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Comparative chloroplast genome analysis of *Camellia oleifera* and *C. meiocarpa*: phylogenetic relationships, sequence variation and polymorphism markers

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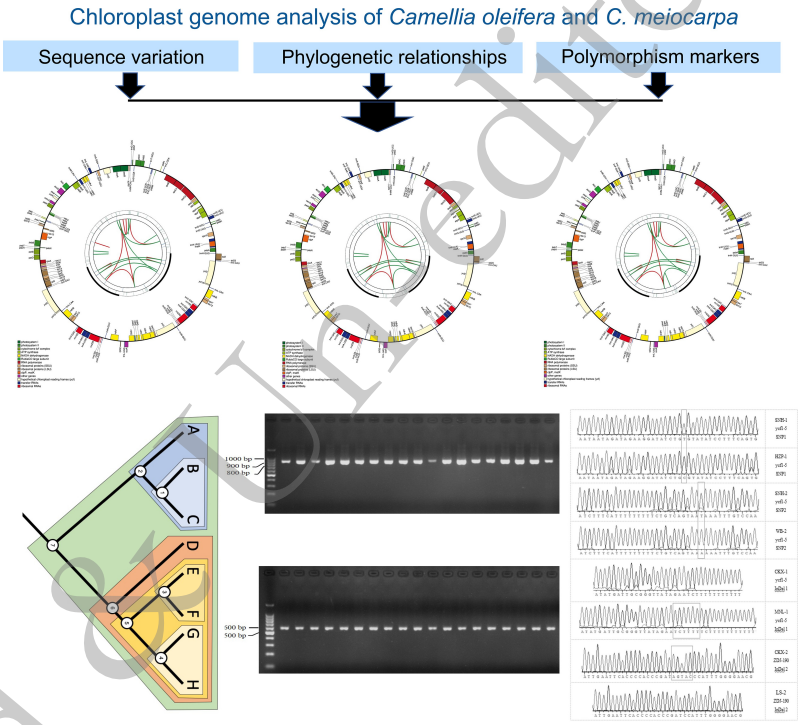
In Brief

Comparative chloroplast genome analysis of Camellia oleifera and C. meiocarpa supported the opinion proposed by Xiansu Hu that C. meiocarpa is an independent species. The development of 17 primers could be used for the resource assessment of Camellia, facilitating molecular phylogenetic analysis, innovation, utilization of tea-oil Camellia germplasm resources, and their production practice.

Graphical abstract



Highlights

- Compared to *C. oleifera* (HZP), there were differences ranging between 460 bp (CKX) and 490 bp (XG) in *C. meiocarpa*.
- *C. meiocarpa* was considered as separated species.
- The development of 17 primers could be used for the resource assessment of *Camellia*.

Citation: Liang H, Qi H, Wang Y, Sun X, Wang C, et al. 2024. Comparative chloroplast genome analysis of *Camellia oleifera* and *C. meiocarpa*: phylogenetic relationships, sequence variation and polymorphism markers. *Tropical Plants* https://doi.org/10.48130/tp-0024-0022

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Comparative chloroplast genome analysis of *Camellia oleifera* and *C. meiocarpa*: phylogenetic relationships, sequence variation and polymorphism markers

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Abstract

Tea-oil *Camellia*, a prominently woody oil crop, serves as a crucial source of edible oil, protein feed, and industrial raw materials. Notably, *C. Oleifera* and *C. meiocarpa* yield higher oil production and larger cultivation areas than other Tea-oil *Camellia* species. However, the taxonomy and phylogenetic relationship between these species remain elusive, complicating their commercial application. Here, we sequenced and analyzed the complete chloroplast genomes of these two species, compared them with related *Camellia* species, and developed chloroplast DNA markers to distinguish between them. The chloroplast genome of *C. Oleifera* was 157,009 bp (HZP) and *C. meiocarpa* was 156,549 bp (CKX) and 156,512 bp (XG) in length. Comparative analysis indicated that distinct differences in the chloroplast genome between HZP and CKX (or XG) than between CKX and XG. The repetitive sequences and interspecific variations among them showed that the differences in number and distribution of CKX and XG were smaller than those in HZP. Phylogenetic analysis showed that *C. meiocarpa* was not closely related to *C. oleifera*. A total of 56 pairs of primers were developed to test the polymorphism among them. After PCR and sequencing verification, variations were detected in the target sequences of 17 primers. The data derived from the chloroplast genomes and the newly developed markers are invaluable for understanding the phylogenetic relationships, and assessing the genetic diversity of tea-oil *Camellia* germplasm resources.

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Introduction

Tea-oil *Camellia* refers to a group of plants within *Camellia* genus of Theaceae family, known for their high oil content in their fruits and their cultivation value[1]. Tea-oil is rich in unsaturated fatty acids, comprising up to around 90%, which is higher than olive oil[2]. This makes it a premium edible oil with significant health and medicinal benefits. Besides that, it is collectively referred to as one of the world's four major woody oil crops, along with *Elaeis guineensis*, *Olea europaea* and *Cocos nucifera*[3]. In China, approximately 30 species within the *Camellia* genus are all referred to as tea-oil *Camellia*[4]. Due to its strong adaptability, long growth cycle, tolerance to infertile soils, suitability for cultivation in mountainous and hilly areas, tea-oil *Camellia* is a key woody oil crop actively promoted in China[5]. Currently, the cultivation area of tea-oil *Camellia* in China is approximately 5.3 million hectares. *C. oleifera*, followed by *C. meiocarpa*, represents the majority of this cultivation, primarily in the southern provinces such as Hunan, Jiangxi, Guangxi, Guangdong, Zhejiang and Fujian. In addition, Wang et al. found that *C. oleifera* and *C. meiocarpa* are distributed in the tropical regions of China (within Wuzhishan in Hainan)[6].

Due to the complexity of nuclear genomes, diverse ploidy levels, rich phenotypic variations and the presence of interspecific hybridization, the phylogeny within Tea-oil *Camellia* present significant challenges. In order to clarify the relationships among them, scholars have employed morphological and molecular classification methods to conduct phylogenetic analysis of tea-oil *Camellia* species[7–11]. However, the phylogenetic relationships among tea-oil *Camellia* remain controversial, for example, the relationships between *C. meiocarpa* and *C. oleifera*. Initially identified by Mr. Xiansu Hu, *C. meiocarpa* was considered as separated species[12]. In Taxonomy of Chang system, it was considered as a variant of *C. oleifera*, and named *C. oleifera* var. *monosperma*[13]. But in Taxonomy of Ming system[14] and Flora of China[15], *C. meiocarpa* was merely a cultivated species of *C. oleifera*, not a distinct taxonomic species. It shares many fundamental characteristics with *C. oleifer,* such as branches, leaves, flowers, and fruits, with the primary distinction being the smaller size of these features in *C.*

meiocarpa. Moreover, Yao and Huang used microsatellite molecular markers to analyze the difference for *C. oleifera* and *C. meiocarpa* and indicated that there was low genetic differentiation between these two species, suggesting that frequent interspecific hybridization and gene introgression blur their low genetic distinctions, supporting the notion that *C. meiocarpa* is a variant of *C. oleifera*[16]. However, most producers and researchers still consider *C. meiocarpa* has a significant difference in morphology and oil quality, compared to *C. oleifera*, affirming its status as a distinct species. These controversies have created inconveniences for the breeding and production of tea-oil Camellia. Moreover, the Camellia oil from *C. meiocarpa* is nutritionally superior to that from *C. oleifera*, and shoddy goods are often overdue[17]. The strategies of developing DNA markers can differentiate them effectively, based on comparative genomes[18].

