RESEARCH ARTICLE

Complete chloroplast genome of *Hordeum brevisubulatum*: Genome organization, synonymous codon usage, phylogenetic relationships, and comparative structure analysis

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Abstract

**Background**

*Hordeum brevisubulatum*, known as fine perennial forage, is used for soil salinity improvement in northern China. Chloroplast (cp) genome is an ideal model for assessing its genome evolution and the phylogenetic relationships. We *de novo* sequenced and analyzed the cp genome of *H. brevisubulatum*, providing a fundamental reference for further studies in genetics and molecular breeding.

**Results**

The cp genome of *H. brevisubulatum* was 137,155 bp in length with a typical quadripartite structure. A total of 130 functional genes were annotated and the gene of *accD* was lost in the process of evolution. Among all the annotated genes, 16 different genes harbored introns and the genes of *ycf3* and *rps12* contained two introns. Parity rule 2 (PR2) plot analysis showed that majority of genes had a bias toward T over A in the coding strand in all five *Hordeum* species, and a slight G over C in the other four *Hordeum* species except for *H. bogdanii*. Additionally, 52 dispersed repeat sequences and 182 simple sequence repeats were identified. Moreover, some unique SSRs of each species could be used as molecular markers for further study. Compared to the other four *Hordeum* species, *H. brevisubulatum* was most closely related to *H. bogdanii* and its cp genome was relatively conserved. Moreover, inverted repeat regions (IRa and IRb) were less divergent than other parts and coding regions were relatively conserved compared to non-coding regions. Main divergence was presented at the SSC/IR border.
Conclusions

This research comprehensively describes the architecture of the *H. brevisubulatum* cp genome and improves our understanding of its cp biology and genetic diversity, which will facilitate biological discoveries and cp genome engineering.

Introduction

Soil salinity is a serious threat to plant growth. Consequently, the breeding of salt-tolerant plants plays a vital role in the exploitation and utilization of saline land [1]. *Hordeum brevisubulatum* (Trin.) Link, or wild barley, is widely distributed in northern China and is known for its use as fine perennial forage. The plant is characterized by its adaptability, good palatability for livestock, and high regeneration capacity. Wild barley can also be used ecologically for soil improvement since it is one of the most salt-tolerant Triticeae species [2]. *H. brevisubulatum* has been domesticated for nearly 50 years in China. Since 1976, *H. brevisubulatum* has been promoted in Jilin Province because of its higher biomass and seed yield than *H. bogdanii* [3, 4]. The first cultivar of *H. brevisubulatum*, 'Junxu No.1', was approved in China in 2003 [5]. However, it took nearly 10 years to breed this variety by conventional breeding methods, which included chemical mutagenesis, fine individual selection and cultivation, variety comparison testing, regional testing, and promotion testing. The use of conventional breeding to create new cultivars generally requires at least 10 years. In contrast, molecular breeding is economical and effective, shortens breeding time, and has been used extensively in various plants including cereals, vegetables, fruits, cash crops, and ornamental plants [6]. Molecular breeding is based on genetic information and has helped developing many new cultivars with high yield, quality, and resistance to stress. Until now, research on *H. brevisubulatum* has focused mainly on its biological characteristics, salt tolerance mechanisms, and disease resistance [1, 7, 8]. The lack of genetic information on *H. brevisubulatum* slows the pace of cultivation of different varieties. In addition, although *H. brevisubulatum* has been studied as a model to understand the salt-tolerance mechanisms of barley cereal crops, the genetic relationship between barley and *H. brevisubulatum* is still unclear. This knowledge would enable scientists to exploit *H. brevisubulatum* germplasm and accelerate the pace of breeding [9].

Owing to advancements in high-throughput sequencing, nearly 22,000 chloroplast (cp) genomes have been completely identified and deposited in the National Center for Biotechnology Information (NCBI) as of early April 2020. Cp is the most important plastid and semi-autonomous organelle containing independent DNA information. Among the three independent genomes with genetic information in plants, the cp genome is conservative and relatively small in size. In general, cp genomes are usually between 107 and 218 kb and encode 120–130 unique genes in a typical quadripartite cycle comprising a pair of inverted repeat regions (IRa and IRb). The regions are separated by a large single copy (LSC) region and a small single copy (SSC) region [10]. The cp genome in conifers, algae, and some legume species is rearranged repeatedly mainly due to expansion, contraction, or the loss of IR regions [10–13]. For the majority of higher plants, the cp genome is highly conservative in gene number, arrangement order, and function [10, 14], since information carried on the cp genome is inherited only from the female parent, and fewer nucleotide substitutions and genome structure rearrangements have occurred compared to the nuclear genome [15–17]. Hence, as an ideal model to unravel genome evolution and the phylogenetic relationships in complex angiosperm families [18, 19], the cp genome has been used widely in gene mapping, variety identification, plant
barcode sequence screening, population genetics, gene diversity studies, and molecular assisted breeding [10, 20]. Therefore, analysis of cp genome organization in *H. brevisubulatum*, its phylogenetic relationship and structure comparative analysis with other *Hordeum* species would be both beneficial and interesting.

In the current study, the whole cp genome of *H. brevisubulatum* was constructed by initially using next-generation sequencing and applying a combination of *de novo* and reference-guided assembly. Then, the whole cp genome sequence of *H. brevisubulatum* was described, repeat sequences and simple sequence repeats (SSRs) were analyzed. Furthermore, an evolutionary phylogenetic tree was constructed and the cp genome structure of *H. brevisubulatum* was compared with another four *Hordeum* species. Results from this study could provide fundamental genetic reference for future biological research and molecular breeding programs of *H. brevisubulatum*.

**Materials and methods**

**Plant material**

Fresh leaf samples of *H. brevisubulatum* were collected in July 2019 from the Lanzhou Scientific Observation and Experiment Field Station of the Ministry of Agriculture for Ecological Systems in the Loess Plateau area (36°01’N, 103°45’E, and altitude 1700 m above sea level), Lanzhou Institute of Husbandry and Pharmaceutical Science, Chinese Academy of Agricultural Sciences, Gansu, China. The voucher specimen was formally identified by an expert on plant taxonomy and kept in the Herbarium of Lanzhou Institute of Husbandry and Pharmaceutical Science, Chinese Academy of Agricultural Sciences (CYSLS-HbWang20190722). Samples were frozen immediately in liquid nitrogen, conserved in drikold and delivered to Beganen Tech Solution CO., Ltd (Wuhan, China) for cp genome extraction and sequencing, and then the data were assembled and further analyzed by Genepioneer Biotechnologies Co., Ltd (Wuhan, China).

