Comparative analysis of the complete plastid genomes in *Prunus* subgenus *Cerasus* (Rosaceae): Molecular structures and phylogenetic relationships

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Abstract

*Prunus* subgenus *Cerasus* (cherry) is an economically important group that distributed in temperate regions of the northern hemisphere. However, shared interspecific morphological traits and variability across taxa of *Cerasus* are among the impediments to taxonomic efforts to correctly delimit taxa. This is further complicated by a lack of genetic information on these taxa, with no focused genomic or phylogenetic studies being done on *Cerasus*. In this study, we conducted comparative analysis on the complete plastid genomes (plastomes) of 20 *Cerasus* species to gain a greater understanding of the attributes of the plastome of these taxa while helping resolve their phylogenetic placement in *Prunus sensu lato* and interspecific relationships within the subgenus. Our results displayed that (1) the plastomes of the 20 *Cerasus* species studied exhibited a typical quadripartite structure with conserved genome arrangement, structure, and moderate divergence. (2) The average size of complete plastomes for the *Cerasus* taxa studied was 157,861 bp, ranging from 157,458 to 158,024 bp. A total of 134 genes were annotated, including 86 protein-coding genes, 40 tRNAs, and 8 rRNAs across all species. In simple sequence repeat analysis, we found *Cerasus* had a comparable number of dispersed and tandem repeats to those identified in other angiosperm taxa, with only *P. pseudocerasus* found to contain trinucleotide repeats. Nucleotide diversity analysis revealed that the *trnG-GCC* gene and *rpl32-trnL* region had the highest Pi value showing potential as phylogenetic markers. (3) Two phylogenetic trees of the plastomes verified the monophyletic relationship of *Cerasus* and provided a more resolved species-level phylogeny. Our study provides detailed plastome information for exploring the phylogeny of subg. *Cerasus*. We identified various types of repeats and nucleotide diversity hotspots, which can be a reference for species identification and reconstruction of phylogenetic relationships.
Prunus L. subg. Cerasus (Mill.) A. Gray contains approximately 150 species that mainly distributed in temperate and subtropical regions of the northern hemisphere [1–5]. Subg. Cerasus provides various edible cherries and ornamentals of economic value, and has great potential for development and application, the research on this economically important group is becoming more and more extensive. Through long-term hybridization and domestication of subg. Cerasus species, a large number of economically significant species, such as sweet cherry (P. avium), Chinese cherry (P. pseudocerasus), and variety of ornamental specie has been widely planted. In recent years, many new cultivars have been developed, indicating that the subg. Cerasus has great potential for development and utilization. However, interspecific hybridizations have complicated the taxonomy of this subgenus [6], making it necessary to investigate the phylogenetic relationships of wild subg. Cerasus species resources. Although some studies have clarified the deep phylogenetic relationships and diversification history of Rosaceae and Prunus s.l. [7–10], only one phylogenetic study has included 13 taxa from subg. Cerasus and was based on a minimal number of molecular markers [8].

The annotation and bioinformatics analysis of plastid genome play an important role in the classification and evolution of plant species, due to its unique genetic characteristics of material inheritance. A typical plastid genome (plastome) consists of a pair of inverted repeats (IR) regions that is separated by a large single copy (LSC) region and a small single copy (SSC) region, with standard plastome sizes ranging between 120 and 170 kb in length [11]. Compared with the nuclear genome, the plastome in angiosperms is a circular DNA molecule which has a highly conserved genomic structure with a small size, single-parental inheritance, and low nucleotide substitution rate. Based on these advantages, the plastome is used in diverse studies focused on species identification, population genetic analyses, and phylogenetic analyses, with phylogenetic studies using the complete plastome undertaken in many angiosperm groups [12–16].

In this study, we compared and analyzed the published complete plastomes of 20 subg. Cerasus species from GenBank, aiming to reveal the complete structure of plastomes and hotspot regions among these 20 species while clarifying their phylogenetic placement. This information will be valuable for further evolutionary studies on subg. Cerasus and Rosaceae in general.

Materials and methods

Sampling, DNA isolation and sequencing

There are no specific permits required for obtaining the healthy and fresh leaves of P. jamasakura and P. discoidea, since they are not endangered or protected species and were collected from the fields that are not privately owned or protected. We collected fresh young leaf samples for P. jamasakura and P. discoidea from Katano, Japan (34° 45’ 53.7”N, 135° 42’ 10.5”E) and Huangshan, China (30° 4’ 14.08”N 118° 5’ 25.54”E). We prepared the voucher specimen for two samples used and deposited them in Nanjing Forestry University (voucher numbers: NF161093652, NF161093753). The leaf samples were quickly dried with silica gel in a zip lock plastic bag upon the sampling and stored at room temperature until further use.

Total Genomic DNA were extracted from each of the two subg. Cerasus plants using a DNeasy Plant Mini Kit (Qiagen Co., Hilden, Germany) following the manufacturer’s protocol. The extracted DNA were quantified in NanoDrop ND1000 (Thermo Fisher Scientific, Massachusetts, USA; quality cutoff, OD 260/280 ratio between 1.7–1.9) and visualized in a 1% agarose-gel electrophoresis for the quality check. Illumina paired-end (PE) libraries (read length: 2 × 125 bp) with insert sizes of 270 to 700 bp for each of the two Cerasus species were
constructed and sequenced on MiSeq platform (Illumina Inc., San Diego, CA) by Nanjing GenePioneer Biotechnologies Inc. (Nanjing, China). We removed poor quality reads (PHRED score of < 20) using the quality trim function implemented in CLC Assembly Cell package v. 4.2.1 (CLC Inc., Denmark).