The chloroplast genome is notably conserved and its uniparental (maternal) inheritance has been extensively utilized in classification and phylogenetic studies[19–22]. Its lack of recombination and maternal transmission render it an invaluable tool for tracing the phylogenetic relationships among the complexity of nuclear genomes[23–25]. Unlike limited genomic segments, the chloroplast genome contains a vast repository of genetic data, provideing abundant variation loci information for the study of phylogeny and taxonomy[26]. Currently, despite their significance, there have been no reports on the chloroplast genome of *C. meiocarpa*, nor has there been a comparative chloroplast genomic analysis conducted between *C. oleifera* and *C. meiocarpa*[27–30].

In this study, we reported the complete chloroplast genome sequences of *C. oleifera* and *C. meiocarpa*, and compared them with other tea-oil *Camelli*a chloroplast genomes. Our objectives were to: 1) reconstruct the phylogenetic relationship between *C. meiocarpa* and *C. oleifera*, and 2) develop molecular markers to test the polymorphism within these species. The results are expected to provide a theoretical foundation for variety identification, breeding, and resource utilization.

Materials and methods

Plant materials, DNA extraction and genome sequencing

Fresh leaves of *C. oleifera* (HZP) were collected from Tianyang in Guangxi province (E 107.073836, N 24.007963, 554m). In *C. meiocarpa*, XG was collected from Sanjiang in Guangxi province (E 109.422086, N25.710639,139 m,) and CKX was from germplasm garden of Guangxi Forestry Research Institute. Quickly frozen in liquid nitrogen, and stored at ultra-low-temperature refrigerator at −80 °C until use. Total DNA extraction was carried out using the modified CTAB method[31]. Following the protocol provided by Illumina (San Diego, CA, USA), double-stranded (PE) libraries were constructed using sheared low-molecular-weight DNA fragments. The complete chloroplast genomes of the aforementioned materials were sequenced on the Illumina NovaSeq platform using the PE150 sequencing strategy and a 350 bp insert size.

Assembly and annotation

The raw reads were filtered for adapter sequences and low-quality reads using the NGSQC Toolkit software (v2.3.3) to obtain high-quality reads[32]. The chloroplast genome was assembled using SPAdes software v3.14[33], and annotation was performed using cpGAVAS2 with manual correction[34]. Subsequently, the sequencing reads were mapped to the reference genome *C. luteoflora* to validate the assembly results.

Comparative analysis of the chloroplast genomes

The eight tea-oil *Camellia* species from GeneBank (Supplemental Table S1) were used to do the comparative analysis. mVISTA program (https://genome.lbl.gov/vista/mvista/submit.shtml) was used to visualize chloroplast genome in Shuffle-LAGAN mode with *C. luteoflora* as a reference. Moreover, we compared events of IR expansion and contraction among these accessions, analyzing the junction regions between the IR, SSC, and LSC using the online tool CPjsdraw (https://github.com/xul962464/CPJSdraw).

To identify the mutational hotspot regions for HZP, XG and CKX, we calculated nucleotide diversity (Pi) by DnaSP v5[35]. MAFFT was employed for alignment of the chloroplast genomes to identify the mutations[36].

Identifcation of sequence repeats

In the chloroplast genomes of HZP, XG and CKX, the REPuter[37] software was used to assess and pinpoint forward (F), reverse (R), complemented (C), and palindromic (P) repeats. The repeat identification utilized the following settings: (1) a Hamming distance equal to 3; (2) a minimal repeat size set to 30 bp; (3) a sequence identity of 90% or greater. Simple Sequence Repeats (SSR) loci were identified using MISA[38], with the minimal repeat number set to 10, 6, 5, 5, 5, 5 for mononucleotide (mono-), dinucleotide (di-), trinucleotide (tri-), tetranucleotide (tetra-), pentanucleotide (penta-), and hexanucleotide (hexa-) nucleotide sequences, respectively.

Phylogenetic analysis

Phylogenetic analysis was carried out by utilizing the complete chloroplast genome sequences of HZP, XG, CKX, and other 26 *Camellia* species with one *Polyspora* species serving as outgroups (Supplemental Table S2). The nucleotide sequences were aligned using MAFFT version 7 software[39]. ModelFinder[40] was employed to determine the best-fit model with default settings, and the maximum likelihood (ML) analysis was conducted using RAxML[41] with 1000 bootstrap replications. The Maximum Parsimony (MP) trees were inferred in MEGA7 with default parameters[42]. MrBayes v3.2.7 was used to infer the BI (Bayesian Inference) tree with Markov Chain Monte Carlo (MCMC) method[43]. 1 million generations and sample every 100 generations. The initial 25% of the phylogenetic tree was removed (burn-in), and the majority-rule consensus tree was finally obtained.

Development and validation of molecular markers

Based on SNPs and Indels in the chloroplast genome, polymorphic markers were designed to identify the difference of *C. oleifera* and *C. meiocarpa*. The PCR reaction had a total volume of 25 μL, consisting of 12.5 μL 2× PCR Mix, 1 μL forward and reverse primers (10 pM each), 1 μL genomic DNA, and 9.5 μL ddH2O. The thermal cycling included an initial denaturation at 94 °C for 4 minutes, followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing temperature reference by 50–58 °C for 30 seconds, extension at 72 °C for 30 seconds, and a final extension at 72 °C for 7 minutes. The PCR products were sequenced for further verification. Based on the principle of

improving detection efficiency and reducing sequencing cost, the size of sequences less than 800 bp were used for the Single-read sequencing, and paired-end sequencing for the sequences which were more then 800 bp in size.

Results

Comparison of the chloroplast genome structures and features between C. meiocarpa and C. oleifera

The results (Table 1; Figure 1) showed that the chloroplast genomes of *C. meiocarpa* (XG), *C. meiocarpa* (CKX) and *C. oleifera* (HZP) had a typical circular tetramerous structure like other related plants[44,45]. The genome sizes were 156,512 bp for XG and 156,549 bp for CKX, differing by only 37 bp between them. Compared to *C. oleifera* (157,009 bp, HZP), there were differences ranging between 460 bp and 490 bp. The three chloroplast genomes are divided into four distinctive regions: the LSC (86,263 bp in CKX, 86,224 bp in XG and 86,637 bp in HZP), SSC (18,400 bp in CKX, 18,402 bp in XG and 86,637 bp in HZP) and two IRs (25,943 bp in CKX, 25,943 bp in XG and 26,041 bp in HZP). The overall GC content was nearly identical across the genomes: 37.32% in CKX, 37.33% in XG and 37.29% in HZP. Furthermore, the GC contents were unevenly distributed across regions of the chloroplast genome, with 35.33% in CKX, 35.34% in XG and 35.30% in HZP for the LSC; 30.58% in CKX, 30.57% in XG and 30.52% in HZP for the SSC; and 43.03% in CKX, 43.03% in XG and 42.99% in HZP for the IR regions, respectively (Table 2). These values indicated a conservative nature within the genomes of tea-oil *Camellia*. Additionally, each of the three genomes encoded the same set of 133 functional genes,

Table 1. Features of *C. meiocarpa* and *C. oleifera* chloroplast genomes.