**DNA extraction, sequencing, and assembly**

Genomic DNA was isolated by the Plant Genomic DNA Rapid Extraction Kit (Biomed Gene Technology) by the modified CTAB method [21]. A Qubit Fluorometer (Invitrogen) and 1% agarose gel electrophoresis were used to detect DNA integrity and quality. A library (350 bp) was constructed using pure DNA according to the manufacturer’s instructions (NEBNext Ultra™ DNA Library Prep Kit for Illumina®). The library was sequenced with an Illumina NovaSeq platform and 150 bp paired-end reads were generated. Among the Illumina PCR adapter reads, more than 5% of reads of unknown origin and low-quality reads were filtered using SOAPnuke software (version: 1.3.0). A total of 6.8 GB of clean data was yielded for *H. brevisubulatum*. Bowtie 2 v2.2.4 software was used to compare the cp genome database built by Genepioneer Biotechnologies Co. Ltd and the matching clean reads were selected for subsequent assembly. The corrected sequence was arranged according to the procedure described by Sun *et al.* [22] to obtain the complete circular cp genome sequence.

**Annotation and analysis of the Cp genome sequences**

Three methods were used to annotate the sequences. Prodigal (v2.6.3) software was used to predict coding DNA sequence, Hmmer v3.1b2 was used to predict rRNA and Aragorn v 1.2.38 was used to predict tRNA of the cp genome sequence and obtain the first annotation result. Blast (version: BLAST 2.+, E-value ≤1e-5) software was used to compare the assembled sequence with its closely related species (*H. bogdanii*, NC 043839.1) to remove nuclear genome
sequences and obtain the second annotation result. The final cp genome gene annotation was obtained after manual correction. The circular genome map of *H. brevisubulatum* was drawn using the OGDRAW v1.2 program [23]. The SSRs were analyzed using the Perl script MISA V1.0, and the minimum repeats of mono-, di-, tri-, tetra-, penta- and hexanucleotide were set to 8, 5, 3, 3, 3 and 3, respectively [22]. Vmatch v2.3.0 software identified dispersed repeats including forward, reverse, complement, and palindromic match repeats with a minimal length of 30bp, and hamming distance of 3 [11, 24, 25]. Tandem repeats were identified using Tandem Repeats Finder v. 4.09 [26]. Nucleotide A, T, C and G content at the third position of synonymous codons was acquired via the program Codon W (version 1.3, [https://sourceforge.net/projects/codonw/](https://sourceforge.net/projects/codonw/)). Parity rule 2 (PR2) analysis was employed to examine the nucleotide usage bias in the coding genes of *Hordeum species* [27].

**Phylogenetic analysis**

Thirty-five species, including 5 species from *Hordeum*, were used for phylogenetic analysis. The cp genomes of another 34 species in fasta format were downloaded from NCBI database. Multiple sequence alignment was done using MAFFT software, which was trimmed by trimAl (v1.4.rev15), and then the (GTR)+G model was used in RaxML (v 8.2.10) software, as suggested, by 1000 bootstrap replicates with the default tree search algorithm of hill-climbing. The phylogenetic tree was constructed with the corresponding GeneBank number labeled near each species.

**Genome structure comparison**

Based on the above results of the phylogenetic analysis, the complete cp genomes of five *Hordeum* species were compared using the mVista program with the shuffle-LAGAN mode using the annotation of *H. brevisubulatum* as reference [24]. The IRscope tool [28] was used to visualize the genes on the boundaries of the junction sites of the five *Hordeum* cp genomes.

**Results**

**Features of *H. brevisubulatum* Cp genome**

The complete cp genome of *H. brevisubulatum* was 137,155 bp in length with a typical quadripartite structure, containing a pair of IR regions (43,174 bp), a LSC region of 81,175 bp, and a SSC region of 12,806 bp (Fig 1). The nucleotide composition of the *H. brevisubulatum* cp genome was biased towards A and T. All regions had different AT contents but all were AT rich. The AT content of the LSC, SSC, and IR regions and the whole cp genome were 63.80%, 67.91%, 56.14%, and 61.77%, respectively. It was obvious that the AT content of the IR region was lower than that of the LSC and SSC regions (Table 1). Base composition asymmetry (AT, CG) was found in a single strand of the *H. brevisubulatum* cp genome, except for in the IR region (Table 1).

Fifty, fifty, fifty-one and fifty-one Coding sequences (CDSs) with lengths larger than 300 bp were screened from the cp genomes of *H. bogdanii*, *H. vulgare* subsp. *vulgare*, *H. vulgare* subsp. *spontaneum* and *H. jubatum*, respectively. Together with 51 CDSs of *H. brevisubulatum*, they were used to carry out PR2 plot mapping analysis which was constructed to show the relationship between the values $A_3/(A_3 + T_3)$ and $G_3/(G_3 + C_3)$, and the data were distributed into four quadrants in a scatter diagram (Fig 2A–2E). It could be seen that along the ordinate, all five *Hordeum* species presented similar distributions with a majority of genes located in the third and fourth quadrants (in which the ratio of $A_3/(A_3 + T_3) < 0.5$). However, along the abscissa, there were two types of distributions. A slightly larger number of genes of *H.*
Fig 1. Cp genome map of *H. brevisubulatum*. Genes inside the circle are transcribed clockwise, and those outside are transcribed counter-clockwise. Genes of different functions are color-coded. The darker gray in the inner circle shows the GC content, while the lighter gray shows the AT content. The red and blue lines indicate GC skew, the red means GC skew greater than zero while the blue means GC skew smaller than zero.

![Chloroplast genome of *Hordeum brevisubulatum*](https://doi.org/10.1371/journal.pone.0261196.g001)

Table 1. Base composition in a single strand of the *H. brevisubulatum* cp genome.

| Region | A (%) | T (U) (%) | C (%) | G (%) | A+T (%) | G+C (%) |
|--------|-------|-----------|-------|-------|---------|---------|
| LSC    | 31.74 | 32.06     | 17.92 | 18.28 | 63.8    | 36.2    |
| SSC    | 36.2  | 31.71     | 16.58 | 15.51 | 67.91   | 32.09   |
| IR     | 28.07 | 28.07     | 21.93 | 21.93 | 56.14   | 43.86   |
| Total  | 31    | 30.77     | 19.06 | 19.17 | 61.77   | 38.23   |

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brevisubulatum (Fig 2A), H. vulgare subsp. vulgare (Fig 2B), H. vulgare subsp. spontaneum (Fig 2C) and H. jubatum (Fig 2D) were distributed on the G< side than on the G>C side, while an equal amount genes of H. bogdanii were distributed on both sides (Fig 2E).