**Genome assembly and annotation**

We employed the low-coverage whole-genome sequence (dnaLCW) method [17] to assemble the complete CP genomes using both CLC de novo assembler in CLC Assembly Cell package and SOAPdenovo (SOAP package v. 1.12) with default parameters. Gaps were filled by the Gapcloser function in the SOAP package. To improve the CP genome assembly, we also conducted reference-based genome assembly using the CP genome sequences of *P. cerasoides* (GenBank accession: MF621234). The contigs obtained from the primary de novo assemblies were aligned to the reference CP genome, then the aligned contigs were assembled to each chloroplast genome in Geneious v7 (http://www.geneious.com). We annotated the CP genomes assembled using the online tool, DOGMA (Dual Organellar GenoMe Annotator) [18] with a few adjustments for start and stop codons. Protein-coding genes were defined based on the plastid-bacterial genetic code. We also scanned all tRNAs with tRNAscan-SE [19] using the default settings to confirm the tRNA boundaries identified by DOGMA. Since this study adopted the concept of *Prunus s.* subgenus *Cerasus*, genus name *Prunus* was used to represent the subgenus name *Cerasus*. The circular plastome maps were visualized using OGDRAW v 1.3.1. The other annotated plastome sequences of 18 subg. *Cerasus* species we studied were all downloaded from GenBank and the accession numbers listed in Table 1.

### Table 1. Sample information and summary of plastome characteristics for the 20 subg. *Cerasus* species studied.

| Species                  | Reference Sequence | Total Length (bp) | LSC length (bp) (GC content) | SSC length (bp) (GC content) | IR length (bp) (GC content) | GC content | Total number of genes |
|--------------------------|--------------------|-------------------|------------------------------|-----------------------------|-----------------------------|------------|-----------------------|
| *Prunus avium*           | NC044701           | 157886            | 85990(34.54%)                | 19080(30.24%)               | 26408(42.52%)               | 36.69%     | 131                   |
| *P. campanulata*         | NC044123           | 157938            | 85947(34.59%)                | 19119(30.21%)               | 26436(42.52%)               | 36.72%     | 128                   |
| *P. cerasoides*          | NC035891           | 157685            | 85792(34.59%)                | 19061(30.26%)               | 26416(42.51%)               | 36.71%     | 129                   |
| *P. cerasoides*          | MT374065           | 158019            | 85910(34.60%)                | 19247(30.06%)               | 26431(42.53%)               | 36.70%     | 130                   |
| *P. discoidea*           | MN158647           | 158024            | 85953(34.59%)                | 19113(30.20%)               | 26609(42.50%)               | 36.70%     | 130                   |
| *P. emarginata*          | MN389436           | 157458            | 85657(34.54%)                | 19121(30.12%)               | 26385(42.53%)               | 36.68%     | 129                   |
| *P. itosakura*           | MN695296           | 157813            | 85931(34.62%)                | 19120(30.19%)               | 26381(42.56%)               | 36.73%     | 129                   |
| *P. jamasakura*          | MN652612           | 157905            | 85910(34.59%)                | 19123(30.19%)               | 26436(42.52%)               | 36.71%     | 129                   |
| *P. kumanoensis*         | MN245147           | 157898            | 85926(34.59%)                | 19070(30.26%)               | 26451(42.52%)               | 36.73%     | 129                   |
| *P. leivillana*          | MN913372           | 157935            | 85940(34.58%)                | 19121(30.22%)               | 26435(42.52%)               | 36.71%     | 130                   |
| *P. matura*              | NC045230           | 157928            | 85904(34.59%)                | 19104(30.23%)               | 26600(42.50%)               | 36.71%     | 129                   |
| *P. maximoviczii*        | NC026981           | 157852            | 85847(34.62%)                | 19133(30.12%)               | 26436(42.56%)               | 36.72%     | 129                   |
| *P. pensylvanica*        | MN427872           | 157953            | 86030(34.55%)                | 19135(30.15%)               | 26394(42.52%)               | 36.68%     | 129                   |
| *P. pseudocerasus*       | KX255667           | 157834            | 85954(34.64%)                | 19084(30.36%)               | 26398(42.46%)               | 36.70%     | 131                   |
| *P. rubra*               | NC048528           | 157723            | 85860(34.60%)                | 19081(30.23%)               | 26391(42.55%)               | 36.73%     | 129                   |
| *P. spontanea*           | KP760073           | 157882            | 85968(34.60%)                | 19120(30.22%)               | 26397(42.53%)               | 36.72%     | 129                   |
| *P. speciosa*            | MH998233           | 157916            | 85927(34.59%)                | 19123(30.19%)               | 26433(42.53%)               | 36.71%     | 129                   |
| *P. subhirtella*         | KP760075           | 157833            | 85951(34.59%)                | 19120(30.20%)               | 26381(42.56%)               | 36.73%     | 129                   |
| *P. takesimensis*        | NC039379           | 157948            | 85959(34.59%)                | 19117(30.12%)               | 26436(42.53%)               | 36.71%     | 129                   |
| *P. yedoensis*           | NC026980           | 157792            | 85914(34.64%)                | 19120(30.26%)               | 26379(42.49%)               | 36.74%     | 130                   |

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Comparative genome analysis

The IRSCOPE (https://irscope.shinyapps.io/irapp/) was chosen to compare the boundaries between single copy regions (LSC and SSC) and inverted repeat (IR) regions among the 20 subg. *Cerasus* plastome sequences. The mVISTA (http://genome.lbl.gov/vista/mvista/submit.shtml) visualized the differences between the complete plastid sequences of 20 subg. *Cerasus* species in Shuffle-LAGAN mode with the annotated complete plastome of *P. avium* as a reference. To analyze nucleotide diversity (Pi), we applied the window size of 600bp with a 200bp step size and we extracted the shared 93 genes of 20 species in subg. *Cerasus* after alignment. The Pi value among the 20 subg. *Cerasus* species was calculated using the DnaSP v6 [20], which was utilized to determine the average number of nucleotide differences between all taxa.

Repeat sequences and SSR analysis

REPuter [21] was selected to investigate the tandem repeat sequences and the size of repeat sequences, which included four types of repeats in the plastomes of the 20 subg. *Cerasus* species. For REPuter analysis, we set the parameters with hamming distance of 3 bp and minimal repeat size of 30 bp. We used SSR software MicroSAellite (MISA) (http://pgrc.ipk-gatersleben.de/misa/) to identify SSR sequences and tandem repeats of 1–6 nucleotides were considered microsatellites. The minimum numbers of repeats were set to 10, 5, 4, 3, 3, and 3 for mono-, di-, tri-, tetra-, penta-, and hexa-nucleotides, respectively.

