergence times between genomes [68]. In the 12 cp genomes studied here, C. meiocarpa was inferred to be the closest to C. sasanqua (S/I = 1.0), while C. nanyongensis could have diverged earlier, and thus, had higher S/I values (Table 6). C. oleifera var. 40, 53, and MY6 shared an identical cp genome, which suggested that cp genome sequences of C. oleifera ecotypes originated from the central and southeast of China were highly conserved and were different from that of southwest ecotypes.

4.4. Genome Divergent Hotspot Regions

Molecular evolutionary rates are often associated with life history in flowering plants [69]. The Camellia species possesses a low rate of molecular evolution because of the rather long generation times [6]. It is critical to be able to identify rapidly evolving cp genomic regions through comparative genomic analysis. Regions with relatively high sequence divergence can be developed into plant DNA barcodes. The noncoding trnH-psbA region contains LSRs that are highly variable in size and the sequence has been used as a barcode in the Artemisia plant [20,70]. trnH-psbA region in Camellia cp genomes contains multiple SNPs and had sufficient potential to be used as barcode markers (Table S4). We found that atpH-atpI regions were highly variable regions in many plants, such as Musa, wild rice, wax gourd, Iris, etc., and can be applied to DNA barcode encoding and phylogenetic analysis [49,71–74]. In this study, we also found atpH-atpl region was highly diversity in the Camellia cp genomes, with multiple InDels and SNPs, which was consistent with the results of Huang et al. [6]. This region presents sufficient potential to be used as a barcode marker (Table S5). Usually, most protein-coding genes in cp genomes have low sequence divergence in a limited number of species only [20,75]. Our research showed several potential barcodes in protein-coding genes, for example, the rpoC2 gene harbored six various SSRs and three hexa-nucleotide InDels. Meanwhile, psbK, psbT, psaJ, rpl33, rps8, rpl32, ycf1, rpl16, matK, and psb1 were identified as divergent hotspots based on the Pi values (Figure 6A and Table S6). We speculate that positive selection was operating in the rpoC2 and ycf1 with the Ka/Ks values ≥ 1 in multiple Camellia taxa. Huang et al. indicated rpoC2 and ycf1 to harbor multiple SSRs in the sets of Camellia taxa and asserted that these SSRs could undoubtedly be subjected to assays for detecting polymorphisms at the population level and to compare more distant phylogenetic relationships at the genus level or above [6]. Previously, rpoC2 gene sequence had been used to analyze the phylogenetic position of the genus Astragalus [76]. The ycf1 coding sequence has also been suggested to be a plastid barcode for land plants and has been frequently applied in plant phylogeny and DNA barcoding studies [76–82]. ycf1 plays an important role in flowering plants and is essential for plant viability [83]. According to previous studies, the high sequence divergence of ycf1 in a range of land plants is likely a result of environmental adaptation during evolution [84–86], and thus, this gene may be used for the evolution analysis of cp. Li et al. also found the potential mutational hotspots genes rbcL, matK, and ycf1 could be suitable barcodes for plant classification in Camellia [49].

4.5. Inference of Phylogenetic Relationships

Understanding phylogenetic relationships in the Camellia genus poses a grand challenge because of the high number of species, frequent hybridization, and polyploidization. Plastid phylogenomics offers a novel and deep insight into phylogenetic relationships and the diversification history [15,62]. In this study, we performed PCA on 12 cp genomes based on the SNPs data. Results showed that most taxa were grouped based on geographical origin, instead of section and species (Figure 7B). This may have been the result of interspecies hybridization based on the absence of recombination, as well as the maternal transmission characteristics of the cp genome.

We also constructed the phylogenetic trees of 12 genomes sequenced here and 20 complete cp genomes acquired from GenBank (Table S1). Data from complete cp genomes, coding and noncoding regions were used for the purpose, respectively. The results were consistent with the traditional classification system of Chinese flora [5] at the genus level,
while the intra-genus phylogenetic relationships of Camellia partially agreed with the traditional classification system. According to Sealy’s taxonomic treatment, most of the sect. Oleifera taxa had a closer phylogenetic relationship to each other, while C. nanyongensis had a closer relationship with C. semiserrata. Six species of sect. Camellia were divided into several subclades and mixed with the sect. Oleifera and Thea, respectively. The phenotypic characteristics of C. chekiangoleosa var. Baihua are similar to those of C. chekiangoleosa, except for the white flowers. However, it was closely related to C. oleifera var. 40, 53, and MY6 according to the cp genome data. Whether C. chekiangoleosa var. Baihua is a hybrid of C. chekiangoleosa and C. oleifera still needs further investigation (Figure 8).