Genome feature	CKX	XG	HZP
Genome size(bp)	156,549	156,512	157,009
LSC length(bp)	86,263	86,224	86,637
SSC length(bp)	18,400	18,402	18,290
IR length(bp)	25,943	25,943	26,041
Number of genes	133	133	133
Number of protien-coding genes	87	87	87
Number of pseudo	2	2	2
Number of tRNA genes	37	37	37
Number of rRNA genes	8	8	8
GC content in LSC (%)	35.33	35.34	35.30
GC content in SSC (%)	30.58	30.57	30.52
GC content in IR (%)	43.03	43.03	42.99
Total GC content (%)	37.32	37.33	37.29
GenBank Number	MZ151356	MZ151355	MZ151357

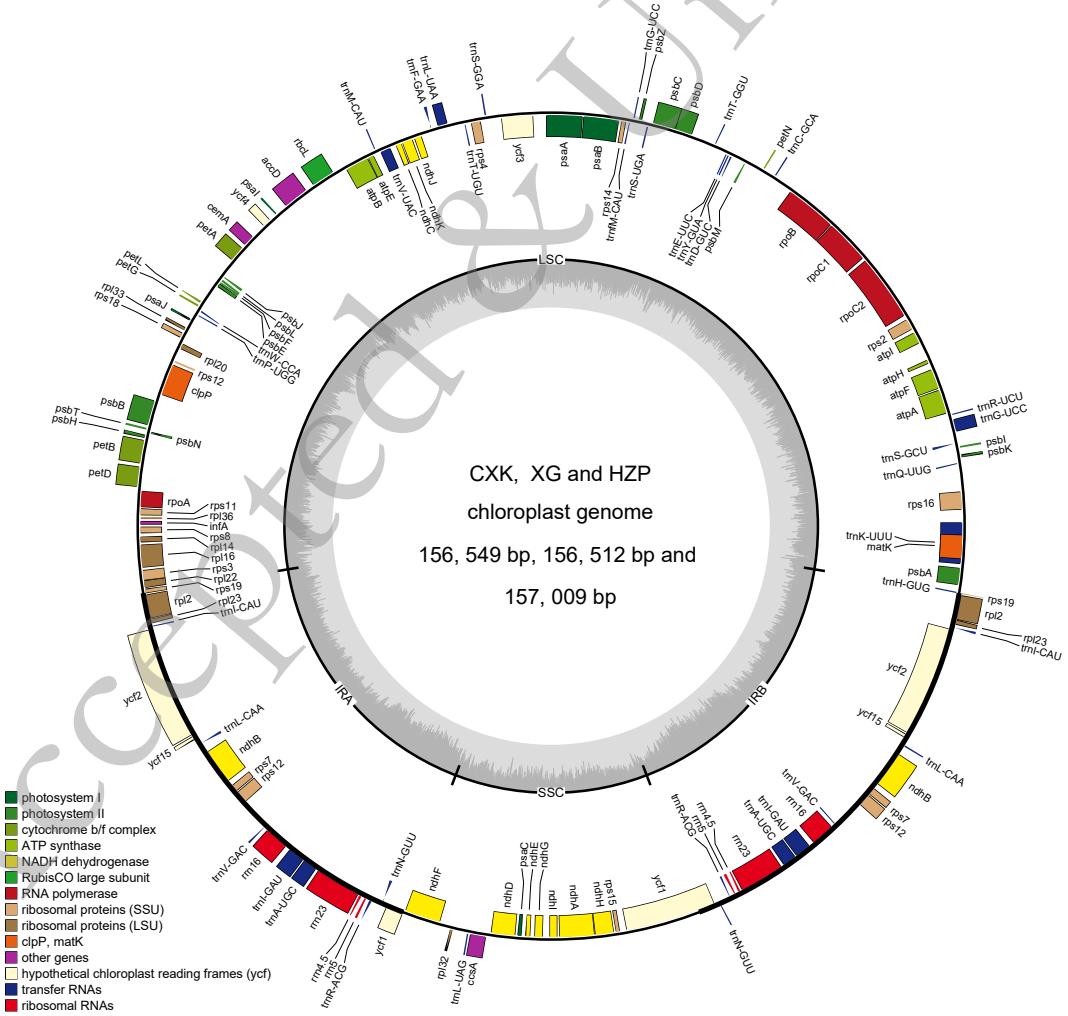

Fig. 1 Chloroplast genome map of *C. meiocarpa* and *C. oleifera*.

Table 2. Features of repetitive sequences in *C. meiocarpa* and *C. oleifera*.

	ZHP	XG	CKX
Total number	49	49	49
Forward	15	16	17
Palindrome	22	20	20
Reverse	9	9	9
Complementary	3	4	3
Gene N	*trnS-GCU*, *trnG-GCC*, *trnS-UGA*, *trnfM-CAU*, *ndhC*, *trnV-UACa*, *petD*, *ycf2*, *ndhA*, *ycf1*,*rpoC2b*, *rpoBc*, *trnA-UGCd*		
SSR Loci(N)	52	48	49
P1 Loci(N)	51	47	49
Pc Loci(N)	1	1	0
LSC	40	35	36
IRA	2	2	2
SSC	8	9	9
IRB	2	2	2

a: special in ZHP; b,c: special in XG; d: special in CKX

including 87 protein-coding genes, 8 rRNA genes and 37 tRNA genes. In Table S3, a total of 18 genes were duplicated, featuring four rRNA genes (*rrn16*, *rrn23*, *rrn4.5* and *rrn5*), two large subunit of ribosomal proteins genes (*rpl2* and *rpl23*), seven tRNA genes *(trnA-UGC*, *trnI-CAU*, *trnI-GAU*, *trnL-CAA*, *trnN-GUU*, *trnR-ACG* and *trnV-GAC*), one subunit of NADH dehydrogenes subunit gene(*ndhB*) and three other genes (*ycf2*, *ycf15* and *ycf1*).