In the cp genome of H. brevisubulatum, a total of 130 functional genes were annotated including 83 protein-coding genes, 39 tRNA genes, 8 rRNA genes. All of these genes were classified into four categories (S1 Table). The protein-coding genes, tRNA genes, and rRNA genes accounted for 63.85%, 30.00%, and 6.15% of all of the annotated genes, respectively. Among the annotated genes, 22 genes were duplicated in the IR regions, including 4 rRNA genes (rrn4.5, rrn5, rrn16, and rrn23), 10 protein coding genes (ndhB, ndhH, rpl2, rpl23, rps15, rps12, rps19, rps7, ycf2 and ycf3), and 8 tRNA genes.

In the H. brevisubulatum cp genome, 16 different genes harbored one intron, including 6 tRNA genes (trnK-UUU, trnG-UCC, trnL-UAA, trnV-UAC, trnL-GAU, and trnA-UGC) and 10 protein coding genes, whereas the protein coding genes of ycf3 and rps12 contained two introns (S1 Table).

Repeat sequence and SSRs analysis

Five categories of repeat sequences were detected and analyzed in the five Hordeum cp genomes (Table 2). The number of repeats was highest in H. jubatum (147) and lowest in H. vulgare subsp. vulgare (126). Tandem, palindromic and forward were the most common repeat sequence type. Complement and reverse repeats were found only in H. jubatum. In five Hordeum species, majority of the tandem repeats were less than 10 bp, more than half forward repeats were between 30–35 bp, and up to a half of palindromic repeats were between 30–35 bp (Table 2).

Except for the IR regions, a total of 52 dispersed repeats were found in H. brevisubulatum cp genome, including 34 forward and 18 palindromic repeats, with no reversed and complement repeats detected (S2 Table). In identified repeats, 35 repeats were 30–39 bp in length, 7 repeats were 40–49 bp, 10 repeats were longer than 50 bp and the longest repeat was 157 bp (S2 Table). As shown in S2 Table, a total of 31, 2, 2, and 2 repeats were detected in the LSC, SSC, IRa, and IRb regions, respectively. Twelve repeats were located in both the LSC and IRa/
IRb regions and 3 repeats were located in both the IRA and IRb regions. The majority of repeat sequences were located in the intergenic spaces (IGS) (37%) and gene coding regions (38%, namely ropC2, rps18, psaA, psaB, infA, rps3, and psbM), while a minority of the repeats were detected in the intron (13%, namely the intron of rps12, ndhB and ycf3). It was worth noting that the ropC2 gene possessed the largest number of repeats.

A total of 182 SSRs were identified in the H. brevisubulatum cp genome using MISA perl script (S3 Table). Among these SSRs, 58 types of repeat units were found, namely, 19 mononucleotides, 5 dinucleotides, 23 trinucleotides, 9 tetranucleotides, and 1 pentanucleotide (CCATA) (S3 Table). Of the 182 total SSRs, 120 SSRs were mononucleotides, accounting for 66%, and only 4 (2 of type C9 and 1 each of type G8 and G9) did not contain A or T (Table 2). Moreover, oligo A and oligo T made up 29% and 35% of the total 182 SSRs, respectively in the H. brevisubulatum cp genome. The SSR varied in number and type depending on the species. H. jubatum had the most SSRs (187) and H. vulgare subsp. spontaneum had the least (180) (Table 2). H. brevisubulatum had the most types (58) and H. vulgare subsp. spontaneum had the least (52) types of repeat units. A15, T14 and T16 only existed in H. brevisubulatum, T21 only existed in H. bogdanii, TA7 only existed in H. jubatum, while GCT3, TAT13 and TTTG3 only existed in H. vulgare subsp. spontaneum (Fig 3).

### Table 2. Summary of repeat sequences and SSRs in five Hordeum chloroplast genomes.

| Species                  | Hordeum brevisubulatum | Hordeum vulgare subsp. vulgare | Hordeum jubatum | Hordeum vulgare subsp. spontaneum | Hordeum bogdanii |
|--------------------------|------------------------|--------------------------------|-----------------|-----------------------------------|------------------|
| Total dispersed repeat   | 53                     | 47                             | 70              | 47                                | 45               |
| Complement               | 0                      | 0                              | 2               | 0                                 | 0                |
| Forward                  | 34                     | 30                             | 43              | 31                                | 29               |
| 30-35bp                  | 21                     | 19                             | 22              | 19                                | 19               |
| 36-45bp                  | 7                      | 8                              | 10              | 9                                 | 7                |
| 46-65bp                  | 3                      | 0                              | 4               | 0                                 | 2                |
| 66-100bp                 | 1                      | 1                              | 4               | 1                                 | 0                |
| >100bp                   | 2                      | 2                              | 3               | 2                                 | 1                |
| Palindromic              | 19                     | 17                             | 23              | 16                                | 16               |
| 30-35bp                  | 8                      | 8                              | 9               | 8                                 | 8                |
| 36-45bp                  | 4                      | 4                              | 4               | 4                                 | 4                |
| 46-65bp                  | 3                      | 0                              | 4               | 0                                 | 2                |
| 66-100bp                 | 1                      | 1                              | 2               | 1                                 | 0                |
| >100bp                   | 3                      | 4                              | 4               | 3                                 | 2                |
| Reverse                  | 0                      | 0                              | 2               | 0                                 | 0                |
| Total tandem repeat      | 85                     | 79                             | 77              | 80                                | 84               |
| <10bp                    | 61                     | 64                             | 59              | 64                                | 61               |
| 10-20bp                  | 19                     | 13                             | 12              | 14                                | 18               |
| 21-30bp                  | 5                      | 2                              | 6               | 2                                 | 5                |
| Total SSR number         | 182                    | 181                            | 187             | 180                               | 182              |
| mononucleotide           | 120                    | 122                            | 128             | 122                               | 119              |
| dinucleotide             | 6                      | 6                              | 6               | 6                                 | 7                |
| trinucleotide            | 45                     | 42                             | 42              | 41                                | 45               |
| tetranucleotide          | 10                     | 10                             | 10              | 10                                | 10               |
| hexanucleotide           | 1                      | 1                              | 1               | 1                                 | 1                |

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Phylogenetic analysis

In the current work, the published cp genomes of 31 species from Triticeae, Gramineae family, namely 10 Aegilops species, 2 Elymus species, 1 Eremopyrum species, 4 Hordeum species, 1 Leymus species, 2 Psathyrostachys species, 1 Secale species, 3 Thinopyrum species, and 7 Triticum species, were downloaded from NCBI to explore the phylogenetic relationship with H. brevisubulatum. In addition, 3 important crops belonging to the Gramineae family, including Zea mays from Maydeae, Sorghum bicolor from Andropogoneae, and Oryza sativa from Oryzeae were used as outgroups to construct the evolutionary phylogenetic tree. The results showed that Hordeum species had a closer relationship with species from Aegilops, Triticum, Secale, Thinopyrum, Elymus, Thinoopyrum, and Eremopyrum than with species from Psathyrostachys and Leymus in Triticeae (Fig 4). H. jubatum, H. brevisubulatum, H. bogdani, H. vulgare subsp. vulgare, and H. vulgare subsp. spontaneum clustered into a big group with much higher internal resolution. Moreover, as shown in Fig 4, all 5 Hordeum species were divided into two subgroups. H. vulgare subsp. vulgare was close to H. vulgare subsp. spontaneum, and separated from H. jubatum, H. brevisubulatum and H. bogdani. In the subgroup, H. brevisubulatum clustered closer to H. bogdani than to H. jubatum.

