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Insight into the Phylogenetic Relationships and Evolutionary History of Pepper Cultivars (*Capsicum annuum* L.) through Comparative Analyses of Plastomes

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Abstract: Pepper (*Capsicum annuum* L.) holds immense global importance, as it is widely cultivated for its economic value in the food industry and its health benefits. Consequently, substantial breeding progress has been made in cultivar development, whole-genome analysis, and transformation techniques aimed at enhancing agricultural traits, including fruit development and capsaicin synthesis. However, research concerning the phylogenetic relationships within *C. annuum* remains insufficient. In this study, we characterized the plastome sequences of seven *C. annuum*, including five hot pepper and two bell pepper cultivars, while also elucidating their phylogenetic relationships. Furthermore, we conducted comparative analyses to gain insight into their evolutionary history. The seven plastomes displayed typical quadripartite structures and ranged from 156,821 to 156,922 bp, displaying highly conserved sequences. In contrast to prior studies, our phylogenomic analyses revealed that *C. annuum* species did not form a monophyletic group. Each subclade was thought to be related to a different evolutionary history, such as hybridization, domestication from wild ancestors, and artificial selection. Therefore, we were able to discern the relationships among cultivars based on their genetic profiles of plastomes. Our findings also revealed that the Korean landraces Younggo 4, 5, 10, and 11 share the most recent common ancestor with Mexican landrace CM334.

Keywords: pepper; *Capsicum annuum*; cultivar; plastome; phylogeny; comparative analysis; artificial selection; genetic profile; Korea landraces Younggo

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1. Introduction

The genus *Capsicum* L. (Solanaceae) is native to the temperate and tropical regions of Central and South America [1], comprising 42 recognized species [2]. It stands out as one of the most widely cultivated plants globally due to its immense economic value in the food industry and its health benefits. Among these species, five species (*C. annuum* L., *C. frutescens* L., *C. chinense* Jacq., *C. baccatum* L., and *C. pubescens* Ruiz. & Pav.) have been extensively cultivated and domesticated [3]. They can be classified into three complexes based on morphological, cytogenetic, and molecular data [4–6]: the *C. annuum*, *C. baccatum*, and *C. pubescent* complexes. The *C. annuum* complex includes *C. annuum*, *C. chinense*, *C. frutescens*, and *C. galapagoense*, whereas the *C. baccatum* complex comprises *C. baccatum*, *C. chaocense*, *C. praetermissum*, and *C. tovarii*. The *C. pubescens* complex consists of *C. cardenasii*, *C. eximium*, and *C. pubescens* [1,5–7]. However, morphological characteristics, such as seed color, corolla color, flower position, and fruit size and shape, have limitations in classifying domesticated species due to overlapping features. In contrast, cytogenetic and molecular studies utilizing partial sequences of chloroplast and nuclear DNA support the existence of these three complexes [1,4,5,8]. Moreover, Madgy et al. [9]

employed 321 whole plastome sequences to elucidate the plastomic boundaries among these *Capsicum* complexes. Although these studies contribute to understanding the taxonomic relationships within *Capsicum*, they have limitations, including low support values or the exclusion of certain species, as well as biased samplings from specific countries.

Capsicum annuum is classified into two distinct types based on pungency and fruit shape: hot pepper and bell pepper. In addition to their agricultural significance, both types of peppers are vital genetic resources that drive research efforts, including the development of monitoring systems for detecting transformation, gene editing and transformation, and enhancement of agricultural traits, such as fruit development, capsaicin synthesis, and disease resistance [10–15]. In recent studies, CRISPR (clustered regularly interspaced short palindromic repeats) tools have been effectively employed for genome editing to improve disease resistance in both hot and bell peppers [11,16]. Interestingly, these biotechnological applications have highlighted contrasting sensitivities among genetic backgrounds, suggesting the existence of molecular disparities [17,18]. Knowing where these differences in molecular responses originate is important in research on improving crop quality.

Research into pepper cultivation in Korea began in the late 1950s. The primary research topics focused on improving the quality of landrace, producing F1 seeds via male sterility and hybridization, and creating disease-resistant cultivars [19]. As an example, researchers identified four landraces (cultivars Younggo 4, Younggo 5, Younggo 10, and Younggo 11, called Subicho, Chilsungcho, Youwolchol, and Tojong, respectively) from different regions in the late 1990s. Each landrace was selected based on specific traits, such as fruit size, spiciness, and flowering time, leading to further refinement of each cultivar. Younggo 4 has the longest fruit, while Younggo 5 is distinguished by its thick flesh compared to Younggo 4. In contrast, Younggo 10 and Younggo 11 exhibit early flowering time, smaller fruit sizes, and a strong spicy flavor relative to the other two landraces [19,20]. The landrace is characterized by a specific adaptation to the environmental conditions of its cultivation areas [21]. Given that landraces offer a distinct source of specialized traits, including disease and pest resistance, nutritional quality, and adaptability to marginal environments [22–24], understanding their genetic information plays a crucial role in genetic research and conservation.

In this study, we determined the complete plastomes of three Korean landraces (Younggo 5, Younggo 10, and Younggo 11), one Mexican landrace (CM334), and three developed cultivars (C15, Dempsey, and Ferrari), all of which have been extensively studied as valuable genetic resources. Additionally, we conducted phylogenetic analyses using the plastome sequences, including seven newly sequenced plastomes and those previously reported. Lastly, comparative analyses were performed to gain insights into plastome variation and the evolutionary history of *C. annuum* cultivars.

2. Materials and Methods

2.1. Sampling and DNA Extraction

Korean landraces (Younggo 5, Younggo 10, and Younggo 11) were provided by Yeongyang Pepper Institute (Yeongyang, Republic of Korea). The commercially available pepper (C15) was obtained from Nongwoo Bio Co. (Suwon, Republic of Korea), and the other peppers, Dempsey and Ferrari, were provided by the Vegetable Breeding Research Center (VBRC, Seoul, Republic of Korea). Seeds of C15 and Younggo 10 were germinated in a growth medium, and fresh leaves were collected from seedlings that reached 68 and 44 days of age, respectively. Following the sample collection, the seedlings were transplanted into 15 cm diameter pots and placed in a growth room. Similarly, fresh leaves from cultivars Younggo 5, Younggo 11, Dempsey, and Ferrari were sampled from seedlings aged 51 days that had been grown under controlled conditions (Figure S1). Genomic DNA was extracted from a 100 mg sample using the Exgene Plant SV mini kit (GeneAll Biotechnology, Seoul, Republic of Korea) according to the manufacturer's instructions. The DNA quantity and quality were confirmed using a spectrophotometer and 1% agarose gel electrophoresis, respectively.