The distribution of repetitive sequences in C. meiocarpa and C. oleifera

The REPuter software results showed that 49 scattered repetitive sequences were detected in HZP, XG and CKX (Fig. 2a; Supplemental Table S4). In Fig. 2a, the repetitive sequences ranging from 15−19 bp were most prevalent, followed by those ranging from 20−24 bp. These two categories respectively constituted 77.55% for CKX and XG, and 75.51% for HZP. The LSC region had the highest distribution of long repetitive sequences, accounting for 59.18% in CKX, XG, and HZP. However, no repetitive sequences between 25−29 bp and 35−39 bp were observed, and the 30-34 bp sequences appeared exclusively in the LSC region. The IRA region followed in sequence distribution. Besides, the 25−29 bp and 35-39 bp repetitive sequences only be found in the IR region. We also identified four repeat types: Forward, Palindrome, Reverse, and Complementary in CKX, XG, and HZP (Table 2 and Supplemental Table S4). Among them, the palindrome type was the most common, comprising 40.82% in both CKX and XG, and 44.90% in HZP, while complementary repeats were the least frequent. Some of repetitive sequences were located in different genes, including *trnS-GCU*, *trnG-GCC*, *trnS-UGA*, *trnfM-CAU*, *ndhC*, *trnV-UAC*, *petD*, *ycf2*, *ndhA*, *ycf1*, *rpoC2*, *rpoB*, and *trnA-UGC*.

We also identified the number and distribution of SSRs in Fig 2b and Supplemental Table S5. In Fig 2c, these SSRs, categorized into single-base repeats (repeating 10 or more times), double-base repeats (6 or more times), and 3-6 base repeats (5 or more times), were found as follows: 49 SSRs each in CKX and XG, and 53 in HZP. Predominantly, these were of the P1 type, with a single C1 type identified in both HZP and XG. The majority were located within the LSC region, representing 73.47% in CKX and XG, and 75.47% in HZP. The SSC region had fewer, with only 4 SSRs repeated in the IR region. All SSR loci were single-base and formed by A/T. In CK and XG, A and T

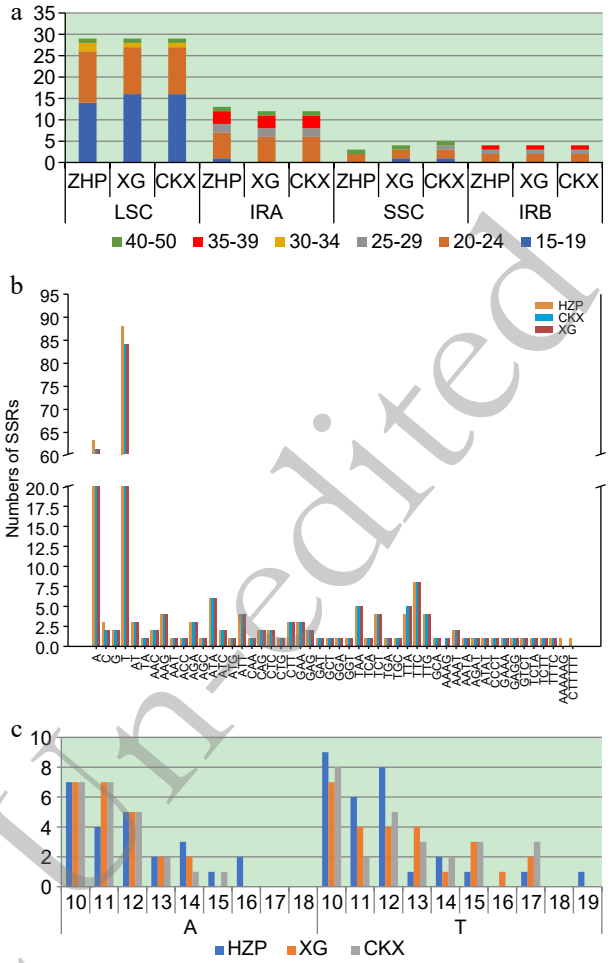

Fig. 2 Comparison of repetitive sequences in *C. meiocarpa* and *C. oleifera*. (a) Number of scattered repetitive by length in different regions. (b) The number and distribution of Simple Sequence Repeats (SSR)s. (c) Frequency of SSRs in A and T.

accounted for 23 (46.94%) and 26 (53.06%), respectively. But in HZP, A and T accounted for 24 (45.28%) and 29 (54.72%), respectively. Notably, 10-12 single-base repeats were most predominant, with 34 in CKX and XG (69.39%), and were less than 39 in HZP (73.58%). This data underscores distinct differences in the repetitive sequence patterns between HZP and the CKX/XG genomes.

IR region expansion and contraction

While chloroplast genomes exhibit high conservation in terms of genomic structure and size, the variations in their lengths are commonly attributed to changes in the position of the IR/SC junctions, caused by the expansion and contraction of these boundary regions[46,47]. The junction regions of the 11 tea-oil *Camellia* chloroplast genomes were examined for comparison. In Fig 3, across these species, the arrangement of genes at each junction point within the IR regions remained consistent. Notably, the gene *rps19* spanned the LSC/IRb region consistently with lengths of 233 bp in the LSC and 46 bp in the IRb across all species. Conversely, the gene *rpl2* located in the IRb region showed contractions, with base number variations ranging from 100 to 106 bp across the species. Similarly, the gene *ycf1* straddled the SSC/IRa boundary with varying lengths,