Comparative analysis of genome structure

The complete cp genome sequence of H. brevisubulatum was compared to four other Hordeum species (Table 3). H. brevisubulatum had the largest cp genome size and the largest LSC and SSC region, while H. vulgare subsp. vulgare had the smallest cp genome, smallest LSC, and the most annotated genes. The LSC length of H. brevisubulatum was 12,806 bp, which was 105 bp, 140 bp, 28 bp and 24 bp longer than H. vulgare subsp vulgare, H. jubatum, H. vulgare subsp. spontaneum, and H. bogdani, respectively. The IR regions of H. brevisubulatum, H. bogdani, H. vulgare subsp. vulgare and H. vulgare subsp. spontaneum were nearly the same length, but H. jubatum was a little shorter. As a domesticated barley variety, H. vulgare subsp. vulgare had the smallest genome size but encoded the most genes, 8–10 genes more than other four species,
Fig 4. Phylogenetic relationships of 35 species based on complete cp genomes. The bootstrap values were based on 1000 replicates and are shown next to the branches. The cp genome accession numbers from GenBank are labeled near the names of plant species.

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which were mainly tRNA genes. The GC content of the cp genomes of five *Hordeum* species differed slightly in four regions, with the highest content in the IR region (43.81–43.87%).

To further present cp genome divergence among the five *Hordeum* species, sequence identity was compared using mVISTA with *H. brevisubulatum* as a reference (Fig 5). Generally, the LSC and SSC regions were more divergent than IR regions and noncoding regions were more divergent than coding regions. Consistent with the results of phylogeny analysis, the cp genomes of these five *Hordeum* species could be divided into three groups. *H. brevisubulatum* was analogical to *H. bogdanii*, and *H. vulgare* subsp. *vulgare* was analogical to *H. vulgare* subsp. *spontaneum*. They were all different from *H. jubatum*. Among the five *Hordeum* species, prominent divergence occurred in the IGS of *trnG-GCC-trnY-GUA*, *psbM-petN*, *petN-trnC-GCA*, *petA-psbI*, *ndhC-trnV-UAC*, *rbcL-pasI*, and *rpl23-ndhD* as well as coding regions of *ccsA*, which could be used as reliable markers for phylogenetic studies. Between *H. brevisubulatum* and *H. bogdanii*, there were significant differences in the IGS of *psbM-petN*, *petA-psbL*, *rpl32-trnL-UAG*, and *ccsA-ndhD*, as well as the coding regions of *infA*, *rps3*, and *ccsA*.

Detailed comparisons of the IR boundaries among the five *Hordeum* species are presented in Fig 6. Seven different genes were located at the juncture of the LSC/IRb (*rps19* and *rpl22*), IRb/SSC (*ndhF*, *rps15* and *ndhH*), SSC/IRA (*ndhH*, *ndhF*, and *rps15*), and IRA/LSC borders (*rps19*, and *psbA*). The cp genomes of these five *Hordeum* species were relatively conserved except for two major diversities—at the IRA/LSC border in *H. jubatum* and at the SSC/IRA border in *H. vulgare* subsp. *spontaneum*. In the cp genomes of *H. brevisubulatum*, *H. vulgare* subsp. *vulgare*, *H. jubatum* and *H. bogdanii*, the *ndhH* gene crossed the SSC/IRA border with the larger part located in the SSC region and the *ndhf* gene located at the IRb/SSC border. In *H. vulgare* subsp. *spontaneum*, the *ndhH* gene crossed the IRb/SSC border with 975 bp located in SSC region and the *ndhf* gene near the SSC/IRA border. Different from other four species which possessed *rps19* in the SSC region with 47 bp away from the IRA/LSC border, there was no *rps19* gene near the IRA/LSC border in *H. jubatum*.

**Discussion**

The cp genome is very useful in identifying closely related, breeding-compatible plant species. In this study, the relationship between 5 *Hordeum* species and among 35 species in Triticaceae

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Table 3. Summary of five *Hordeum* chloroplast genome features.

| Species            | *Hordeum brevisubulatum* | *Hordeum vulgare* subsp. *vulgare* | *Hordeum jubatum* | *Hordeum vulgare* subsp. *spontaneum* | *Hordeum bogdanii* |
|--------------------|--------------------------|-----------------------------------|-------------------|---------------------------------------|-------------------|
| Accession          | NC_008590.1              | NC_027476.1                       | NC_042692.1       | NC_043839.1                           |
| Genome size(bp)    | 137155                   | 136462                            | 136826            | 136536                                | 136968            |
| LSC length(bp)     | 81175                    | 80597                             | 80901             | 80612                                 | 81047             |
| SSC length(bp)     | 12806                    | 12701                             | 12665             | 12778                                 | 12747             |
| IR length(bp)      | 21587                    | 21582                             | 21630             | 21573                                 | 21587             |
| Number of genes    | 130                      | 139                               | 129               | 130                                   | 131               |
| Number of Protein-coding genes | 83                     | 83                                | 82                | 83                                     | 83                |
| Number of tRNA genes | 39                    | 48                                | 39                | 39                                     | 40                |
| Number of rRNA genes | 8                    | 8                                 | 8                 | 8                                      | 8                 |
| All GC content(%)  | 38.23                    | 38.32                             | 38.24             | 38.30                                  | 38.24             |
| LSC GC content(%)  | 36.20                    | 36.31                             | 36.19             | 36.30                                  | 36.20             |
| SSC GC content(%)  | 32.09                    | 32.33                             | 32.32             | 32.25                                  | 32.12             |
| IR GC content(%)   | 43.86                    | 43.83                             | 43.81             | 43.84                                  | 43.87             |
were examined. This could be beneficial for traditional variety breeding in *H. brevisubulatum* since it is mainly breeding cultivated crops with their wild relatives. Furthermore, the publication of the cp genome of *H. brevisubulatum* will enable researchers to build transgene cassettes for further cp genome engineering since it could provide both flanking and regulatory sequences. Furthermore, it would enable the construction of species-specific chloroplast vectors. There were many successful examples of the use of cp genome engineering to improve abiotic resistance. For example, salt tolerance in carrots was enhanced by over-expressing the *badh* gene in its cp genome [29] and tabacco salt resistance was improved by the expression of γ-TMT in its chloroplast [30]. However, some factors, including the need to identify species-
specific vectors, difficulties in homogenization, and the need to examine the rule of cp protein expression have hindered cp genome engineering development [10].