2.2. DNA Sequencing, Genome Assembly, and Annotation

A paired-end DNA library was prepared using the Illumina TruSeq Nano DNA library preparation kit (Illumina, Inc., San Diego, CA, USA), with an insert size of 350 bp. Subsequently, the library underwent sequencing on the Illumina NovaSeq 6000 (Illumina, Inc.) at the Macrogen Corp. in Seoul, Korea, generating approximately 10 GB of raw data for each cultivar. To assemble the raw reads into a complete plastome, we employed the NOVOplasty ver. 4.3 software [25]. The assembly process employed a 39 *k*-mer, with a fragment of the *psbA* gene from *C. annuum* var. *glabriusculum* (accession number KJ619462) as the seed sequence. For cultivar CM334, we downloaded contigs from NCBI (AYRZ00000000) and mapped them to the reference genome of *C. annuum* (MH559327) for assembly. Following this, we employed CPGAVAS2 [26] and tRNAscan-SE 2.0 software [27] with default settings to identify and annotate the plastome genes. The annotation of protein-coding regions was validated through searches for homologous genes using the NCBI Conserved Domain Database (CDD) [28]. The circular map of the plastome was generated using OGDRAW ver. 1.3.1 [29]. The annotated complete chloroplast genome sequences were deposited in GenBank.

2.3. Comparative Plastome Analysis

To compare the plastome sequences of the cultivars, Younggo 4 was also included and analyzed, in addition to the seven cultivars from this study. Genetic distance analyses were conducted using the Kimura 2-parameter model implemented in MEGA X [30]. All ambiguous positions were removed for each sequence pair (pairwise deletion option), leaving a total of 157,212 positions in the final dataset. To explore genetic variability among the newly sequenced cultivars, we utilized the MIcroSAtellite (MISA) identification tool (https://webblast.ipk-gatersleben.de/misa/, accessed on 20 August 2023) and set the motif lengths and minimum numbers of repetitions as follows: 10 repeat units for mononucleotide SSR motifs, six for dinucleotide motifs, and five for trinucleotide to hexanucleotide motifs. For nucleotide diversity (*Pi*) calculation, sliding window analysis was conducted using DnaSP v. 6 [31]. The step size was set to 300 bp, with a 600 bp window length. A comparison of the complete plastomes among the eight *C. annuum* cultivars, including the seven cultivars and previously sequenced Younggo 4, was performed using mVISTA [32] with the Shuffle-LAGAN mode option [33]. Relative synonymous codon usage (RSCU) was calculated for all codons using the statistics panel of Geneious v. 10 [34]. The detection of single nucleotide polymorphisms (SNPs) and insertion-deletion (InDel) polymorphisms was carried out using DnaSP v. 6 [31]. To compute the Ka/Ks values (ratios of non-synonymous to synonymous substitution rates), protein-coding sequences without stop codons were extracted from the plastomes of eight *C. annuum* cultivars, with *C. annuum* var. *glabriusculum* as a reference. Ka/Ks values were calculated using the genetic code 11 (bacterial and plant plastid code) and the model selection (MS) mode, employing KaKs_Calculator 3.0 [35].

2.4. Phylogenetic Analysis

We downloaded plastome sequences of 29 *Capsicum* species, encompassing seven *C. annuum*, six *C. baccatum*, two *C. chacoense*, four *C. chinense*, one *C. eximium*, four *C. frutescens*, two *C. galapagoense*, one *C. lycianthoides*, one *C. pubescens*, and one *C. tovarii*, and included *Nicotiana tabacum* as an outgroup. These sequences were aligned with the newly sequenced *C. annuum* cultivars using MAFFT ver. 7 [36]. Gaps or poorly aligned positions were then removed using Gblocks v. 0.91b [37]. A maximum likelihood (ML) analysis was performed in IQ-TREE v. 1.4.2 [38]. Model selection was conducted using ModelFinder [39] within IQ-TREE, resulting in the choice of the TVM+F+I model based on the Bayesian information criterion. Subsequently, nonparametric bootstrap (BS) analysis was carried out with 1000 replicates. For the Bayesian inference (BI) phylogenetic tree, the analysis was run until the standard deviation of split frequencies dropped below 0.01, using MrBayes v3.1.2 [40]. Sampling in each chain was performed every 100 generations,

and the first 25% of the samples were discarded as burn-in. The remaining data were used to generate a consensus tree.

3. Results

3.1. Plastome Organization and Features

The complete plastome sizes of seven cultivars ranged from 156,821 to 156,922 bp, and all plastomes exhibited a circular quadripartite structure characterized by a pair of inverted repeats (IRs) separated by a small single copy (SSC) and a large single copy (LSC) (Figure 1). The LSC, SSC, and IR regions encompassed a range of 87,256 to 87,395, 17,853 to 17,939, and 25,790 to 25,843 bp, respectively. Across all cultivars, the gene contents, gene order, and GC contents were highly conserved, as indicated in Table 1. In particular, the plastome sequences of Korean landraces Younggo 4, Younggo 10, and Younggo 11 are completely identical.

Table 1. Summary of the plastome characteristics of the eight *Capsicum annuum* cultivars.

Characteristics	C15	CM334	Younggo 5	Younggo 4/10/11	Dempsey	Ferrari
Total size (bp)	156,821	156,881	156,922	156,878	156,895	156,826
LSC (bp)	87,384	87,256	87,391	87,347	87,395	87,380
SSC (bp)	17,853	17,939	17,929	17,929	17,920	17,862
IR (bp)	25,792	25,843	25,801	25,801	25,790	25,792
Total GC content (%)	37.7	37.7	37.7	37.7	37.7	37.7
LSC (%)	35.7	35.8	35.7	35.7	35.7	35.7
SSC (%)	32.0	32.0	32.0	32.0	32.0	32.0
IR (%)	43.1	43.0	43.1	43.1	43.1	43.1
Number of total genes	113	113	113	113	113	113
Number of protein-coding genes	79	79	79	79	79	79
Number of tRNA genes	30	30	30	30	30	30
Number of rRNA genes	4	4	4	4	4	4

3.2. Plastome Sequence Variability

The heatmap in Figure 2 illustrates pairwise comparisons within the plastome of *C. annuum* cultivars, highlighting Dempsey as genetically distinct from other cultivars [41]. Although Dempsey and Ferrari share the common "bell pepper" classification, they are genetically distant. Ferrari is closer to C15, whereas the Mexican landrace CM334 is closely related to the Korean landrace Younggo.