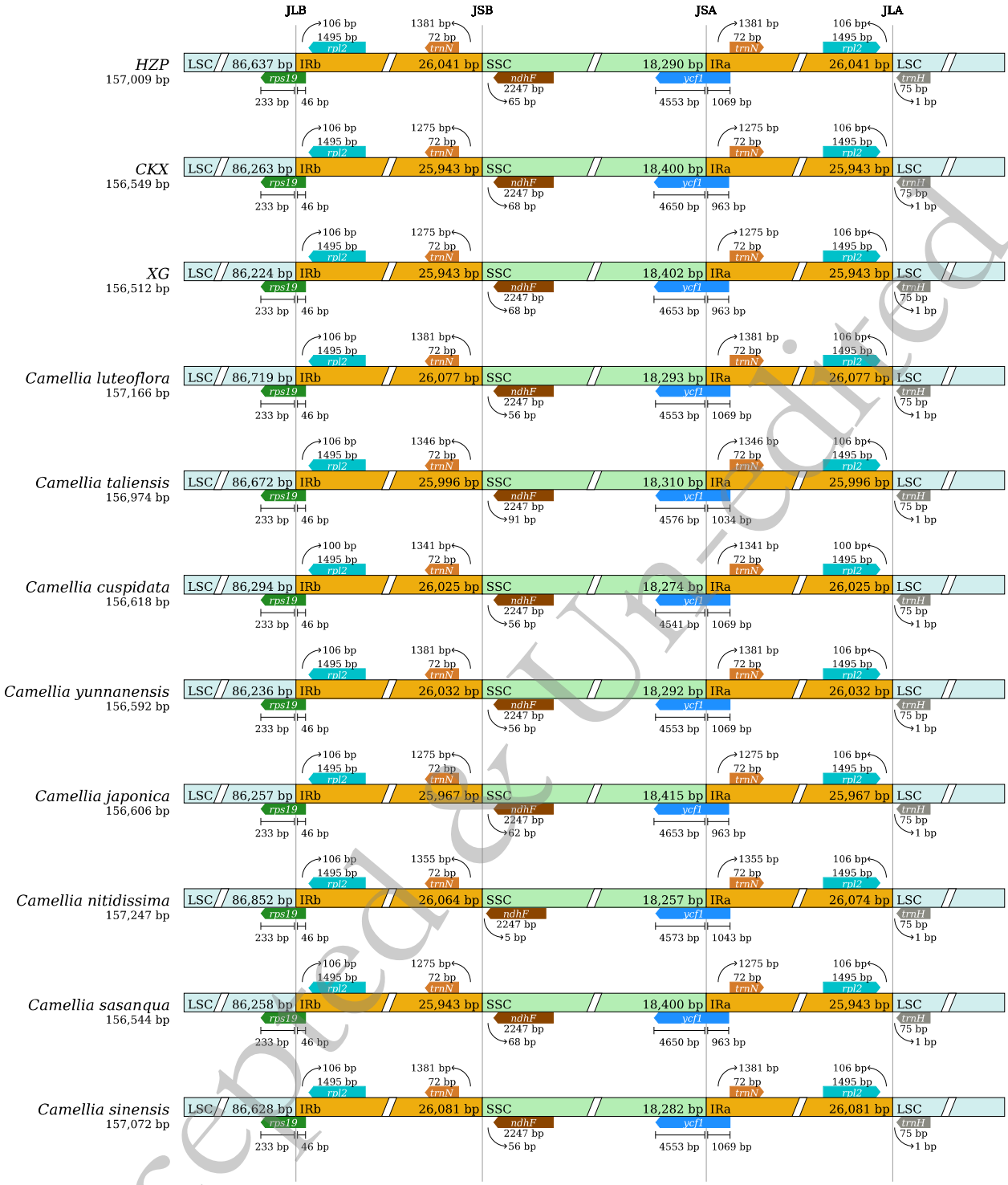

Fig. 3 Comparison of the Large single copy (LSC), Inverted repeat (IR), Small single copy (SSC) junction positions among 11 tea-oil *Camellia* species.

4541 to 4653 bp in the SSC and 963 to 1069 bp in the IRa. The gene *ndhF*, exhibited base contractions varying from 5 to 68 bp. Additionally, the gene *trnN* in the IRa region was consistently positioned 1275-1381bp from the SSC/IRa boundary. In particular, the distances of *trnH* to the SSC/IR junction were 1275 bp for CKX and XG, and 1381 bp for HZP, indicating that *trnH* is located at the edge of the LSC region, merely 1 bp from the SSC/IRa boundary. This analysis highlights that the variations in the expansion and contraction of the IR regions are more pronounced between HZP and CKX (or XG) than between CKX and XG, illustrating distinct genomic adaptations among these tea-oil Camellia species.

Comparative analysis of genome structure

To explore the interspecific variation in chloroplast genome sequences, the identity percentage was graphically repre-

sented for the 11 tea-oil Camellia accessions utilizing the mVISTA program with *C. luteoflora* as the reference. In Fig. 4, the divergence in the SSC region compared to the LSC and IR regions, with non-coding regions exhibiting greater divergence than coding regions. The overall alignment revealed a high degree of sequence similarity among the species. Compared to HZP, the variation of chloroplast genome between CKX and XG was closer. To further understand the variation between *C. meiocarpa* (CKX and XG) and *C. oleifera* (HZP), we calculated nucleotide diversity (Pi) values within them. The results showed (Fig. 5a; Supplemental Table S6) that Pi values were low (ranging from 0 to 0.011, average value was 0.0006). Specifically, the SSC region indicated the highest level of variation (average Pi value of 0.00142), followed by LSC (average Pi value of 0.00060), and the lowest was in IRB (average Pi value of 0.00013). The *ycf1* had the most mutation sites (72, with average Pi value of 0.00294), and *psbM* had the highest level of average Pi. Interestingly, 50 genes exhibited zero nucleotide diversity (Fig. 5b; Supplemental Table S6). Furthermore, we also detected 210 variants, including 72 Indel sites and 138 SNP sites among the three chloroplast genomes (Table 3; Supplemental Table S7). Most Indels were 1 bp in length, constituting 38.89% of all Indel sites, followed by 2 bp lengths at 16.67%, and a single occurrence of a 9 bp Indel. Among the SNPs, transitions from G to A were most frequent (26.09%), followed by C to T changes (23.19%), with C to G being the least common (3.62%). The majority of these variations occurred in intergenic regions (118 sites), with significant occurrences also noted in in 21 genes, such as *accD*, *atpB*, *atpF*, *ccsA*, *clpP*, *infA*, *matK*, *ndhA* and *ycf1* et al. In statistics, a total of 140 Indel and SNP sites were located in LSC, 49 sites in SSC, 13 sites in IRA and 8 sites in IRB. The gene ycf1 had the highest number of variants (22), while the intergenic region between *trnE-UUC* and *trnT-GGU*, along with *petN-psbM*, contained the most variant sites. These findings underscore the genomic organizational differences and the variability between *C. oleifera* (HZP) and *C. meiocarpa* (CKX or XG), highlighting distinct evolutionary trajectories within these species.

Phylogenetic analysis

In this study, we combined the chloroplast genome information from 27 published species of *Camellia* genus within the Theaceae family to reconstruct a phylogenetic tree, thereby