**Cp genome feature of *H. brevisubulatum***

The cp genomes of terrestrial plants are not only conserved in structure but also in gene and intron content. Generally, the cp genome is a typical quadripartite structure, except for IR segment divergence. For example, the cp genome of alfalfa comprises only one copy of the IR region [31], whereas in some algae and conifers, there are no IR regions [11, 12]. With the loss of IR regions or entire gene families, linear cp genomes have been found [32]. The cp genomes of the earliest diverging angiosperms harbor 113 different genes, including 16 duplicated in IR regions, for a total of 129 genes. These genes play vital roles in photosynthesis, self-replication, and biosynthesis related to amino acids, fatty acids, pigments, starch, and other unknown functions. However, the loss of genes has been observed in many angiosperms [14]. Haberhausen and Zetsche [33] found the loss of the *ndh* genes in *Cucuta reflexa*. Millen et al. [34] found the loss of the *infA* gene in *Lemna minor*, and proposed that these genes had been transferred into the nucleus or did not participate in critical life development. In this study, the cp genome of *H. brevisubulatum* was a typical quadripartite structure that harbored 108 different genes with 22 genes duplicated in the IR regions, verifying highly conserved traits of cp genomes in gene content and genome organization. Tang et al. [35] compared gene sequences of *accD*, *ycf1*, and *ycf2* in *Typha latifolia* with 13 other Gramineae species and found that only conserved segments of these genes were retained in these 13 species. This suggested that sequences of *accD*, *ycf1*, and *ycf2* genes were gradually eliminated during the evolution of Gramineae [35]. The *accD* gene was lost in all five *Hordeum* species, while only segments of *ycf1* and *ycf2* occurred in *H. brevisubulatum* and was lost in *H. vulgare* subsp. *vulgare*, *H. jubatum*, and *H. bogdanii*, suggesting gene degradation during evolution.
Among the 129 total genes in the earliest diverging angiosperms, 22 genes contain introns. Intron absence within protein-coding genes usually occurs in monocot and eudicot clades, such as Cicer arietinum, Manihot esculenta, and H. vulgare [10, 14, 35]. In the H. brevisubulatum cp genome, a total of 17 different genes contained introns. Compared with the earliest diverging angiosperms, introns of four genes, namely, clpP, rpoC1, rpoC2, and ycf1 were absent. Due to the intron absence of clpP, which formerly harbored two introns, only the ycf3 gene harbored two introns. This was different from Fagopyrum dibotrys, Quercus acutissima, and Sinapis alba, in which both ycf3 and clpP contained two introns [36–38]. Intron absence in clpP extensively occurred in Onagraceae, Oleaceae, and gymnosperms [14], as well as in Gramineae, except for Anomochloa marantoida [33]. The genes with intron absence might endow H. brevisubulatum with diverse functions in protease, RNA polymerase and ribosomal proteins.

AT was abundant in the H. brevisubulatum cp genome. This was consistent with findings in P. mume, S. alba, Q. acutissima, P. pretense, D. grandiflorum, and Dicliptera species [11, 26, 38–41]. Due to the bias of the replication mechanism, gene orientation and transcribed/non-transcribed strands, as well as cytosine deamination, strand composition asymmetry were universally existed in prokaryotic and eukaryotic chromosomes [42–44]. The asymmetry between A and T and between C and G in a single strand were evident in the LSC, SSC, and cp genome of H. brevisubulatum. Moreover, PR2 analysis further verified that a majority of genes had a bias toward T over A in the coding strand in all five Hordeum species, and a slight bias toward C over G in the coding strand in four other Hordeum species, expect for H. bogdanii. A direct cause of base composition asymmetry was replication mechanism, and nevertheless that asymmetries between the coding and noncoding strands, the cause mechanisms such as transcription-induced mutation or codon choice, are responsible for a large part of the nucleotide skews [45]. Since the leading strand is synthesized continuously, whereas as the lagging strand is replicated in a fragmented manner, the 2 DNA strands can be highly asymmetric in nucleotide composition. Moreover, the deamination events in the nontranscribed strand contribute to C→T substitutions, which accumulates such changes at a two-to threefold higher rate than the complementary transcribed strand [44, 45]. Nevertheless, the impact of the replication mechanism on base bias differed for GC and AT asymmetries. The effect of replication on the GC skew was generally very strong, and the AT skew was exclusively caused by coding sequence-related mechanisms [45–47]. The reasons that lead to the base asymmetry in H. brevisubulatum cp genome need to be studied in depth.

Molecular markers

Repeat sequences are found extensively in plant cp genomes [48]. Repeat sequences differ in type, number, and location depending on species. In four species of the Dicliptera genus and Nasturtium officinale [24, 26], three types—forward, palindromic, and reversed repeats were found. In the Dicliptera peruviana cp genome, there were 56 dispersed repeats, and in Dicliptera montana, there were 41 dispersed repeats. Among the five Hordeum species, H. jubatum possessed the most of dispersed repeats while H. bogdanii had the least. Moreover, H. jubatum had four types of dispersed repeats, and the other four species only had two types. The most tandem repeats were in H. brevisubulatum, and 52 dispersed repeat sequences were detected from the categories of forward and palindromic. The majority of identified dispersed repeats in the H. brevisubulatum cp genome were located in the LSC region, showing an uneven distribution. Moreover, 75% were concentrated in IGS, which are highly variable [26] and gene coding regions. In N. officinale, most were located in IGS and introns. Repeat sequences could be used as indicators of mutational hotspots [49]. In this study, the rpoC2 gene possessed the
largest number of repeats, and similar phylogeny relationships were obtained when using only this gene to construct a new phylogeny tree. Hence, we reasoned it might be used as a potential molecular marker for further studies in Hordeum species.