Taxonomic studies on the Camellia genus are very controversial, especially on the definition of species and sections. The number of Camellia species and sections differ according to the different classification systems [1,5]. With the emergence of more complete cp genome sequences, the controversial taxonomy of Camellia can be addressed using cp genome analysis. Although this method is also insufficient to fully resolve all taxonomic controversies, our results suggest that it has the potential to provide a solution to many taxonomical disputes. With the rapid development of DNA sequencing technologies and a dramatic fall in the sequencing costs, complete cp genome analysis should be noted as a powerful tool to carry out taxonomic and phylogenetic studies in Camellia. The barcodes mined from the hotspot regions of cp genomes also have a significant potential to improve our ability to identify species or distinguish between them, and thus, can contribute to the phylogenetic relationships study.

In recent years, many studies have used chloroplast whole-genome sequence for phylogenetic analyses and taxonomic classification [6,11,16]. This had been proven to be particularly powerful in revealing phylogenetic relationships of plants with large nuclear genomes [21,22]. Finding more regions with a higher evolution rate in the chloroplast genome sequence could be helpful to distinguish closely related species or genus, which is of great significance to the study of DNA barcodes [87].

5. Conclusions

The genome size, structure, and gene number and order were shown to be well-conserved among 12 cp genomes in this study. Meanwhile, there were slight differences in SSC/IR boundaries and in ycf1 gene with different expansion lengths in different species. The whole plastome encoded 132 genes, including 87 protein-coding, 37 tRNA, and 8 rRNA genes. The cp genomes in this study possessed abundant variations, including LSRs, SSRs, SNPs, and InDels. A total of nine intergenic sequence divergent hotspots and 14 protein-coding genes with high sequence polymorphism were identified and had sufficient potential to be used as barcode markers in phylogenetic and taxonomic studies. PCA results showed that cp genomes had closer relationships with the same geographical origin rather than the same section. Phylogenetic trees suggested that C. nanyongensis had a rather distant phylogenetic relationship with the other taxa of sect. Oleifera. The complete cp genomes in this study will facilitate further research on Camellia and enhance our understanding of Theaceae family plastome evolution. It also provides a reference for the phylogenetic analysis of other plants.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biom12101474/s1, Table S1: A list of taxa of the chloroplast genome sequences used in this study; Table S2: Details of the LSRs in the 12 sequenced Camellia cp genomes; Table S3: Details of the SSRs in the 12 sequenced Camellia cp genomes; Table S4: Details of the SNPs in the 12 sequenced Camellia cp genomes; Table S5: Details of the InDels in the 12 sequenced Camellia cp genomes; Table S6: Details of Pi values for each gene across 19 Camellia cp genomes; Table S7: Ka Ks values for protein-coding genes using C.chekiangoleosa var. Baihua as a reference; Figure S1: The distribution of InDel types in the 12 sequenced Camellia cp genomes (jpeg 767 kb).
Author Contributions: X.Y. conceived the research objectives. P.L., H.Y. and K.W. processed the genome sequence data. H.G. and L.L. were involved in the preparation of DNA and RNA samples. P.L. and H.Y. drafted the manuscript. All authors contributed and approved the paper. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by The National Key R&D Program of China (2018YFD1000603-2) and the National Science Foundation of China (31770719).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The complete chloroplast genomes generated during the cuttent study were submitted to the NCBI database and are available with Genbank accession numbers OL689014~OL689025.

Acknowledgments: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

cp: chloroplast; Pi: nucleotide diversity; IR: inverted repeat region; LSC: large single copy region; SSC: small single copy region; tRNA: transfer RNA; rRNA: ribosomal RNA; LSRs: long sequence repeats; IGS: intergenic sequences; Ka: nonsynonymous mutation rate; S/I: the ratio of SNPs to InDels; Ks: synonymous mutation rate; PCA: principal component analysis; ML: maximum likelihood; GTR model: general time reversible model; MCMC: Markov Chain Monte Carlo.

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