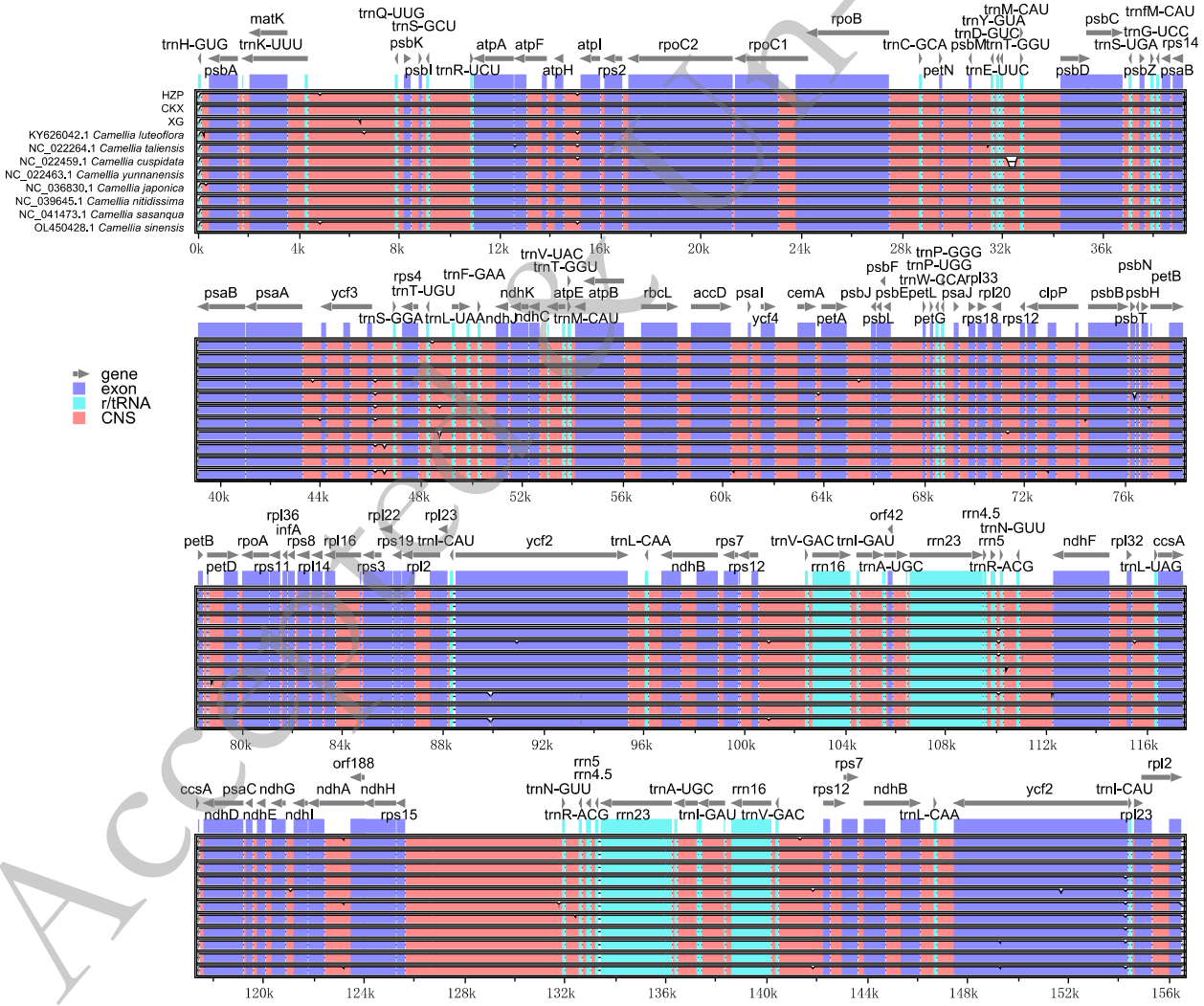

Fig. 4 Identity plots comparing the chloroplast genomes of 11 *Camellia* accessions. The vertical scale indicates the percentage of identity, ranging from 50 to 100%. The horizontal axis indicates the coordinates within the chloroplast genome. Genome regions are color coded, including protein-coding, rRNA, tRNA, intron, and conserved non-coding sequences (CNS).

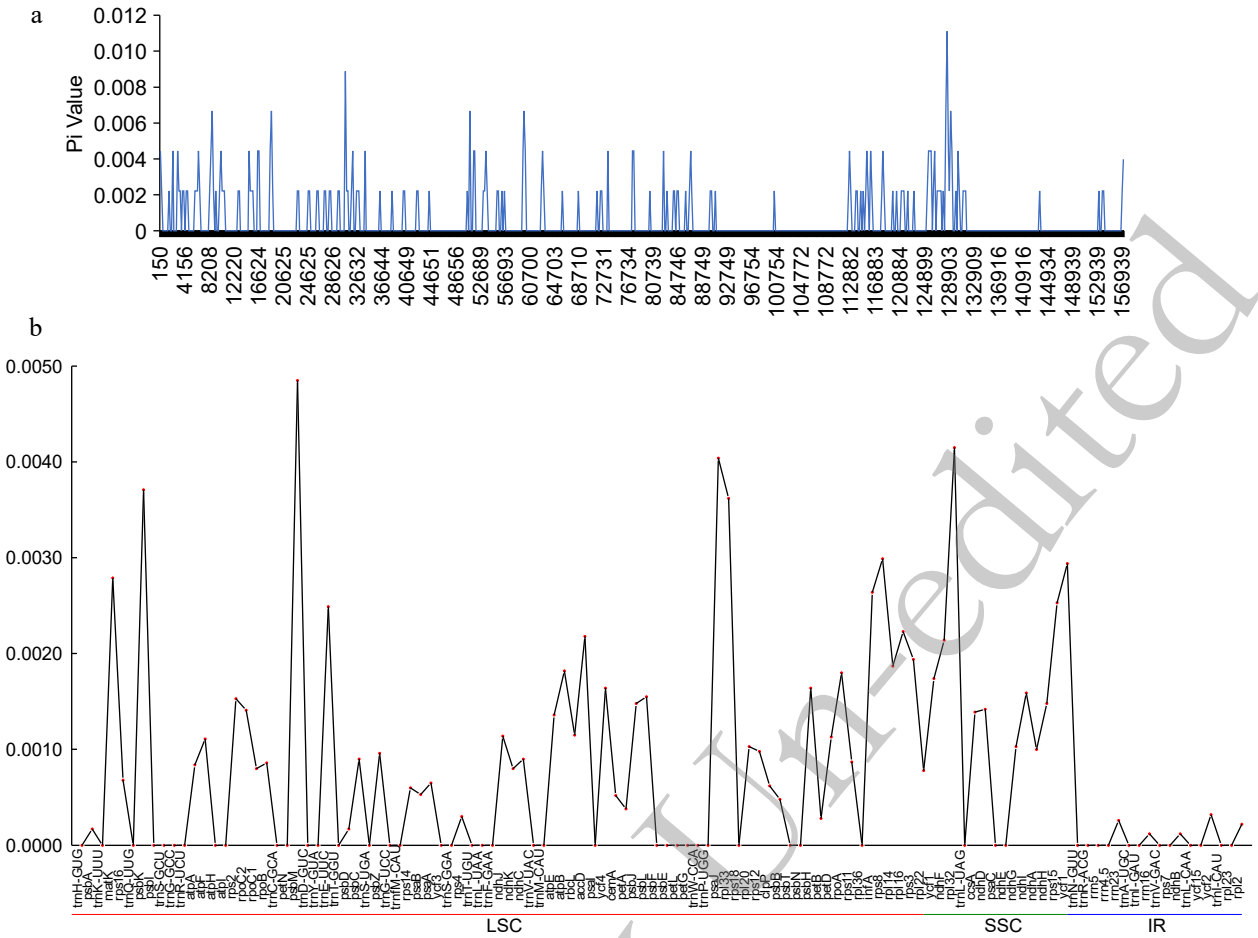

Fig. 5 The nuclear divergence in *C. meiocarpa* and *C. oleifera*. chloroplast genomes by (a) sliding window analysis of the whole genomes; (b) gene regions.

Table 3. Indel and SNP types among three chloroplast genomes.