In the plastomes of eight *C. annuum* cultivars, we identified a variable range of 31 to 35 SSRs, including mono-, tri-, and tetranucleotide motifs (Figure 3A). All motifs exclusively consisted of adenine and thymine nucleotides. Mononucleotide SSRs consisted solely of A/T repeat units. The trinucleotide motif AAT/TTA was consistently present in the *ycf1* gene, with five repeats across all cultivars except Dempsey, which displayed seven repeats. Furthermore, the ATAA/TATT motif was observed only in the intergenic region of the *psaA* and *ycf3* genes in cultivar C15. Most of the SSRs were located within the LSC region, ranging from 72.7% in Ferrari to 80% in Dempsey (Figure 3B). Additionally, SSRs were predominantly found in intergenic spacers (IGS), constituting over 60%, followed by introns and exons (Figure 3C).

While the sliding window analysis indicates a predominance of conserved sequences, with an average nucleotide diversity value of 0.0003, the *psaA-ycf3* IGS stands out with a comparatively high *Pi* value of 0.007 (Figure 4).

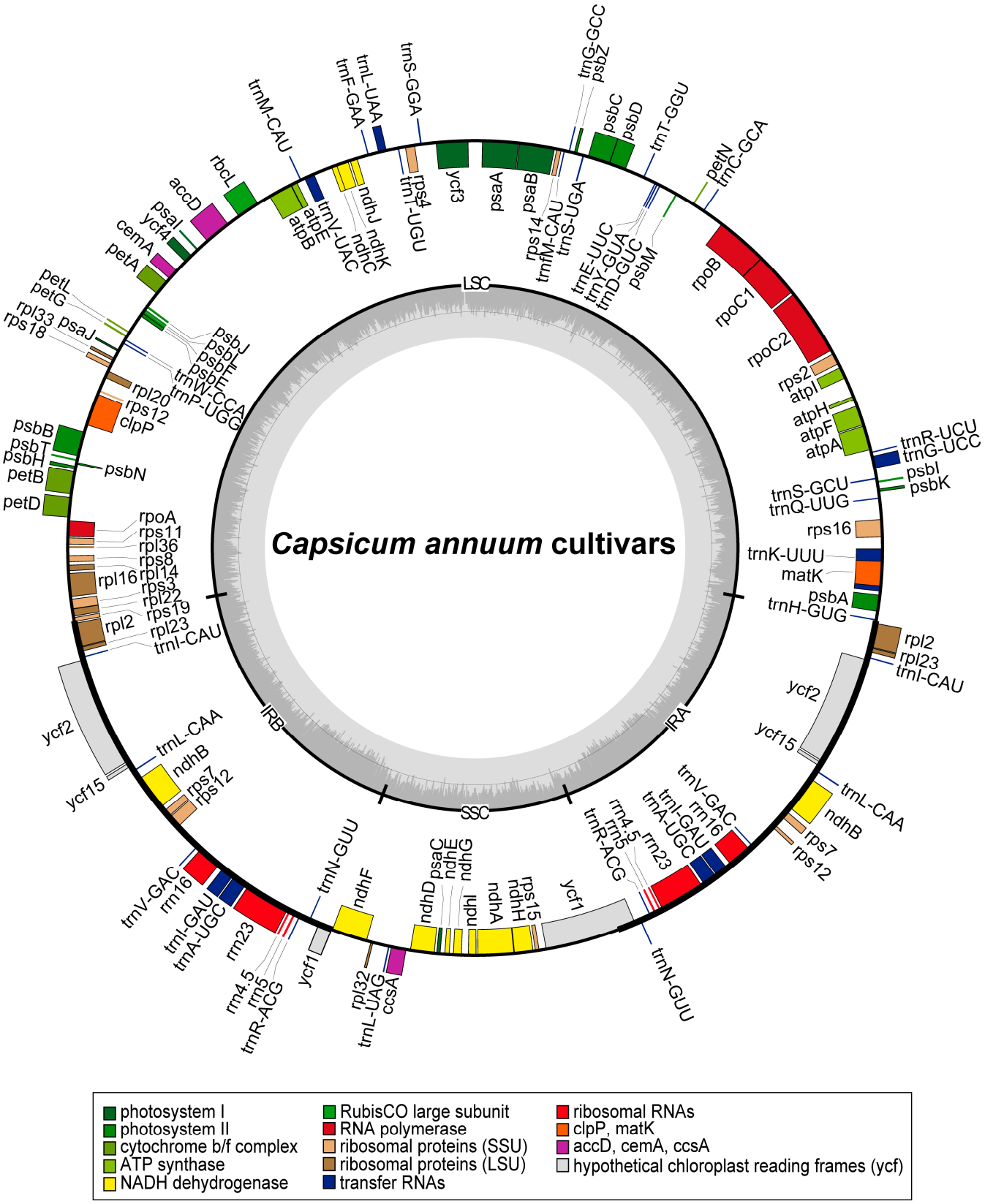

Figure 1. The plastome map of the seven *Capsicum annuum* cultivars. Genes positioned outside the circle are transcribed in a counterclockwise direction, whereas genes within the inner circle are transcribed clockwise. The GC content is depicted by the dark grey inner circle.

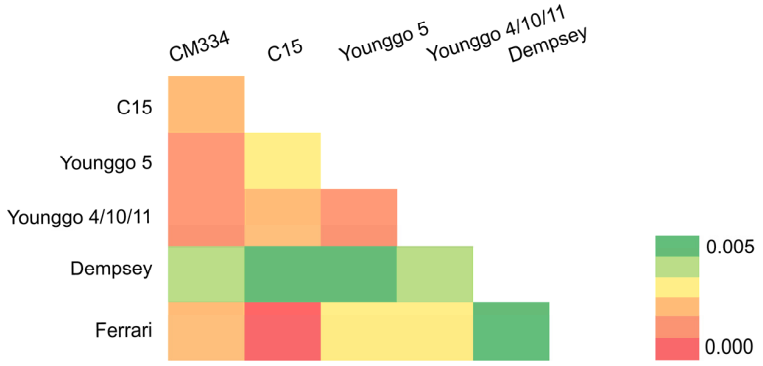

Figure 2. Pairwise genetic distance between eight *Capsicum annuum* cultivars. Red and green indicate low and high distances, respectively.