SSRs, also termed microsatellites, are rich in cp genomes [24, 26, 36, 50, 51] and composed of one or a few consecutive repeated nucleotides. Since genetic information loaded on the cp genome is only inherited from the maternal line, SSRs in cp genomes are sensitive to population genetic effects [52] and have been used widely to trace the maternal gene flow in populations and in research in population evolution and polymorphism [49]. SSRs exhibit variations in number and type according to species. In five Hordeum species, only one pentanucleotide (CCATA) SSR was found, while two pentanucleotide and two hexanucleotide SSRs were found in S. alba [36] and only mononucleotide, dinucleotides, trinucleotides, and tetranucleotides were detected in Dicliptera species [26], suggesting genetic diversity among different species [36]. In the cp genome of H. brevisubulatum, oligo adenine and oligo T repeats accounted for 64% of the total SSRs. These results were consistent with the findings in S. alba, N. officinale and Raphanus sativus [24, 36, 49], verifying that oligo adenine and oligo T repeats were common features of cp genomes. Only 4 SSRs did not contain A or T nucleotide, providing further evidence for A and T nucleotide bias in H. brevisubulatum. Among the five Hordeum species, except for H. vulgare subsp. vulgare, there were unique SSRs for each species, which could be used as molecular markers to distinguish different species. Therefore, the analysis of repeat sequences and SSRs in H. brevisubulatum and the other four Hordeum species further illustrated its cp genome characteristics and provided strong evidence in identifying molecular markers for further study in mutational hotspots, phylogenetic studies, species delimitation, haplotype recognition, population genetic analysis, and crop breeding [24, 49–51, 53].

Phylogenetic relationships and genome structure comparison

The cp genome is considered to be a perfect model for evolutionary research and has been used widely in studies in plants including in cereals, cottons, trees, vegetables, ornamental plants, medical plants, and forages [24, 26, 37–39]. A robust phylogenetic tree would help improve target plants and facilitate sustainable conservation strategies [10]. To examine the relationship between H. brevisubulatum and other species in Triticeae, a phylogenetic tree based on cp genomes was constructed among 35 species. The results demonstrated a significant relationship among five species of the Hordeum genus (Fig 4). H. brevisubulatum had a closer relationship with H. bogdanii than did H. jubatum and H. vulgare subsp. vulgare was close to H. vulgar subsp. spontaneum. This was in agreement with Bernhardt et al., who reported that H. vulgar was far from H. jubatum [54]. Although cp genomes are relatively conserved in gene composition and structure, important alterations occur due to gene or intron fragment loss, gene rearrangement, and expansion, extraction or loss of the IR region [35, 55, 56]. To fully demonstrate genome divergence among five closely related Hordeum species, including H. brevisubulatum, H. bogdanii, H. vulgar subsp. vulgar, H. jubatum and H. vulgar subsp. spontaneum, the genome structure was compared by analyzing genome features, sequence identity, and IR borders.

Low divergence among these five Hordeum species was found, revealing that the cp genomes were conserved. IR regions were more conserved than LSC and SSC regions and coding regions had less divergence than noncoding regions. This might be related to copy corrections during gene conversion [26] and has been reported in other angiosperms, such as in the Prunus, Sinapis, Quercus, Dicliptera, and Nasturtium genera [11, 24, 26, 36, 38]. The ndhH gene crossed the SSC/IRA border in H. brevisubulatum, H. vulgar subsp. vulgar, H. jubatum, and H. bogdanii. However, in Quercus species and N. officinale, the ycf1 gene crossed the SSC/
IRa region [24, 38] and in Brassica juncea, there was no gene that crossed the SSC/IRa border [36], suggesting genetic diversity in different species. The genome size variation of the five Hordeum species was due mainly to variation in IR regions and divergence at the SSC/IRa border (Table 3, Fig 6). The contraction or expansion of IR regions at the borders is widespread [41] and has been regarded as an evolutionary indicator that explains correlations among taxa [57, 58]. Significant changes in the gene location of \( rps19 \) were found between \( H. \) jubatum and the other four species, due mainly to the contraction of the IR region after its expansion during evolution [35, 59]. Unlike the other four species, in \( H. \) vulgare subsp. spontaneum, the \( ndhH \) gene crossed the IRb/SSC border with a pseudo part at the SSC/IRa border. This kind of gene conversion during speciation was thought to occur from IR variations [26].

**Conclusions**

In this study, the architecture of the \( H. \) brevisubulatum cp genome, including basic features, repeat sequence, SSRs, and phylogenetic relationships was described comprehensively. Then, the cp genome of \( H. \) brevisubulatum was compared with four other Hordeum species. The cp genome of \( H. \) brevisubulatum had a typical quadripartite structure and 130 functional genes were annotated. The gene of \( accD \) was lost in all five Hordeum species when compared with the earliest diverging angiosperms, and only segments of \( ycf1 \) and \( ycf2 \) were retained in \( H. \) brevisubulatum and absent in \( H. \) vulgare subsp. vulgare, \( H. \) jubatum, and \( H. \) bogdannii. Moreover, introns of \( clpP \), \( rpoC1 \), \( rpoC2 \), and \( ycf1 \) were absent in \( H. \) brevisubulatum. Base asymmetry were found in all five Hordeum species. In the coding strand, the bias of T over A existed in all five Hordeum species, while the bias of G over C differed. LSC and SSC regions were more divergent than IR regions, and IGS was more divergent than coding regions. Moreover, main divergence occurred at the SSC/IR borders in Hordeum. These findings, in combination with identified introns, repeat sequences, and SSRs enrich our knowledge on cp biology and genetic diversity of \( H. \) brevisubulatum and lay a strong foundation for further studies on molecular marker development, phylogenetic analysis, population studies and cp genome engineering.

**Supporting information**

S1 Table. List of genes annotated in the cp genome of \( H. \) brevisubulatum. Gene*: Gene with one intron; Gene**: Gene with two introns; #Gene: Pseudo gene; Gene (2): Number of copies of multi-copy genes.

(XLS)

S2 Table. Repeat sequences in \( H. \) brevisubulatum cp genome. F, forward repeats; R, reversed repeats; IGS, intergenic spaces. Note: The first nucleotide position was located at the beginning of the LSC region, and the nucleotide position was counted along with the direction of LSC-IRa-SSC-IRb.

(XLS)

S3 Table. Distribution of SSRs in the \( H. \) brevisubulatum cp genome.

(XLS)

S1 File. The full annotation of \( H. \) brevisubulatum cp genome.