We also analyzed codon usage to examine the distribution of codon usage using CodonW v1.4.2 (http://codonw.sourceforge.net/) with RSCU ratio for all protein-coding genes.

Phylogenetic analysis

We selected the 20 previously sequenced plastomes from subg. *Cerasus* in GenBank (Table 1), and combined these with 29 published plastomes of Rosaceae and four other angiosperm species that were set as an outgroup for the phylogeny. The plastomes of Rosaceae were conserved in gene construction, so the alignment was straightforward. We utilized different databases, including complete plastome and CDS regions to construct the phylogenetic tree. Before building the phylogenetic tree with plastomes, 53 sequences were aligned with MAFFT v7.467 [22] and manually adjusted in BioEdit [23], and the phylogenetic analyses were performed by Maximum likelihood (ML) and Bayesian inference (BI) methods, respectively. Maximum likelihood (ML) analyses were performed using IQ-TREE v2.1.1 [24] with 1000 bootstrap replications. MrBayes v3.2.7 [25] was used to conduct Bayesian inference (BI) analyses. The Markov chain Monte Carlo (MCMC) algorithm was run for two million generations with trees sampled every 500 generations. The first 25% of generations were discarded as burn-in. A 50% majority-rule consensus tree was constructed from the remaining trees to estimate posterior probabilities (PPs). To determine the best fitting substitution model, the phylogenetic trees were visualized using Figtree v1.4 (http://tree.bio.ed.ac.uk/software/figtree/) [26].

Results

Plastome structure

The plastomes of the subg. *Cerasus* taxa studied have the typical quadripartite structure comprising long single copy (LSC), inverted repeat (IR) and small single copy (SSC) regions (Fig 1). The average length of complete plastomes among these subg. *Cerasus* species is 157,861bp, ranging from 157,458 to 158,024 bp in length (Fig 1 and Table 1), with IRs of 26,379–26,469 bp, LSCs 85567–86,030 bp and SSCs 19,061–19,247 bp. The entire GC content of these plastome sequences is 36.68–36.74%, and the GC contents of the LSC, SSC, and IR regions are
Fig 1. Plastid map of 20 subg. Cerasus species. The colored boxes represent conserved plastid genes. Genes shown inside the circle are transcribed clockwise, whereas genes outside the circle are transcribed counter-clockwise. Genes are color-coded to indicate functional groups. The dark gray area in the inner circle corresponds to GC content while the light gray corresponds to the AT content of the genome. The small (SSC) and large (LSC) single copy regions and inverted repeat (IRA and IRb) regions are noted in the inner circle.

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Table 2. List of genes within plastomes of the 20 subg. Cerasus species studied.

| Category                      | Group of gene          | Name of gene              |
|-------------------------------|------------------------|---------------------------|
| Photosynthetic                | Photosystem I          | psaA, psaB, psaC, psaI, psaL |
|                               | Photosystem II         | psbA, psbB, psbC, psbD, psbE, psbF, psbH, psbI, psbJ, psbK, psbL, psbM, psbN, psbT, psbZ |
|                               | NADH dehydrogenase     | ndhA*<sup>+</sup>, ndhB<sup>+</sup>, ndhC, ndhD, ndhE, ndhF, ndhG, ndhH, ndhI, ndhJ, ndhK |
|                               | Cytochrome b6/ complex  | petN, petA, petL, petG, petB<sup>+</sup>, petD<sup>+</sup> |
|                               | ATP synthase           | atpA, atpB, atpE, atpF<sup>+</sup>, atpH, atpI |
|                               | Rubisco large subunit  | rbcL |
| Transcription & Translation   | Ribosomal protein, LSU | rpl2<sup>+</sup>(x2)<sup>+</sup>, rpl14<sup>+</sup>, rpl16<sup>+</sup>, rpl20, rpl22, rpl23<sup>+</sup>(x2), rpl32, rpl33, rpl36 |
|                               | Ribosomal protein, SSU | rps2, rps3, rps4<sup>+</sup>(x2), rps5<sup>+</sup>(x2), rps8, rps11, rps12<sup>+</sup>(x2), rps14, rps15, rps16<sup>+</sup>, rps18, rps19<sup>+</sup>(x2) |
|                               | RNA polymerase         | rpoA, rpoB, rpoC<sup>+</sup>, rpoC2 |
|                               | Ribosomal RNAs         | rrn23<sup>+</sup>(x2), rrn16<sup>+</sup>(x2), rrn5<sup>+</sup>(x2), rrn4<sup>+</sup>(x2) |
|                               | Transfer RNAs          | trnA-UGC<sup>+</sup>(x2), trnC-GCA, trnD-GUC, trnE-UUC, trnF-GAA, trnM-CAU, trnG-GCC, trnG-UCC, trnH-GUG, trnL-CAU<sup>+</sup>(x2), trnL-UUU, trnL-CAA<sup>+</sup>(x2), trnK-AAA, trnL-UAG, trnM-CAU<sup>+</sup>(x2), trnN-GUU<sup>+</sup>(x2), trnP-UGG, trnQ-UGU, trnR-ACG<sup>+</sup>(x2), trnR-UCU, trnS-AGA, trnS-GCU, trnS-GGA, trnS-UGA, trnT-GGU<sup>+</sup>(x2), trnT-UGU, trnV-GAC<sup>+</sup>(x2), trnT-UAC, trnW-CCA, trnY-GUA |
| Biosynthesis                  | Maturase               | matK |
|                               | Protease               | clpP<sup>+</sup> |
|                               | Envelope membrane protein | cemA |
|                               | Acetyl-CoA carboxylase | acsD |
|                               | c-type cytochrome synthesis gene | ccsA |
| Unknown function              | Conserved hypothetical plastid Reading Frames | ycf1<sup>+</sup>(x2), ycf2<sup>+</sup>(x2), ycf3<sup>+</sup>, ycf4 |

×2 refers to genes duplicated in the IR regions; Genes marked with asterisks are with single (<sup>+</sup>) or double (<sup>+</sup>) introns.