Indel (bp)	1	2	3	4	5	6	9	10-20	21-	Total
Number (N)	28	12	5	2	10	5	1	6	3	72
Proportion (%)	38.89	16.67	6.94	2.78	1.39	6.94	1.39	8.33	4.17	
SNP type	G/A	C/T	A/C	G/T	C/G	T/A				
Number (N)	36	32	27	27	5	11				138
Proportion (%)	26.09	23.19	19.57	19.57	3.62	7.97				

inferring the phylogenetic relationships among tea-oil *Camellia* with two *Polyspora* species serving as outgroup species. (Fig. 6; Supplemental Fig. S1). In Fig. 6, CKX, XG and HZP formed a cluster within one clade (PP = 1), aligning closely with *C. japonica* and *C. chekiangobleosa* in group I (PP = 0.74, Fig. 6a); and in Fig. 6b, CKX and XG also clustered in one clade and then with *C. japonica*, *C. chekiangobleosa* and *C. polyodonta* (PP = 1), Group I and Group II were clustered in Clade A (PP = 1). Within Group II, *C. oleifera* (HZP) formed the basal clade, subsequently clustering with *C. azalea, C. granthamiana, C. gauchowensis, C. vietnamensis,* and *C. suaveolens* (PP = 1). Despite these classifications, the phylogenetic relationships within *Camellia* remained complex; for instance, *C. crapnelliana* was identified as the basal clade in Group I (PP = 1, as depicted in Fig. 6a), yet it appeared as a sister taxon to *C. gigantocaroa* in Fig. 6b (PP = 0.99). Nonetheless, MP and ML trees (Supplemental Fig. S1) still consistently supported the notion that *C. meiocarpa* (CKX and XG) was not closely related to *C. oleifera* (HZP).

Development of polymorphic marker

Based on the above results, 56 primers (Supplemental Table S8) were designed to include as many polymorphic sites as possible. The lengths of these target sequences ranged from 99 bp to 1553 bp, covering 128 polymorphic sites, which included 89 SNPs and 39 Indels. Each primer pair was capable of detecting 1 to 9 polymorphic sites, with primer ZDJ78 identifying up to 9 sites. Specifically, seven primers ZDJ05, ZDJ43, ZDJ64, ZDJ66, ZDJ67, ZDJ68, and ZDJ75 were each able to detect 4 polymorphic sites. A total of 20 pair of primers (10 targeted region in genes and 10 in intergenic regions) had only one mutation site, including 10 SNPs and 10 Indels. These 56 pair of primers were distributed across 29 genes and 32 intergenic regions, with *ycf1* having the highest number of markers (5 markers). Sanger sequencing was employed to further verify these regions. We confirmed that 17 of these primers were effective for assessing the polymorphic sites. For example, ZDJ76 detected 3 polymorphic sites (2 SNP sites and 1 Indel site), ZDJ01 detected 2 polymorphic sites (1 SNP site and 1 Indel site), and a series of primers: ZDJ03, ZDJ15, ZDJ51, ZDJ54, ZDJ55, ZDJ59, ZDJ69, ZDJ72, ZDJ77, ZDJ80, ZDJ83 and ZDJ85, each detected one SNP site. Additionally, ZDJ45, ZDJ60 and ZDJ84 each identified one Indel site (see Table 4 for detailed

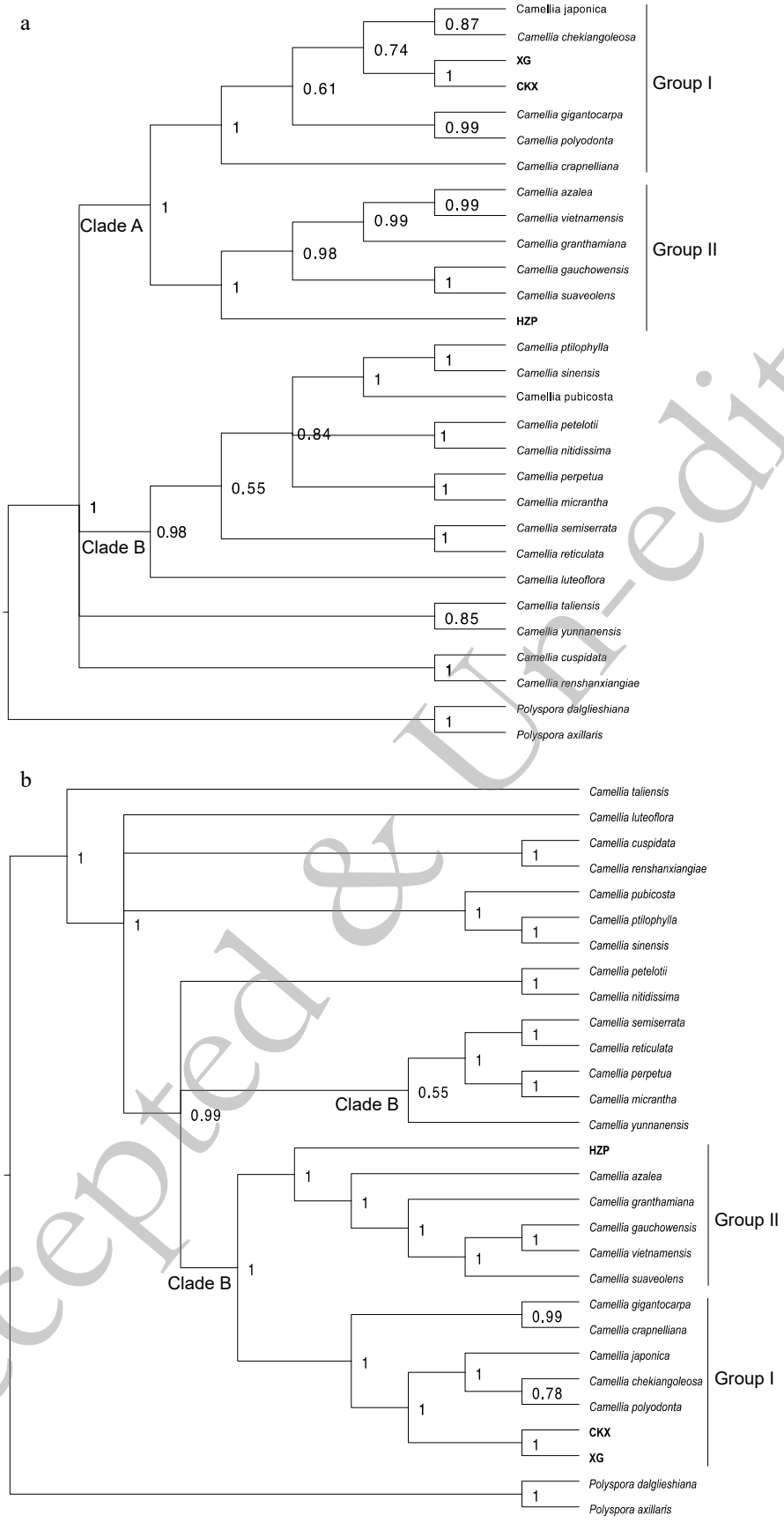

Fig. 6 Phylogenetic tree reconstruction of 27 *Camellia* species based on (a) protein-coding genes and (b) whole chloroplast genome sequences by Bayesian method.

Table 4. The SNP and Indel in the targeted regions.