(TBL)

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References
1. Lu YM, Li YF, Cao MF, Li XW, Zhang YL, Yang BM, et al. Physiological-biochemical and molecular biological analysis of salt-tolerant new lines of *Hordeum brevisubulatum* (Trin.) Link. Scientia Agricultura Sinica. 2002; 35: 282–286.
2. Wang CM, Zhang Q, Zhang HS, Wang XB, Zhu XQ, Xia ZR, et al. Research Progress in salt tolerance of *Hordeum brevisubulatum*. Chinese Herbivore Science. 2013; 33: 48–52.
3. Jing DW, Wang ZS. The wild forage grass in Jilin province, wild barley. Jilin Agriculture Science. 1981; 2: 73–76.
4. Bao XY, Wang YZ, Wu LN, Se LJ, Zhang XZ. Production test of wild barley. Inner Mongolia Prataculture. 1999; 4: 60–63.
5. Li YF, Shen JL, Li XW, Zhang YL, Cheng XR, Yang BM, et al. Studies on breeding of *Hordeum brevisubulatum* Junxu No.1. Jilin Animal Husbandry and Veterinarian. 2004; 8: 31–33.
6. Yang R, Ren DL, Yu XL. Literature analysis of molecular breeding research in China between 1980 and 2017. Molecular Plant Breeding, 2018; 22: 7452–7459.
7. Wang CM, Xia ZR, Wu GQ, Yuan HJ, Wang XR, Li JH, et al. The coordinated regulation of Na\(^+\) and K\(^+\) in *Hordeum brevisubulatum* responding to time of salt stress. Plant Science. 2016; 252: 358–366. https://doi.org/10.1016/j.plantsci.2016.08.009 PMID: 27717472
8. Zhang LL, Wang YX, Zhang QK, Jiang Y, Zhang HW, Li RF. Overexpression of HbMBF1a, encoding multiprotein bridging factor 1 from the halophyte *Hordeum brevisubulatum*, confers salinity tolerance and ABA insensitivity to transgenic *Arabidopsis thaliana*. Plant Molecular Biology. 2020; 102: 1–17. https://doi.org/10.1007/s11103-019-00926-7 PMID: 31655970
9. IBSC, A physical, genetic and functional sequence assembly of the barley genome. Nature. 2012; 491: 711–717. https://doi.org/10.1038/nature11543 PMID: 23079545
10. Daniell H, Lin CS, Yu M, Chang WJ. Chloroplast genomes: diversity, evolution, and applications in genetic engineering. Genome Biology. 2016, 17: 134. https://doi.org/10.1186/s13059-016-1004-2 PMID: 27339192
11. Xue S, Shi T, Luo WJ, Ni XP, Iqbal S, Ni ZJ, et al. Comparative analysis of the complete chloroplast genome among *Prunus mume*, *P. armeniaca*, and *P. salicina*. Horticulture Research. 2019; 6: 89. https://doi.org/10.1038/s41438-019-0171-1 PMID: 31666958
12. Strauss SH, Palmer JD, Howe GT, Doerkson AH. Chloroplast genomes of two conifers lack a large inverted repeat and are extensively rearranged. Proceedings of National Academy of Sciences of the United States of America. 1988; 85: 3898–3902. https://doi.org/10.1073/pnas.85.11.3898 PMID: 2836862
13. Palmer JD, Osorio B, Thompson WF. Evolutionary significance of inversions in legume chloroplast DNAs. Current Genetics. 1988; 14: 65–74. https://doi.org/10.1007/BF00405856
14. Jansen RK, Cai ZQ, 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. Proceedings of National Academy of Sciences of the United States of America. 2007; 104: 19369–19374. https://doi.org/10.1073/pnas.0709121104 PMID: 18048330
15. Ma J, Yang BX, Zhu W, Sun LL, Tian JK, Wang XM. The complete chloroplast genome sequence of *Mahonia bealei* (Berberidaceae) reveals a significant expansion of the inverted repeat and phylogenetic
relationship with other angiosperms. Gene. 2013; 528: 120–131. https://doi.org/10.1016/j.gene.2013.07.037 PMID: 23900198

16. Zhang YZ, Ma J, Yang BX, Li RY, Zhu W, Sun LL, et al. The complete chloroplast genome sequence of Taxus chinensis var. mairei (Taxaceae): loss of an inverted repeat region and comparative analysis with related species. Gene. 2014; 540: 201–209. https://doi.org/10.1016/j.gene.2014.02.037 PMID: 24583178

17. Smith DR. Mutation rates in plastid genomes: they are lower than you might think. Genome Biology and Evolution. 2015; 7: 1227–1234. https://doi.org/10.1093/gbe/evv069 PMID: 25869380

18. Wicke S, Schnieweiss GM, dePamphilis CW, Muller KF, Quandt D. The evolution of the plastid chromosome in land plants: gene content, gene order, gene function. Plant Molecular Biology. 2011; 76: 273–297. https://doi.org/10.1007/s11103-011-9762-4 PMID: 21424777

19. Duchene D, Bromham L. Rates of molecular evolution and diversification in plants: chloroplast substitution rates correlate with species-richness in the Proteaceae. BMC Evolutionary Biology. 2013; 13: 65. https://doi.org/10.1186/1471-2148-13-65 PMID: 23497266

20. Parks M, Cronn R, Liston A. Increasing phylogenetic resolution at low taxonomic levels using massively parallel sequencing of chloroplast genomes. BMC Biology. 2009; 7: 84. https://doi.org/10.1186/1741-7007-7-84 PMID: 19954512

21. Doyle JJ, Doyle JL. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemistry. 1987; 19: 11–15.

22. Sun CQ, Chen FD, Teng NJ, Xu YC and Dai ZL. Comparative analysis of the complete chloroplast genome of seven Nymphaea species. Aquatic Botany. 2021; 170. https://doi.org/10.1016/j.aquabot.2021.103353

23. Lohse M, Drechsel O, Kahlau S, Bock R. Organellar Genome DRAW—a suite of tools for generating physical maps of plastid and mitochondrial genomes and visualizing expression data sets. Nucleic Acids Research. 2013; 41:W575–W581. https://doi.org/10.1093/nar/gkt289 PMID: 23609545

24. Yan C, Du J, Gao L, Li Y, Hou X. The complete chloroplast genome sequence of watercress (Nasturtium officinale R., Br.): genome organization, adaptive evolution and phylogenetic relationships in Cardamineae. Gene. 2019; 699: 24–36. https://doi.org/10.1016/j.gene.2019.02.075 PMID: 30849538

25. Kurtz S, Philippy A, Delcher AL, Smoot M, Shumway M, Antonescu C, et al. Versatile and open software for comparing large genomes. Genome Biology. 2004; 5: R12. https://doi.org/10.1186/gb-2004-5-2-r12 PMID: 14759262