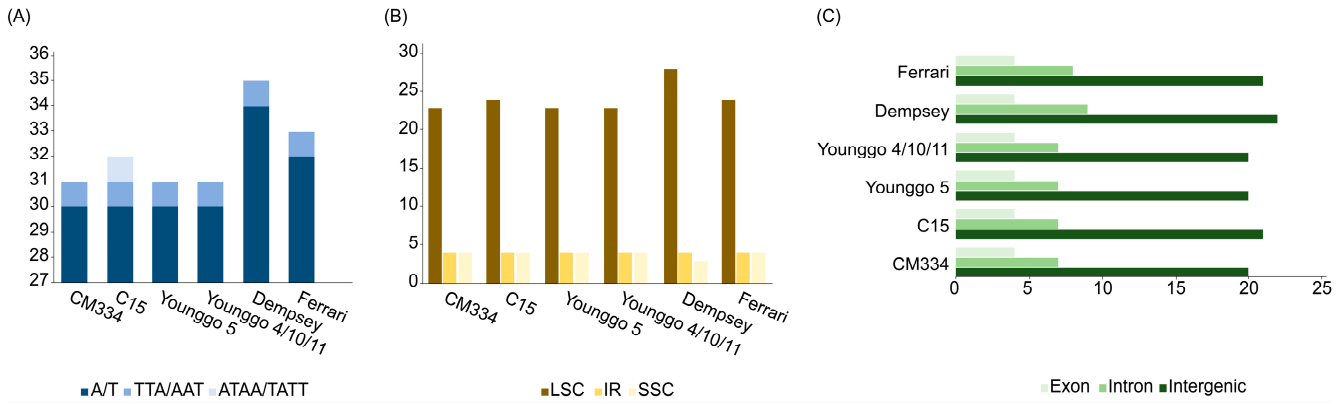

Figure 3. The type and distribution of simple sequence repeats (SSRs) in plastomes of eight *Capsicum annuum* cultivars. (**A**) Numbers of different SSR types; (**B**) location of SSRs in large single copy (LSC), inverted repeat (IR), and small single copy (SSC) regions; (**C**) location of SSRs in exon, intron, and intergenic spaces.

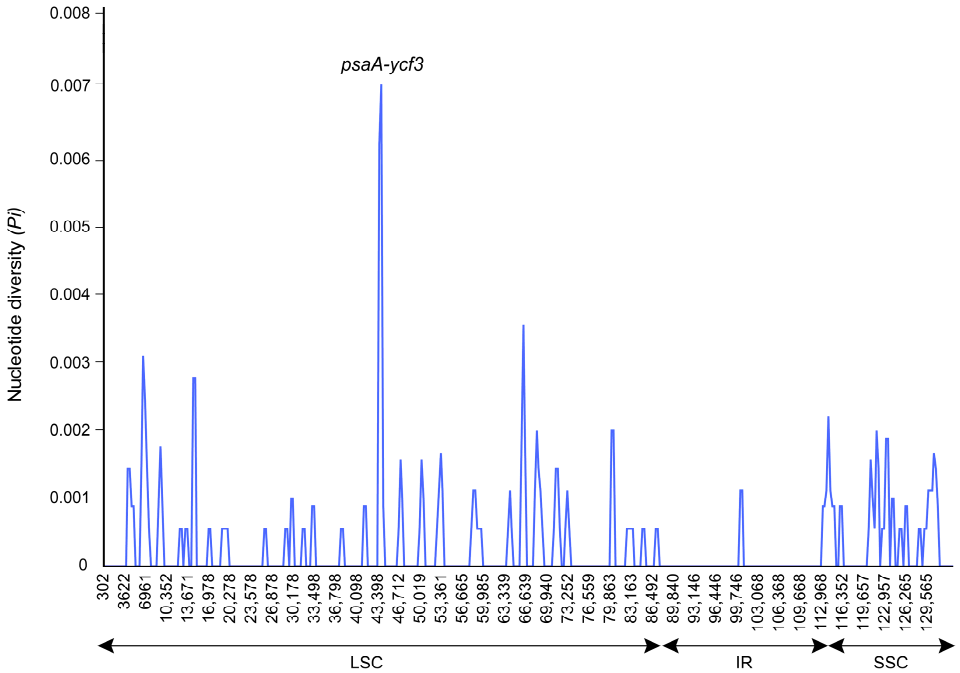

Figure 4. Plastome sliding window analysis for the eight *Capsicum annuum* cultivars. Nucleotide diversity is shown on the *y*-axis, and the position of the plastome is represented on the *x*-axis.

The plastome sequences of eight *C. annuum* cultivars were plotted using mVISTA, with *C. annuum* var. *glabriusculum* hypothesized as the reference (Figure 5). The results indicated that the LSC region was the most divergent, with non-coding regions showing more divergence and variability than coding regions. Among the divergent regions, sequence variations were identified among the eight cultivars, including eight IGS (*rps16-trnQ*, *trnS-trnG*, *trnE-trnT*, *trnL-trnF*, *rbcL-accD*, *rpl16-rps3*, *ndhB-rps7*, and *trnN-ndhF*) and two coding genes (*accD* and *ycf1*) (Figure 5).