Primers	Loci	SNP	Indel
ZDJ01	TCCACTATTT[C/A]AATTATAAAA	1	0
ZDJ01	CAACCCATAA[C/-]CCATAAAAAT	0	1
ZDJ03	CCCAAAAAAT[G/A]GATTTTGGTT	1	0
ZDJ15	TCAATGGCCC[T/C]CCTACGTAGT	1	0
ZDJ45	TCCCATATAT[T/-]AAATATTAAA	0	1
ZDJ51	ATTGAAAGCT[A/G]GGATTTCTAG	1	0
ZDJ54	AATCCTTGTT[T/G]CGGAGTCGAT	1	0
ZDJ55	ACCAAAAAAT[A/C]TTTTTTGCTT	1	0
ZDJ59	TTCATCTATT[T/C]CATGACCGGA	1	0
ZDJ60	GACCAAGAAG[G/-]ATTCTCTTTC	0	1
ZDJ69	ATAAAAAATT[A/T]CCCCCTGCAA	1	0
ZDJ72	AAAATCATGT[G/A]TTGGTCCAGA	1	0
ZDJ76	TTCAAAATGG[C/-]TTTCAAATTA	0	1
ZDJ76	AAAGAATAGT[A/C]AATTTTTGCA	1	0
ZDJ76	AGAATAATTT[G/T]AATCTTAAAA	1	0
ZDJ77	GTATAACCCC[C/T]TTTTGCTTTC	1	0
ZDJ80	TAAGAATGGG[G/T]GACGGTATTC	1	0
ZDJ83	GAATTCTGTG[A/G]AAAGCCGTAT	1	0
ZDJ84	AAGAGAATCC[T/-]TCTTGGTCGT	0	1
ZDJ85	TCCGGTCATG[A/G]AATAGATGAA	1	0

In Loci, the variant in left side was *C. oleifera* and the right side was *C. meiocarpa*

results).

Discussion

The phylogenetic relationship of C. meiocarpa and C. oleifera

The taxonomic status and phylogenetic relationships of *C. meiocarpa* and *C. oleifera* continue to be hotly debated, significantly affecting germplasm innovation, breeding of new varieties, and industrial development. In the production process of tea-oil *Camellia*, the fruits of *C. meiocarpa* are smaller and a bear a single seed. Compared to *C. oleifera*, it exhibits advantages such as a thin fruit peel, high oil content, high seed extraction rate, strong adaptability, disease resistance and a relatively stable yield. Currently, *C. meiocarpa* occupies the second largest cultivation area after *C. oleifera*, leading some researchers to recognize it as a distinct species[48,49]. In this study, we assembled and annotated a reference-quality chloroplast genome for both *C. meiocarpa* and *C. oleifera*, revealing a typical quadripartite structure similar in size, gene count, and GC content to other tea-oil *Camellia*[50,51]. This comparative genomic analysis provides new insights into the phylogeny of tea-oil Camellia, suggesting that despite complex morphological classifications, their chloroplast genomes are relatively conserved[52–54].

Whether *C. meiocarpa* should be considered as a variety of *C. oleifera* previous study still remains controversial in previous studies[48,55,56]. Here, we were committed to clarify the relationship between *C. meiocarpa* and *C. oleifera* amid ongoing controversies. In morphology, the distinct morphological features such as the number of seeds per fruit and the size of flowers, fruits, and leaves differentiate the species, with *C. meiocarpa* generally having 1-3 seeds per fruit and smaller morphological features compared to *C. oleifera*'s typically 4 or more seeds. In cytology, *C. meiocarpa* is tetraploid, while *C. oleifera* is hexaploidy[12]. Recent phylogenetic trees constructed from three nuclear regions placed *C. meiocarpa* with *C. vietnamensis*, distinct from *C. oleifera,* which forms the basal clade[57]. Our findings from the chloroplast genomes indicate significant genomic differences, with over 450 bp variation in size between *C. meiocarpa* (XG and CKX) and *C. oleifera* (HZP). The analysis of genomic structures and variant sites indicated that genetic divergence between XG and CKX is less pronounced than between either of these and HZP. The phylogenetic trees (Fig. 6; Supplemental Fig. S1) showed *C. meiocarpa* and *C. oleifera* did not group together. Instead, XG and CKX clustered closely, distinctly separate from HZP. Combining the evidence of morphology and cytology, we supported the opinion proposed by Xiansu Hu that *C. meiocarpa* is an independent species[58]. It facilitates a better understanding and innovative utilization of *C. meiocarpa* and *C. oleifera* by taxonomists and breeders. This approach is also beneficial for the development of the *Camellia* oil industry.

Molecular marker development and application in C. meiocarpa and C. oleifera

In the production practice of Camellia oil, the seedlings of *C. meiocarpa* and *C. oleifera* are hard to distinguish. Many substitutes and fake seedling will bring heavy losses in yield and quality in *Camellia* oil. The application of molecular markers can help to solve this problem by enabling the rapid and accurate identification of specific polymorphisms[59,60]. In contrast to classification systems based on morphological traits, molecular markers provide insights into genetic differences at the DNA level and prove effective in assessing genetic diversity within breeding programs[61]. Among these, chloroplast DNA markers have shown exceptional utility, emerging as a superior tool for the identification and classification of complex species[62]. The diversity of chloroplast genomes is the base for the polymorphic DNA marker development[63]. However, the markers still not yet been developed for *C. meiocarpa* and *C. oleifera*, and that is seriously affecting the production of Tea-oil and appraisal of plasm resources of Tea-oil *Camellia*. Although the chloroplast genomes of these species show relative conservation, the presence of numerous variations, such as SNPs and Indels, provides a rich source for marker development. In this study, we developed 56 pairs of primers to test polymorphisms in both species. PCR and sequencing results showed that only 17 primers existed mutations, demonstrating their potential to aid in resource evaluation and differentiation between *C. monosperma* and *C. oleifera*. The above analysis results provided references for the classification and evaluation between these two species as well as for practical production.

Conclusions

The present study primarily investigated the chloroplast genomes of *C. meiocarpa* and *C. oleifera* as well as conducted a comparative analysis with other related species within tea-oil *Camellia*. The genomic size, gene structure, and organization were observed to be conservative and consistent with previous studies in *Camellia*. Based on the evidence of the chloroplast genome, we supported the idea proposed by Xiansu Hu, that *C. meiocarpa* is an independent species. The development of 17 primers could be used for the resource assessment of *Camellia*, facilitating molecular phylogenetic analysis, innovation, utilization of tea-oil *Camellia* germplasm resources, and their production practice. Our study provided the high-quality chloroplast

genomes and reliable molecular markers resources for future tea-oil *Camellia* researches.

Author contributions

D.Z. designed and supervised the project. H.L. and H.Q. wrote the manuscript. H.L. annotated and analyzed the genomes H.Q., X.S., C.W., T.X., and J.C. prepared the samples and performed the experiments. Y.W., H.Y., X.F., S.X. and Y.G. analyzed the data. D.Z. and H.L. revised the manuscript. All authors contributed to the article and approved the submitted version.

Data availability

The three chloroplast genome sequences of *Camellia* are deposited in GenBank of the National Center for Biotechnology Information (NCBI) repository, accession numbers MZ151355 (XG), MZ151356 (CKX) and MZ151357 (HZP).

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Conflict of interest

The authors declare that they have no conflict of interest.

Supplementary Information accompanies this paper at (XXXXXX)

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