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34.54–34.64%, 30.06–30.36%, and 42.46–42.56%, respectively. The eight rRNA genes are distributed in the IR region, resulting in the highest GC content in this region.

Plastome annotation

The subg. Cerasus plastomes contained 134 genes, which consisted of 78 protein coding genes, 31 tRNA- and 4 rRNA-coding genes (Table 2). There was a single functional protein encoding gene (rps19) deletion found in P. avium resulting in 77 protein coding genes for this species, with rps19 repeated twice in P. pseudocerasus (Lindl.) G. Don and P. yedoensis (Mats.) Yü et Li. The ycf1 gene also repeats twice in P. confodiae, P. discoides, P. pseudocerasus and P. yedoensis. Compared with trRNAs in other subg. Cerasus species, P. campanulata and P. yedoensis lacked the trnM-CAU gene. P. avium encodes the most abundant tRNA of all subg. Cerasus species studied. And this species contains the unique trnS-AGA gene. At the same time, trnM-CAU & trnT-GGU are repeated twice in P. avium. Ten protein coding genes (ndhB, ndhA, petB, petD, rpl2, rpl16, rps12, rps19, rps16, rpoC1) contained one intron, two genes (clpP, ycf3) contained two introns (Table 3).

Comparative plastome structure and polymorphism

Though the plastome is usually well conserved, contraction and expansion of IR regions is the main cause of plastome differences among different plant species [13]. The IR and SC boundaries of 20 subg. Cerasus species are shown in Fig 2. The gene arrangement and content of these species are similar in the IR/SC boundaries, which were located in the rps19 and ycf1 genes. The rps19 gene, positioned within the boundary of the LSC/IRb region of these species...
except for *P. avium*, showed the same fragment size of 279 bp in all species (Fig 2). The fragment size ranges from 175 to 239 bp in the IRb region while the tail section of the gene located in the LSC region ranged from 40 to 104 bp (Fig 2). The *ycf1* gene is a critical gene that crossed the SSC/IRa border with 4,546–4,580 bp in the SSC regions and 1,041–1,069 bp in the IRa regions. The *ndhF* gene ranged from 2,237 to 2,285 bp and was located in the boundary of the IRb/SSC region in these tested species. However, the whole *ndhF* gene was situate in the SSC region in *P. campanulata*, *P. conradinae*, *P. discoidea*, *P. emarginata*, *P. itosakura*, *P. jamasakura*, *P. kumanoensis*, *P. leveilleana*, *P. maximowiczii*, *P. pensylvanica*, *P. pseudocerasus*, and *P. spontanea*. In addition, the *trnH* gene was located in the LSC region of all tested species, with the distance of the *trnH* gene from the IRa/SSC boundary ranging from 1 to 128bp. Furthermore, the *rps19* pseudogenes spanned two regions in *P. campanulata*, *P. conradinae* and *P. pseudocerasus* at the IRa/LSC boundary, with fragment sizes of 217, 217 and 210 bp, respectively (Fig 2). The *ycf1* pseudogene with similar fragment size across the IRb/SSC boundary was also found in *P. avium*, *P. campanulata*, *P. conradinae*, *P. discoidea*, *P. pseudocerasus*, and *P. spontanea*. The pseudogene fragment sizes of *ycf1* in the above different species are 1,055, 1,045, 1,043, 1,052, 1,058, and 1,058 bp, respectively (Fig 2).

By comparing the IR and SC regions, it is clear that the full-length differences of the 20 subg. *Cerasus* plastome sequences are caused by the differences in the size of the genes at the IR/SC boundaries.

The Shuffle-LAGAN mode of mVISTA online software was employed to compare the sequence discrepancy of 20 plastomes of subg. *Cerasus* with the sequence of *P. avium* as reference (Fig 3). The plastome alignments were more conserved in the coding regions than the non-coding regions. Moreover, there was more variation in the intergenic spacer (IGS) regions: *trnK-rps16*, *rps16-trnQ*, *trnR-atpA*, *atpF-atpH*, *trnC-petN*, *trnT-psbD*, *trnT-trnL*, *ndhC-trnV*, *petA-psbJ*, *psbF-petL*, *rpl16-rps3*, *rpl32-trnS*, *trnS-trnL* (Fig 3). In addition, the coding regions with slight variation contained *accD*, *psbN*, *ndhF*, *rpl32*, *trnS*, *rps15* and *ycf1* (Fig 3). *P. emarginata* and *P. pensylvanica* exhibited polymorphisms on the intergenic region between *trnC* and *petN*, which were mainly manifested in the deletion of partial sequence fragments.

### Table 3. Information on 10 intron-containing genes in the plastome of the 20 subg. *Cerasus* species studied.

| Gene     | Location | Start   | End     | Exon I (bp) | Intron I (bp) | Exon II (bp) | Intron II (bp) | Exon III (bp) | Intron III (bp) |
|----------|----------|---------|---------|-------------|---------------|--------------|----------------|---------------|----------------|
| rps12    | IRa      | 99918   | 100711  | 26          | 536           | 233          |                |               |                |
| rps16    | LSC      | 5217    | 6349    | 30          | 863           | 40           |                |               |                |
| atpF     | LSC      | 11606   | 12907   | 410         | 747           | 145          |                |               |                |
| rpoC1    | LSC      | 20998   | 23809   | 1617        | 760           | 435          |                |               |                |
| ycf3     | LSC      | 43927   | 45919   | 153         | 777           | 228          | 709            | 126           |                |
| rps12    | IRb      | 143240  | 144033  | 232         | 536           | 26           |                |               |                |
| clpP     | LSC      | 71714   | 73781   | 228         | 668           | 289          | 812            | 71            |                |
| petB     | LSC      | 76703   | 78109   | 6           | 759           | 642          |                |               |                |
| petD     | LSC      | 78297   | 79522   | 8           | 743           | 475          |                |               |                |
| rpl16    | LSC      | 83036   | 84454   | 399         | 1011          | 9            |                |               |                |
| rpl2     | IRa      | 86241   | 87742   | 434         | 683           | 385          |                |               |                |
| ndhB     | IRa      | 96877   | 99089   | 756         | 680           | 777          |                |               |                |
| ndhA     | SSC      | 122795  | 125028  | 539         | 1142          | 553          |                |               |                |
| ndhB     | IRb      | 144889  | 147101  | 777         | 680           | 756          |                |               |                |
| rpl2     | IRb      | 156236  | 157737  | 385         | 683           | 434          |                |               |                |

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Fig 2. Comparisons of LSC, SSC and IR region boundaries among the plastomes of the 20 subg. Cerasus species studied.