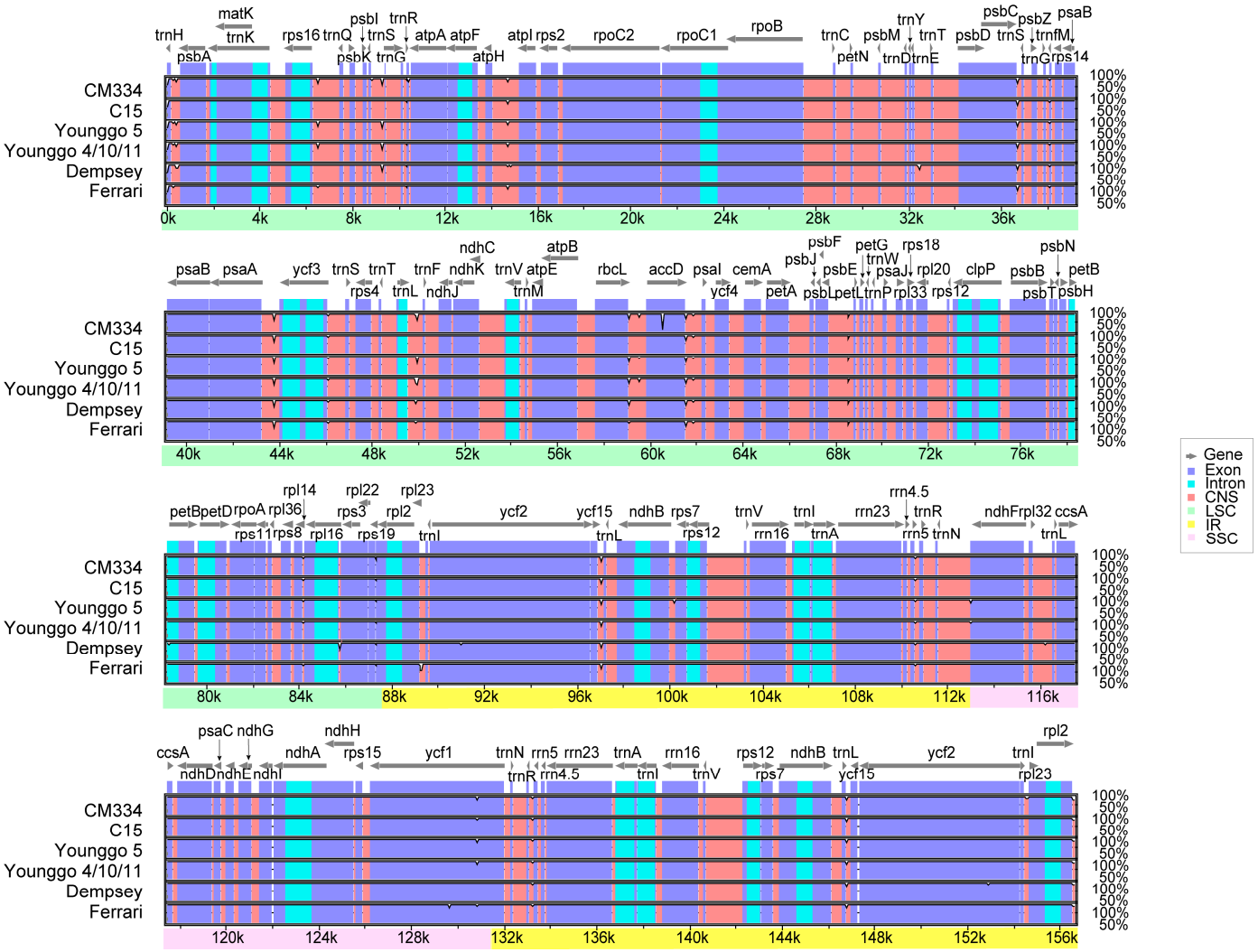

Figure 5. Visualization of alignment of eight *Capsicum annuum* plastomes. The *x*-axis and *y*-axis, respectively, indicate the coordinates in the plastome and the percentage of sequence identity within aligned regions, ranging from 50 to 100 %. *Capsicum annuum* var. *glabriusculum* (KJ619462) was used as the reference.

To confirm the sequence variation patterns based on pepper fruit types, specifically hot peppers and bell peppers, we compared plastome sequences accordingly. The majority of variations were found in the LSC and IGS in both types. Furthermore, our results indicated greater sequence divergence within bell peppers, which exhibited 80 SNPs and 41 InDels, compared to hot peppers, which had 41 SNPs and 34 InDels (Figure 6).

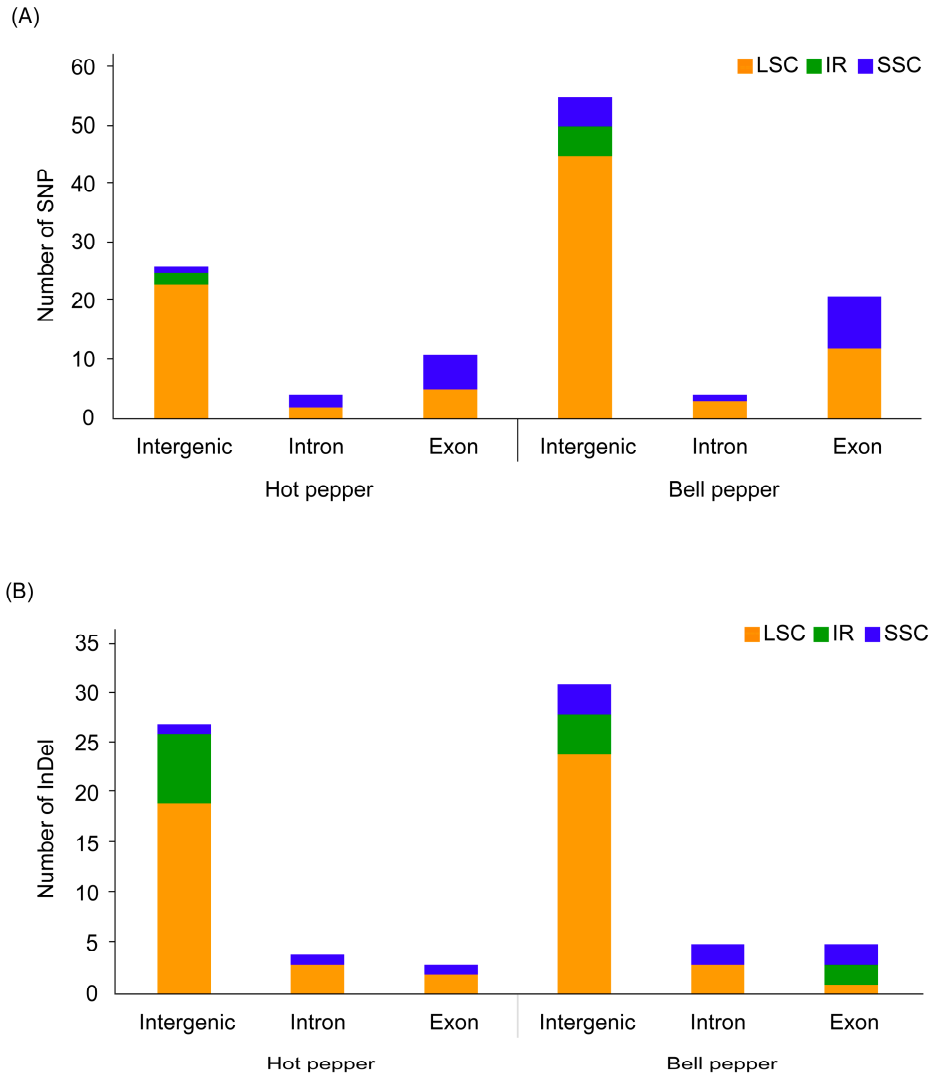

Figure 6. Number and distribution of single nucleotide polymorphisms (SNPs) (**A**) and insertion-deletions (InDels) (**B**) categorized by pepper types: hot and bell peppers. Hot peppers include cultivars CM334, C15, Younggo 4, Younggo 5, Younggo 10, and Younggo 11, whereas bell peppers encompass cultivars Dempsey and Ferrari.

Based on the sequences of 79 protein-coding genes, we found consistent patterns in codon usage across cultivars (Figure 7). RSCU analysis revealed an average codon usage ranging from 23,039 (C15) to 23,061 (Younggo 5) (Table S1). Leucine (2453–2459) is the most frequently occurring amino acid, followed by isoleucine (1935–1939) and serine (1729–1734). Conversely, cysteine (255) is the least frequently encountered amino acid. The UUA codon for leucine exhibited the highest RSCU value (1.96–1.97), followed by GCU for alanine (1.79) and UCU for serine (1.74–1.75). The AGC codon for serine displayed the lowest RSCU value (0.35), along with CUG and CUC codons for leucine (0.38–0.39). AUG and UGG codons, encoding methionine and tryptophan, respectively, showed no bias, with an RSCU value of 1.00. In addition, the RSCU analysis showed a high encoding efficacy of the codons that contained A/T at 3′ position with an RSCU $\geq$ 1.00 compared with codons ending with C/G at 3′ position, which had an RSCU < 1.00.