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Fig 3. Sequence alignment of 20 subg. *Cerasus* plastomes with *P. avium* as the reference. The y-axis represents the percent similarity between 50% and 100%. Different colors represent different genetic regions.

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We identified a total of 99 shared genes and IGS regions from the plastomes of the 20 subg. *Cerasus* species studied (see S1 Table and S1 Fig). The nucleotide diversity (Pi) of the coding sequence (CDS) ranged from 0 to 0.05526 and the IGS ranged from 0.00070 to 0.00822. Among these, tRNA *trnG-GCC* had the most nucleotide diversity (0.05526), while the protein coding gene *ndhI*, which is located in the LSC and SSC region, has the most nucleotide variation (0.00414). The IGS regions *rpl32-trnL* and *trnR-atpA* showed highly variable polymorphism (Pi > 0.05), with the values of nucleotide diversity 0.00822 and 0.00567, respectively (S1 Table). Therefore, these four sequences could be used to develop useful makers for phylogenetic analysis and distinguishing taxa in subg. *Cerasus*.

**Codon usage pattern**

According to the codon usage analysis, overall 64 codons that encode 20 amino acids (AAs) are present across the 20 subg. *Cerasus* species studied. The protein-coding sequences of the plastomes of the 20 species consist of 26158, 26061, 26516, 26172, 26490, 26525, 26163, 26152, 26160, 26158, 26156, 26158, 26162, 26680, 26171, 26158, 26164, 26152, 26158, 26565 codons, respectively (S2 Table). Among the encoded amino acids, leucine is most frequent and cysteine least frequent. Of the 20 subg. *Cerasus* species studied, *P. campanulata* and *P. pseudocerasus* have 31 codons less frequently used than the expected usage at equilibrium (RSCU < 1) while the other 18 subg. *Cerasus* taxa showed codon usage bias (RSCU < 1) in 32 codons. All 20 subg. *Cerasus* species had 30 codons more frequently used than the expected usage at equilibrium (RSCU > 1). Codons with A and/or U in the third position take up ~43% and ~47% of all codons respectively (S2 Table). The frequency of use for the start codons AUG and UGG, encoding methionine and tryptophan, showed no bias (RSCU = 1) in all tested species. The codon UCC encoding serine in *P. campanulata* and *P. pseudocerasus* also showed no bias (RSCU = 1) (S2 Table).

**Tandem repeats and simple sequence repeats (SSRs)**

SSRs, also called microsatellites, were extensively distributed in the plastome of all species studied. Using MISA software, we found the total number of SSRs in the plastomes of the subg. *Cerasus* taxa range from 70 to 94 (S3 Table and S2 Fig). Of the mono-, di-, tri-, tetra-, penta-, and hexanucleotide categories of SSRs present in the plastomes of the subg. *Cerasus* taxa, mononucleotide repeats are the most abundant, ranging from 60.00% (*P. yedoensis*) to 71.27% (*P. leveilleana*). Pentanucleotide repeats were detected in *P. avium*, *P. kumanoensis*, *P. rufa*, *P. speciose*, *P. subhirtella*, *P. takesimensis* and *P. yedoensis*, which accounts for the low proportion of SSRs in these seven species. Except for *P. campanulata*, *P. subhirtella* and *P. yedoensis*, the rest of these species all contain hexanucleotide repeats. Among all tested subg. *Cerasus* taxa, only one trinucleotide repeat is detected in *P. pseudocerasus*.

Different types of compound SSRs were detected across the 20 subg. *Cerasus* species studied. Of all the subg. *Cerasus* species studied, *P. avium* contained the largest number of repeat types (Fig 4 and S4 Table). Among the four repeat types known, the most common repeat type is palindromic repeats, which ranged from 37.70% in *P. avium* to 59.09% in *P. rufa*, which explains why *P. rufa* contains no complement repeats.

**Phylogenetic analysis**

In order to resolve the phylogenetic placement of taxa of subg. *Cerasus* in *Prunus s.l.* and interspecific relationships within subg. *Cerasus*, we used Maximum Likelihood (ML) analysis and Bayesian Inference (BI) analyses to perform phylogenetic analyses using the complete plastome data and CDS regions of 53 published complete plastid sequences (S5 Table and S3 Fig).
Similar phylogenetic topologies were obtained in both ML and BI analyses. In these topologies, *Prunus* taxa formed a monophyletic group which diverged into two major clades with strong bootstrap support, with one clade formed of all 20 subg. *Cerasus* taxa and the other clade of 5 other *Prunus* taxa belonging to *Prunus s.l.* The 20 subg. *Cerasus* taxa formed a monophyletic group that were divided, albeit on very short branch lengths but with high support, into two principal clades (Figs 5 and S3). Clade I was basal in the monophyly of subg. *Cerasus* and contained *P. cerasoides* and *P. rufa* from central and southern Asia. Clade II was further divided, again on very short branch lengths and with mixed support, into three sub-clades. Clade IIb contained only one taxa *P. avium*, with high support in the ML tree on plastome data but weak support in trees run with CDS data, while the branch collapsed in BI analyses.

When comparing topologies based on just plastome data or CDS regions, Clade IIa and Clade IIc exhibited differences (Fig 5). Using just plastome data (Fig 5A), Clade IIc included 12 taxa, while when using data from just CDS regions (Fig 5B) it contained 14 taxa, with the additional two species, *P. emarginata* and *P. pensylvanica*. In both topologies, *P. emarginata* and *P. pensylvanica* are shown to be closely related and forming a clade, but the position of this clade, whether in Clade IIa or IIc, is unresolved. In addition, *P. matuurae* exhibits a closer
relationship with \textit{P. speciosa} in the phylogenetic trees constructed with CDS regions (Fig 5B), while the topology constructed on plastome data places it in a clade with \textit{P. maximowiczii} (Fig 5A). In both topologies, a number of moderate or strongly supported relationships were retrieved, albeit with short branch lengths: a) \textit{P. itosakura}, \textit{P. yedoensis} and \textit{P. subhirtella} formed a clade in Clade IIa; b) \textit{P. pseudocerasus} is positioned at the basal node in Clade IIc; c) \textit{P. kumanensis} and \textit{P. takesimensis} formed a sub-clade in Clade IIc; d) \textit{P. campanulata}, \textit{P. spontanea} and \textit{P. discoidea} formed a sub-clade in Clade IIc.

**Discussion**

**Variations in plastome structure**

Analyzing the genetic background and diversity of Subg. \textit{Cerasus} is a challenge. The phylogenetic, diversity, and genetic relationships based on simple chloroplast markers and sequence data were previously reported in Subg. \textit{Cerasus} [8,9].

The plastomes of angiosperms exhibit a highly conserved structure and organization of gene content [26,27]. The subg. \textit{Cerasus} plastomes also exhibit the typical quadripartite structure and similar complete plastome sequence size of angiosperms, ranging from 157,458 bp in \textit{P. emarginata} to 158,024 bp in \textit{P. discoidea} (Table 1 and Fig 1). Previous studies on angiosperms have revealed that the number of genes in plastomes ranges from 120 to 130 [26]. However, in this study, subg. \textit{Cerasus} plastomes contained 134 genes, including 78 protein coding genes, 31 tRNA- and 4 rRNA-coding genes (Table 2). The plastomes among these species were similar in intron and GC contents (Tables 1 and 3). The number of genes containing introns was 12, suggesting that the intron contents in subg. \textit{Cerasus} are also similar to those of most flowering plant clades [16]. However, the GC content in the IR regions was significantly higher than that in the LSC and SSC regions. The main reason for this is that all eight rRNA genes with high GC contents are distributed in the IR region. In general, the IR region is the most conserved region of the plastome [15], and expansion and contraction of the IR regions are the main causes of different plastome lengths [13,28]. In this study, we found only small changes in the organization of genes in the IR region and boundary between the IR and LSC and SSC regions in the 20 plastomes studied. The distribution of genes across the SC-IR boundaries, such as \textit{rps19}, \textit{ndhF}, \textit{ycf1}, etc. are similar to that of other \textit{Prunus} species [15] (Fig 2).
Variation of SSRs
SSRs are highly polymorphic and codominantly inherited, and are seen as valuable markers for studies involving gene diversity, population genetics and gene mapping. Previous research found that SSRs can be widely used as important resources of molecular markers and these have been broadly applied in phylogenetic and biogeographic studies [29,30]. We counted the quantities of SSRs for the 20 species in subg. *Cerasus*, with the largest number of SSR types being mononucleotide repeats, which is consistent with results from other studies of angiosperms [12,15,16]. In the plastomes of subg. *Cerasus* taxa, the number of SSRs was found to be significantly higher than that in other angiosperms, and the content of A/T repeats is far greater than that of G/C repeats, similar to the results of Melotto-Passarin and other studies [31,32] (S3 Table).

Polymorphism in IGS and CDS
Additionally, we also analyzed the sequence polymorphism of these 20 subg. *Cerasus* taxa in both IGS and CDS regions (S1 Table and S1 Fig). Consistent with the diversity patterns found in most angiosperms [16,33,34], sequence divergence in IGS regions was higher than that in CDS regions. We then calculated the Pi value of the coding genes and six IGS regions in the complete sequence and screened out the Pi value more than 0.05. Among these sequences, *trnG-GCC*, *ndhI*, *rpl32-trnL* and *trnR-atpA* have been previously known as hypervariable regions in *Prunus* species [15], and we speculate that these fragments with high polymorphism can be used as hot spots for developing molecular markers, allowing a more efficient study of phylogenetic relationships within subg. *Cerasus*.

Phylogenetic relationships
Both molecular and morphological evidence suggests that subg. *Cerasus* had a complex evolutionary history. In order to obtain a clearer understanding of phylogenetic relationships within Rosaceae and *Prunus s.l.*, previous studies selected either a few gene fragments or the complete plastome to reconstruct the phylogenetic framework [7–9,35–40].

Our study indicates that taxa of subg. *Cerasus* with corymbs are monophyletic and with a clear sister relationship to other single-flowered and racemose groups within *Prunus s.l.* (Figs 5 and S3). Our selection of outgroups for the phylogenetic analysis was based on the large-scale phylogenomic study of Rosaceae [7], with the topological structure retrieved in this study similar to our own. These similarities include high support for a sister relationship of Amygdaleae and Sorbarieae (S3 Fig) and subg. *Cerasus* being positioned in Amygdaleae as a monophyletic group. This result is also consistent with other previous studies on the phylogeny of *Prunus s.l.* [8].

Plastid phylogenomics provides one possible solution for studies focused on problematic phylogenetic relationships [41]. The relationships between taxa of subg. *Cerasus* retrieved in our study differed from previous studies that did not incorporate plastid phylogenomics. For example, Shi et al. found *P. avium* and *P. pseudocerasus* to be closely related, while our study suggests *P. avium* to be basally positioned (Clade IIb) in the monophyletic subg. *Cerasus* clade, with *P. pseudocerasus* placed basal in Clade IIC [8]. In their study, subg. *Cerasus* was divided into two clades. However, the phylogenetic topology in our study based on two methods (ML and BI) found that subg. *Cerasus* was also divided into two major clades with high support but a short branch length. Clade I contains *P. cerasoides* and *P. rufa*, with a central and southern Asian distribution.

Placement of certain taxa within Clade II was also unresolved due to inconsistencies when comparing topologies based on complete plastome or CDS datasets. This includes the *P.
emarginata + P. pensylvanica clade being placed in Clade IIa or IIc, depending on complete plastome or CDS data, respectively. CDS data also provided greater resolution on certain relationships, such as placing P. matuurae with P. speciosa in a clade that is rooted in a large polytomy in clade IIc. While the topology based on complete plastome shows no such relationship, with both species held in the large polytomy. The causes of the insignificant divergence of subg. Cerasus taxa may be due to quantum speciation or low resolution of the markers. Widespread interspecific hybridisations also have complicated the taxonomy of this subgenus [6].