The nucleotide substitution rate varied across plastome genes, with Ka and Ks values ranging from 0 to 0.015 and 0 to 0.045, respectively. Among the 79 genes, we identified 13 genes with Ka/Ks value > 1, which suggests positive selection (Figure 8). These genes can be categorized into functions related to photosynthesis, transcription, and other functions. Specifically, seven genes are associated with photosynthesis, including subunits of

photosystem (*psaA* and *psbL*), cytochrome b/f complex (*petA*), *ndh* complex (*ndhD*, *ndhH*, and *ndhI*), and Rubisco (*rbcL*). Additionally, three genes are linked to self-replication, such as a large subunit of the ribosome (*rpl20*) and the subunits of the RNA polymerase (*rpoB* and *rpoC2*). We also detected the envelope membrane protein *cemA* and protease *clpP*, which had Ka/Ks values > 1. All cultivars had Ka/Ks values > 1 for *ndhD* and *psbL*, while the *cemA*, *clpP*, *ndhH*, *rbcL*, *rpl20*, *rpoB*, and *rpoC2* genes were observed in all cultivars other than Dempsey. The *ndhI* and *psaA* genes were found only in C15 and Ferrari, and *ycf1* and *petA* were exclusive to Dempsey and Younggo, respectively. The remaining genes had a Ka/Ks ratio < 1.

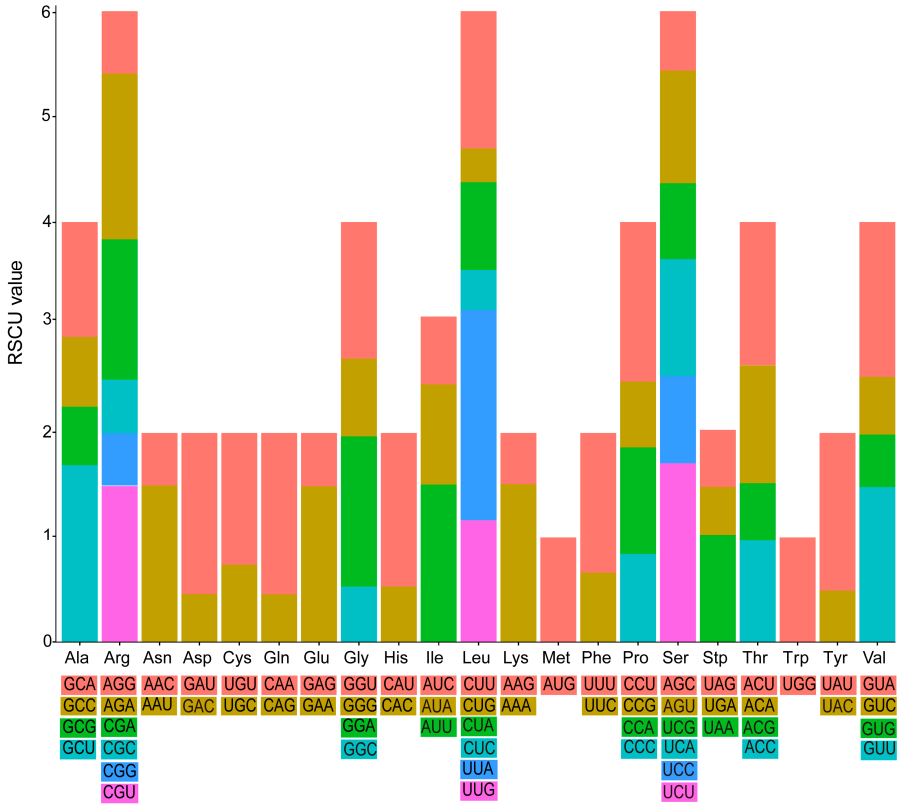

Figure 7. Relative synonymous codon usage (RSCU) in the plastomes of eight *Capsicum annuum* cultivars. Each codon is marked with a different color.

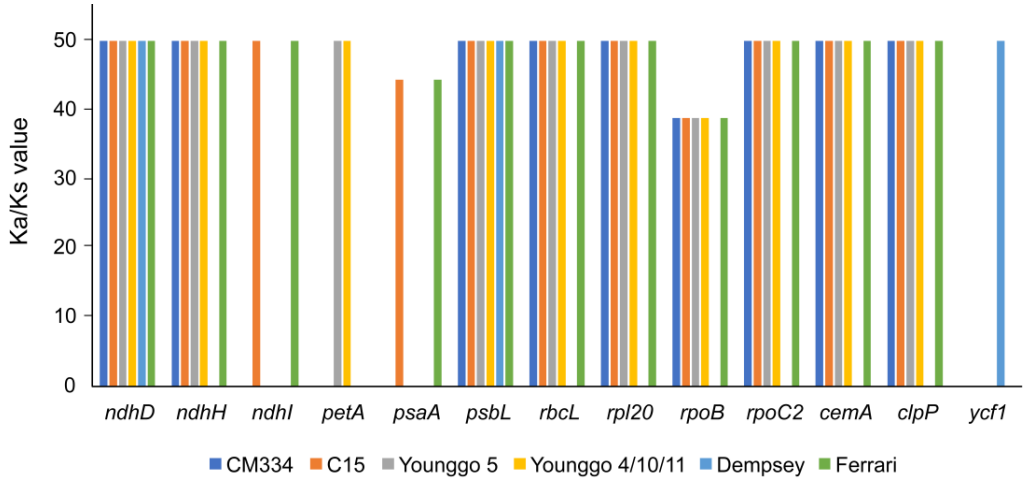

Figure 8. The Ka/Ks values of 13 protein-coding genes from eight *Capsicum annuum* cultivars.