Nevertheless, certain relationships in Clade II were well-supported and consistent. Despite an unresolved placement within Clade II, the relationship between P. emarginata and P. pensylvanica was notable, with these species sharing similar geographical distribution and habitat in North American. High support for the basal position of P. pseudocerasus in Clade IIc was also found in all analyses. Prunus campanulata, P. spontanea and P. discoidea formed a sub-clade in Clade IIc. P. kumanoensis and P. takesimensis, these species with an eastern Asian distribution, also consistently formed a well-supported sub-clade in Clade IIc in all analyses. While their relationship with P. emarginata and P. pensylvanica is still unresolved, P. itosakura, P. yedoensis and P. subhirtella showed a well-supported relationship and were consistently grouped in Clade IIa.

Conclusions
In this study, we revealed the plastome size, GC content, and gene number and order among 20 subg. Cerasus species. We identified highly polymorphic regions of nucleotide variation that are potential molecular markers for identifying and overcoming phylogenetic problems at the species level. We found that SSRs in our plastomes might also provide intraspecific level polymorphic markers that can be used for population genetic studies.

The phylogenetic trees constructed based on complete plastome and CDS regions all exhibited similar topologies, albeit with certain irregularities and discords and a lack of resolution within certain clades. We find support for P. cerasoides and P. rufa as basal in the monophyly of subg. Cerasus, as well as a close relationship between certain groups of taxa. Undoubtedly, taxa of subg. Cerasus are a monophyletic group sister to taxa of Prunus s.l., but some infrageneric relationships within subg. Cerasus remain unresolved, as can be seen by certain low bootstrap values and changing positions of taxa depending on datasets used. Thus, it is particularly important to explore characteristics of repeat elements and in-depth information mining of plastomes in future research as well as inclusion of greater taxon sampling to help clarify these relationships.

Supporting information
S1 Fig. Comparative analysis of the nucleotide diversity by Pi values of the 20 subg. Cerasus species studied.
(PDF)

S2 Fig. Number of motif types in SSRs in 20 subg. Cerasus complete plastomes.
(PDF)

S3 Fig. Combined ML and BI phylogenetic trees of 20 subg. Cerasus species based on either (a) complete plastome data or (b) coding region (CDS) data. The support value is displayed above the branch in the order of Maximum Likelihood bootstrap support and Bayesian Inference posterior probability. “-” indicates the branch collapse in the Bayesian tree.
(PDF)
S1 Table. The statistics of nucleotide diversity (Pi) between the species of 20 subg. *Cerasus* chloroplast genome.

(DOCX)

S2 Table. Codon features of 20 Subg. *Cerasus* complete plastomes.

(DOCX)

S3 Table. Number of analyses of simple sequences repeats (SSRs) in 20 subg. *Cerasus* complete plastomes.

(DOCX)

S4 Table. The statistics of four repeat types in 20 subg. *Cerasus* complete plastomes.

(DOCX)

S5 Table. Summary for all the information of 33 species.

(DOCX)

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**References**

1. Koehne E. *Prunus* L. In: Sargent CR, editor. Plantae Wilsonianae. 2: Portland: Dioscorides Press; 1913. pp. 196–282.

2. Krussmann G. Manual of Cultivated broad-leaved trees and shrubs. Timber Press. 3: Portland: Timber Press; 1986. https://doi.org/10.1073/pnas.83.24.9581 PMID: 16593791

3. Rehder A. Manual of Cultivated Trees and Shrubs Hardy in North America Exclusive of the Subtropical and Warmer temperate Regions: MacMillan, New York; 1940.

4. Wang XR. An Illustrated Monograph of Cherry Cultivars in China: Science Press, Beijing; 2014.

5. Yu TT, Lu LT, Ku TC, Li CL, Chen SX. Rosaceae (3) Prunoideae. In: Yu TT, editor. Flora Reipublicae Popularis Sinicae, Tomus 38: Science Press, Beijing; 1986. pp. 1–133.

6. Ohta S, Yamamoto T, Nishitani C, Katsuki T, Iketani H, Omura M. Phylogenetic relationships among Japanese flowering cherries (*Prunus* subgenus *Cerasus*) based on nucleotide sequences of chloroplast DNA. Plant Sys Evol. 2007; 263(3): 209–225. https://doi.org/10.1007/s00606-006-0474-1

7. Zhang SD, Jin JJ, Chen SY, Chase MW, Soltis DE, Li HT. Diversification of Rosaceae since the Late Cretaceous based on plastid phylogenomics. New Phytol. 2017; 214(3): 1355–1367. https://doi.org/10.1111/nph.14461 PMID: 28186635.
8. Shi S, Li J, Sun J, Yu J, Zhou S. Phylogeny and Classification of *Prunus sensu lato* (Rosaceae). J Integr Plant Biol. 2013; 55(11): 1069–1079. https://doi.org/10.1111/jipb.12095 PMID: 23945216.

9. Potter D, Eriksson T, Evans RC, Oh S, Smedmark JEE, Morgan DR, et al. Phylogeny and classification of Rosaceae. Plant Syst Evol. 2007; 266(1): 5–43. https://doi.org/10.1007/s00607-007-0539-9

10. Sun K, Liu QY, Wang A, Gao YW, Zhao LC, Guan WB. Comparative Analysis and Phylogenetic Implications of Plastomes of Five Genera in Subfamily Amyridoideae (Rutaceae). Forests. 2021; 12: 277. https://doi.org/10.3390/f12030277

11. Wicke S, Schneeweiss GM, de Pamphilis CW, Müller KF, Quandt D. The evolution of the plastid chromosome in land plants: gene content, gene order, gene function. Plant Mol Biol. 2011; 76(3): 273–297. https://doi.org/10.1007/s11103-011-9762-4 PMID: 21424877.

12. Watanabe O, Yamada Y, Ohara H, Tanaka Y, Hori C, et al. Comparative analysis of plastid genomes among *Prunus mume*, *P. armeniaca*, and *P. salicina*. Hortic Res. 2019; 6: 89. https://doi.org/10.1038/s41438-019-0171-1 PMID: 31666958.

13. Lee SR, Kim K, Lee BY, Lim CE. Complete chloroplast genomes of all six *Hosta* species occurring in Korea: molecular structures, comparative, and phylogenetic analyses. BMC Genomics. 2019; 20(1): 833. https://doi.org/10.1186/s12864-019-6215-y PMID: 31706273.

14. Kurtz S, Choudhuri JV, Ohlebusch E, Schleiermacher C, Stoye J, Giegerich R. REPuter: the manifold applications of repeat analysis on a genomic scale. Nucleic Acids Res. 2001; 29(22): 4633–4642. https://doi.org/10.1093/nar/29.22.4633 PMID: 11713313.

15. Huelsenbeck JP, Ronquist J, Fredrik J, MrBayes. Bayesian inference of phylogenetic trees. Bioinformatics. 2001; 17(8): 754–755. https://doi.org/10.1093/bioinformatics/17.8.754 PMID: 11524383.

16. Daniell H, Lin CS, Yu M, Chang WJ. Chloroplast genomes: diversity, evolution, and applications in genetic engineering. Genome Biol. 2016; 17(1): 134. https://doi.org/10.1186/s13059-016-1004-2 PMID: 27339192.

17. Palmer JD. Comparative organization of chloroplast genomes. Annu Rev Genet. 1985; 19(1):325–354. https://doi.org/10.1146/annurev.ge.19.120185.001545 PMID: 3936406.

18. Ravi V, Khurana JP, Tyagi AK, Khurana P. An update on chloroplast genomes. Plant Syst Evol. 2008; 271: 101–222. https://doi.org/10.1007/s00606-007-0608-0
29. Pauwels M, Vekemans X, Godé C, Frérot H, Castric V, Saumitou-Laprade P. Nuclear and chloroplast DNA phylogeography reveals vicariance among European populations for the study of metal tolerance, Arabidopsis halleri (Brassicaceae). 2012; 193(4): 916–928. https://doi.org/10.1111/j.1469-8137.2011.04003.x PMID: 22225532.

30. Powell W, Morgante M, McDevitt R, Vendramin GG, Rafalski JA. Polymorphic simple sequence repeat regions in chloroplast genomes: applications to the population genetics of pines. Proc Natl Acad Sci USA. 1995; 92(17): 7759–7763. https://doi.org/10.1073/pnas.92.17.7759 PMID: 7644491.

31. Martin G, Baurens FC, Cardi C, Aury JM, D'Hont A. The complete chloroplast genome of banana (Musa acuminata, Zingiberales): insight into plastid monocotyledon evolution. PLoS One. 2013; 8(6): e67350–e. https://doi.org/10.1371/journal.pone.0067350 PMID: 23840670.

32. Melotto-Passarin DM, Tambarussi E, Dressano K, De Martin V, Carrer H. Characterization of chloroplast DNA microsatellites from Saccharum spp and related species. Genet Mol Res. 2011; 10(3): 2024–2030. https://doi.org/10.4238/vol10-3gmr1019 PMID: 21948764.

33. Gao X, Zhang X, Meng H, Li J, Zhang D, Liu C. Comparative chloroplast genomes of Paris Sect. Marmorata: insights into repeat regions and evolutionary implications. BMC Genomics. 2018; 19(10): 878. https://doi.org/10.1186/s12864-018-5281-x PMID: 23840670.

34. Ruisen L, Li P, Qiu YX. The Complete Chloroplast Genomes of Three Cardiocrinum (Liliaceae) Species: Comparative Genomic and Phylogenetic Analyses. Front Plant Sci. 2017; 7: 2054. https://doi.org/10.3389/fpls.2016.02054 PMID: 28119727.

35. Bortiri E, Oh SH, Gao FY, Dan P. The phylogenetic utility of nucleotide sequences of sorbitol 6-phosphate dehydrogenase in Prunus (Rosaceae). Amer J Bot. 2002; 89(10): 1697–1708. https://doi.org/10.3732/ajb.89.10.1697 PMID: 21665596.

36. Chang KS, Chang CS, Park TY, Roh MS. Reconsideration of the Prunus serrulata complex (Rosaceae) and related taxa in eastern Asia. Bot J Linn Soc. 2007; 154(1): 35–54. https://doi.org/10.1111/j.1095-8339.2007.00631.x

37. Lee S, Wen J. A phylogenetic analysis of Prunus and the Amygdaloideae (Rosaceae) using ITS sequences of nuclear ribosomal DNA. Amer J Bot. 2001; 88(1): 150–160. https://doi.org/10.2307/2657135 PMID: 11159135.

38. Morgan D, Solits D, Robertson K. Systematic and Evolutionary Implications of rbcL Sequence Variation in Rosaceae. Amer J Bot. 1994; 81(7): 890–903. https://doi.org/10.2307/2445770

39. Shimada T, Hayama H, Nishimura K, Yamaguchi M, Yoshida M. The genetic diversities of 4 species of subg. Lithocerasus (Prunus, Rosaceae) revealed by RAPD analysis. Euphytica. 2001; 117(1): 85–90. https://doi.org/10.1023/A:10043327542.

40. Bortiri E, Oh SH, Jiang J, Baggett S, Granger A, Weeks C, et al. Phylogeny and Systematics of Prunus (Rosaceae) as Determined by Sequence Analysis of ITS and the Chloroplast trnL-trnF Spacer DNA. Syst Bot. 2001; 26(4): 797–807. https://doi.org/10.1023/A:1004193327542

41. Jansen RK, Cai Z, Raubeson LA, Daniell H, Depamphilis CW, Leebens-Mack J, et al. Analysis of 81 genes from 64 plastid genomes resolves relationships in angiosperms and identifies genome-scale evolutionary patterns. Proc Natl Acad Sci USA. 2007; 104(49): 19369–19374. https://doi.org/10.1073/pnas.0709121104 PMID: 18048330.