3.3. Phylogenetic Analysis

The plastid phylogenomic tree, incorporating seven cultivars from this study, supported previously reported species relationships. The genus *Capsicum* was divided into two major lineages closely aligned with the *C. annuum* and *C. baccatum* complexes, each with robust support (100% BS; 1 posterior probability, PP). The *C. baccatum* complex, consisting of *C. chacoense* and *C. baccatum*, shared a common ancestor and formed a monophyletic group. In contrast, the *C. annuum* complex exhibited a more complex pattern. Species within the *C. annuum* complex, including *C. annuum*, *C. chinense*, *C. frutescens*, and *C. galapagoense*, did not form a monophyletic group. Concerning the positioning of *C. annuum*, this species was grouped into two distinct subclades with strong support (Figure 9). Three of fourteen *C. annuum* accessions belonged to subclade I, while the rest belonged to subclade II. Notably, landraces CM334 and Younggo were grouped into subclade II-1 and were closely related (BS = 98, PP = 1). It was consistent with the genetic distance result (Figure 2). The bell pepper cultivars Dempsey and Ferrari did not form a monophyletic group; instead, they belonged to subclades I and II, respectively. C15, Ferrari, and most of the previously reported *C. annuum* accessions were situated in subclade II-2 with the basal lineage of *C. galapagoense* (MH559322).

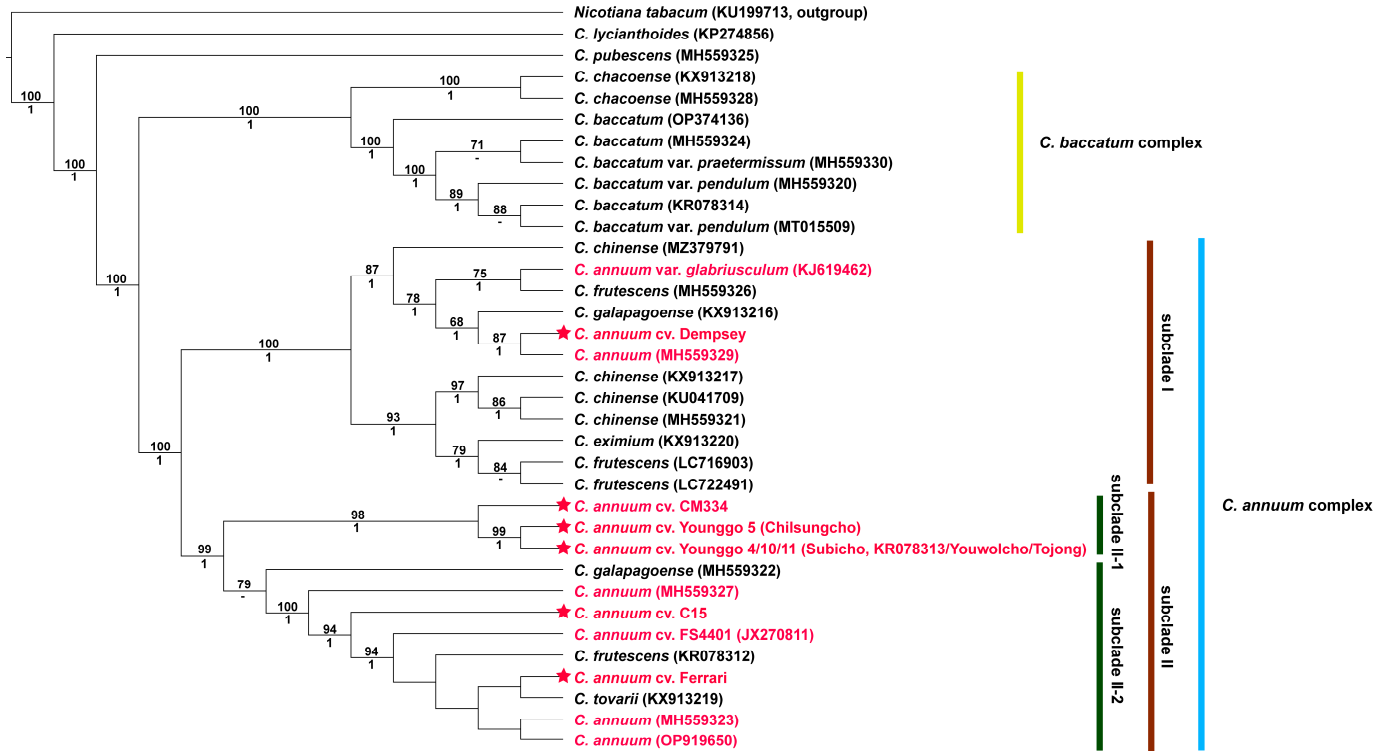

Figure 9. Maximum likelihood tree was inferred from a dataset comprising 36 species within the genus *Capsicum* and an outgroup, *Nicotiana tabacum*. Based on 1000 replicates, Bootstrap values are presented at each node, and Bayesian inference posterior probability (PP) is indicated below the corresponding node. The absence of values or a dash at the node indicates an insufficient support value for that particular node. *Capsicum annuum* species are marked in red, and accession with a star symbol indicates the cultivars sequenced in this study.

4. Discussion

4.1. Plastome Feature

In this study, the plastomes of seven *C. annuum* cultivars were sequenced, revealing highly conserved genes and structures. This structural consistency corresponded with the enduring characteristics observed in angiosperms, encompassing gene content, gene order, and GC content [42,43]. Interestingly, we found that Younggo 10 and Younggo 11 have

completely identical sequences compared to the previously reported Younggo 4 (KR078313). The difference in fruit length between Younggo 5 and the remaining Younggo members was attributed to six InDels within the LSC region. Among these, five InDels occurred in IGS, namely *trnS-trnG*, *trnL-trnF*, *ndhC-trnV*, *rbcL-accD*, and *rpl14-rpl16*, ranging from 5 to 21 bp. Furthermore, a 12 bp insertion occurred within the *rpl20* gene of Younggo 5 without inducing a frameshift mutation. Short InDels, recognized as major contributors to structural genetic diversity, have been documented to play pivotal roles in influencing flowering time and the variegated colors of flowers [44–46]. In addition, an exonic InDel of the *rpl20* gene in the plastome has been associated with leaf development in *N. tabacum* [47]. These studies underscore the prominent influence of InDels in shaping distinct phenotypic traits. Given the variations in morphology within the Younggo cultivars, it would be necessary to delve deeper into the connection between divergences in sequences—particularly within genes like *rpl20*—and their potential impact on morphological traits.

4.2. Sequence Divergence

The similarity of plastome sequences among *C. annuum* cultivars was remarkably high, exceeding 99%, despite their various breeding and domestication backgrounds. This finding aligns with the trend toward lower diversity in pepper, tomato, potato, and sweet potato cultivars compared to their wild relatives [48–51]. Interestingly, our analysis revealed that the cultivar Dempsey stands out in terms of genetic variation. This result may be attributed to Dempsey's hybrid origin, which can be traced back to a three-way cross involving hot peppers PI264281 and PI163192, as well as the bell pepper Jupiter [41]. The introduction of these two hot peppers as paternal gives resistance to tobacco etch potyvirus and bacterial spot. In contrast, the other bell pepper Ferrari is a transgenic plant regenerated via the inducible activation of the BABY BOOM transcription factor [52]. Although they commonly have bell-shaped fruit, the observation of a high genetic distance between them, their position in different subclades, and the presence of distinct genetic backgrounds collectively indicate that these two bell peppers likely originated from different maternal sources.

The detected SSRs were predominantly located within the LSC region and IGSs, consistent with prevalent characteristics observed in other angiosperms [53–55]. As SSRs show high polymorphism between individuals, SSR markers have been widely used in determining genetic diversity and conducting phylogenetic studies [56,57]. However, in the case of newly sequenced cultivars, they exhibited nearly identical repeat numbers and motifs, presenting a challenge for the development of SSR markers on plastomes. We identified the notably variable region *psaA-ycf3* among the seven cultivars examined, which corroborates the findings reported by Magdy et al. [9]. Nevertheless, the average nucleotide diversity value, which remained below 0.01, highlights the highly conserved plastome evolution across *Capsicum* cultivars. These high similarities can be attributed to domestication or artificial selection, as well as the low cross-pollination rates resulting from the tendency toward self-pollination in cultivated *Capsicum* species [7].

4.3. Plastome Evolution

Codon usage bias can be used to understand the molecular evolution and environmental adaptation of species and the superior agronomic performance of the cultivated species [58]. However, we could not find any significant differences among cultivars. Relative synonymous codon usage and amino acid frequency revealed high similarities among them. The prevalence of leucine as the most encoded amino acid and cysteine as the least was consistent with observations in other Solanaceae species [59–61].

While most plastome genes evolve under purifying selection (Ka/Ks < 1) due to their critical roles in photosynthesis and self-replication functions, our analysis revealed that 3 to 11 genes within the cultivars experienced positive selection pressure. In particular, three genes *(ndhD*, *ndhH*, and *ndhI*) of the *ndh* family, which are associated with photosynthesis, were identified as being under positive selection. Chloroplast *ndh* monomers are known to be sensitive to high light stress, suggesting that the *ndh* genes likely play a role in stress

acclimation [62]. The phenomenon of positive selection in the *rbcL* gene of land plants is commonly observed [63]. This widespread adaptive evolution may signify an effort to optimize its performance under changing thermal conditions and in response to coevolution with proteins in the Rubisco complex. Additionally, the positive selection observed in the *rpoB* and *rpoC2* genes, encoding the β subunit of RNA polymerase, could potentially lead to changes in cell wall metabolism, possibly due to alteration in transcription [64]. As evident from the examples above, positive selection is often interpreted as an indication of adaptation to changing environments, ecological niches, or coevolutionary processes [60]. In this context, we postulated that the positive selection observed in the genes of *C. annuum* cultivars is associated with their adaptation to diverse environments. It may also be attributed to artificial selection during domestication from wild ancestor *C. annuum* var. *glabriusculum* [65], as well as during subsequent cultivation and development of new cultivars. A more comprehensive understanding of the relationship between positive selection in genes and the adaptation of *C. annuum* cultivars requires further investigation.

4.4. Phylogenetic Relationship

Studies aimed at elucidating the relationships among *Capsicum* species have employed various molecular approaches, including isozyme [66], random amplified polymorphic DNA [67,68], amplified fragment length polymorphism [67,69,70], microsatellite genotyping [71,72], SNP [73], molecular markers [4,6], and complete plastomes [9]. These studies provide robust support for distinguishing the two major complexes within the genus *Capsicum*, *C. annuum* and *C. baccatum*. Our phylogenetic tree also revealed that each complex forms a monophyletic group with high support values (BS = 100%, PP = 1). The *C. annuum* complex included not only *C. annuum* but also *C. tovarii* and *C. eximium*, which are categorized within the *C. baccatum* and *C. pubescens* complexes, respectively. Tong and Bosland [74] suggested that *C. tovarii* shares a closer genetic relationship with the *C. baccatum* complex than with other *Capsicum* complexes. Similarly, Ince et al. [68] also placed *C. tovarii* within the *C. baccatum* clade. However, Magdy et al. [9] reported *C. tovarii* (KX913219) and *C. frutescens* (KR078312) as variants of *C. annuum* var. *annuum*, and Shiragaki et al. [4] suggested that *C. eximium* should be reclassified as *C. frutescens* based on morphological and molecular traits. Based on these studies, we concluded that subclade II-2 primarily includes *C. annuum* species.

Unlike previous studies [4,9] showing the monophyly of *C. annuum,* the *C. annuum* species was divided into three subclades in this study: subclade I consists of *C. annuum*, *C. annuum* var. *glabriusculum*, *C. frutescens*, and *C. galapagoense*; subclade II-1 includes landrace *C. annuum*; and subclade II-2 comprises another cluster of *C. annuum* and *C. galapagoense*. Although the precise lineage of previously sequenced *C. annuum* remains elusive, we inferred that these three subclades would correspond to distinct genetic histories: subclade I includes the hybrid cultivar, Dempsey. Subclade II-1 comprises the *C. annuum* landrace, which has been domesticated and cultivated for a long period, while subclade II-2 encompasses cultivars that were chosen either through specific breeding strategies or genetic modification techniques. For instance, in subclade II-2, Ferrari is a transgenic plant, as described above, and is renowned for its efficiency in transformation and regeneration following genetic engineering. The cultivar C15 has been reported as a transformable inbred line and served as a parental source for developing new cultivars [75,76]. Therefore, we were able to discern the relationships among cultivars based on their plastome genetic profiles. However, further validation involving a broader range of cultivars with diverse genetic backgrounds and the examination of nuclear or mitochondrial genomes is warranted.

Due to the distinct characteristics observed within the *C. annuum* cv. Younggo, such as variations in flowering time, fruit pungency, and fruit length, they have been treated as separate cultivars [77]. Our findings revealed robust support that the Korean landraces Younggo formed a monophyletic group, indicating a shared maternal lineage. To further investigate these results, phylogenetic analysis using nuclear DNA is warranted.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9101092/s1. Figure S1: Photos of seven *Capsicum annuum* cultivars sequenced in this study; Table S1: The codon usage frequency and ratio of relative synonymous codon usage in eight *Capsicum annuum* cultivars; Table S2: The non-synonymous (Ka) and synonymous (Ks) substitution ratio.

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Data Availability Statement: The annotated plastomes of six *Capsicum annuum* cultivars have been archived on the National Center for Biotechnology Information (NCBI) website, which can be accessed at https://www.ncbi.nlm.nih.gov. These plastome sequences are associated with the following accession numbers: OR538721 (Dempsey), OR538722 (Younggo 11), OR538723 (Younggo 10), OR538724 (Younggo 5), OR538725 (C15), and OR538727 (Ferrari).

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