26. Huang SN, Ge XJ, Cano A, Salazar BGM, Deng YF. Comparative analysis of chloroplast genomes for five Dicliptera species (Acanthaceae): molecular structure, phylogenetic relationships, and adaptive evolution. Peer J. 2020; 8:e8450 (2020). https://doi.org/10.7717/peerj.8450 PMID: 32071806

27. Wei L, He J, Jia X, Qi Q, Liang ZS, Zheng H, et al. Analysis of codon usage bias of mitochondrial genome in Bombyx mori and its relation to evolution. BMC Evolutionary Biology. 2014; 14: 262. https://doi.org/10.1186/s12862-014-0262-4 PMID: 25515024

28. Amiryousefi A, Hyvönen J, Poczai P. IRscope: an online program to visualize the junction sites of chloroplast genomes. Bioinformatics. 2018; 34: 3030–3031. https://doi.org/10.1093/bioinformatics/bty220 PMID: 29659705

29. Kumar S, Dhingra A, Daniell H. Plastid-expressed betaine aldehyde dehydrogenase gene in carrot cultured cells, roots, and leaves confers enhanced salt tolerance. Plant Physiology. 2004; 136:2843–54. https://doi.org/10.1104/pp.104.045187 PMID: 15347789

30. Jin S, Daniell H. Expression of gamma-tocopherol methyltransferase in chloroplasts results in massive proliferation of the inner envelope membrane and decreases susceptibility to salt and metal-induced oxidative stresses by reducing reactive oxygen species. Plant Biotechnology Journal. 2014; 12:1274–85. https://doi.org/10.1111/pbi.12224 PMID: 25051898

31. Tao XL, Ma LC, Zhang ZS, Liu WX, Liu ZP. Characterization of the complete chloroplast genome of alfalfa (Medicago sativa) (Leguminosae). Gene Reports. 2017; 6: 67–73. https://doi.org/10.1016/j.genrep.2016.12.006

32. Oldenburg DJ, Bendich AJ. The linear plastid chromosomes of maize: terminal sequences, structures, and implications for DNA replication. Current Genetics. 2015; 62: 431–432. https://doi.org/10.1007/s00294-015-0548-0 PMID: 26650613

33. Haberhausen G, Zetsche K. Functional loss of ndh genes in an otherwise relatively unaltered plastid genome of the holoparasitic flowering plant Cuscuta reflexa. Plant Molecular Biology. 1994; 24: 217–222. https://doi.org/10.1007/BF00040588 PMID: 8111019

34. Millen RS, Olmstead RG, Adams KL, Palmer JD, Lao NT, Heggie L, et al. Many parallel losses of infA from chloroplast DNA during angiosperm evolution with multiple independent transfers to the nucleus. Plant cell. 2011; 13:645–658.
35. Tang P, Yuan QY, Peng C. Phylogeny in structure alterations of Poaceae cpDNA. Zhongguo Nong Xue Tong Bao. 2011; 27: 171–176.

36. Du XY, Zeng T, Feng Q, Hu LJ, Luo X, Weng QB, et al. The complete chloroplast genome sequence of yellow mustard (Sinapis alba L.) and its phylogenetic relationship to other Brassicaceae species. Gene. 2020; 731: 144340. https://doi.org/10.1016/j.gene.2020.144340 PMID: 31923575

37. Wang XM, Zhou T, Bai GQ, Zhao YM. Complete chloroplast genome sequence of Fagopyrum dibotrys: genome features, comparative analysis and phylogenetic relationships. Scientific Reports. 2018; 8: 12379 (2018). https://doi.org/10.1038/s41598-018-30398-6 PMID: 30120274

38. Li X, Li YF, Zang MY, Li MZ, Fang YM. Complete chloroplast genome sequence and phylogenetic analysis of Quercus acutissima. International Journal of Molecular Science. 2018; 19: 2443. https://doi.org/10.3390/ijms19082443 PMID: 30126202

39. Cui GX, Lu Y, Wei XX, Wang XL, Wang CM, Gao YQ, et al. Characterization of the complete chloroplast genome of Phleum pretense L. cv. Minshan. Mitochondrial DNA PartB-Resources. 2019; 4: 4180–4181. https://doi.org/10.1080/23802359.2019.1692706 PMID: 33663732

40. Duan HR, Lu Y, Duan XR, Zhou XH, Wang CM, Tian FP, et al. Characterization of the complete chloroplast genome of Delphinium grandiflorum L. Mitochondrial DNA PartB-Resources. 2020; 5: 35–36. https://doi.org/10.1080/23802359.2019.1692707 PMID: 3366410

41. Huang H, Shi C, Liu Y, Mao SY, Gao LZ. Thirteen Camelina chloroplast genome sequences determined by high-throughput sequencing: genome structure and phylogenetic relationships. BMC Evolutionary Biology. 2014; 14: 151. https://doi.org/10.1186/1471-2148-14-151 PMID: 25001059

42. Mugal CF, von Grünberg HH, Peifer M. Transcription-induced mutational strand bias and its effect on substitution rates in human genes. Molecular Biology and Evolution. 2008; 26: 131–142. https://doi.org/10.1093/molbev/msn245 PMID: 18974087

43. Francisco MP, Ochman H. Deamination as the Basis of strand-asymmetric evolution in transcribed Escherichia coli sequences. Mol. Biol. Evol. 18: 1147–1150, 2001. https://doi.org/10.1093/molbev/msm148 PMID: 17646257

44. Neacsuiea A, Lobry J. A new method for assessing the effect of replication on DNA base composition asymmetry. Molecular Biology and Evolution. 2007; 24: 2169–2179. https://doi.org/10.1093/molbev/msm148 PMID: 17646257

45. MRAZEK J, Karlin S. Strand compositional asymmetry in bacterial and large viral genomes. Proc Natl. Acad. Sci. USA. 1998, 95:3720–3725. https://doi.org/10.1073/pnas.95.7.3720 PMID: 9520433

46. Wang FP, Wang ZJ, Li YX. Comparison of GC contents in genomes of three model microbes. Genomics and Applied Biology. 2019; 38:2215–2220.

47. Romiguier J, Roux C. Analytical biases associated with GC-content in molecular evolution. Frontiers in Genetics. 2017; 8: 16. https://doi.org/10.3389/fgene.2017.00016 PMID: 28261263

48. Nie XJ, Lv SZ, Zhang YX, Du XH, Wang L, Biradar SS, et al. Complete chloroplast genome sequence of a major invasive species,crofton weed (Ageratina adenophora), PLoS ONE. 2012; 7: e36869. https://doi.org/10.1371/journal.pone.0036869 PMID: 22606302

49. George B, Bhatt BS, Awasthi M, George B, Singh AK. Comparative analysis of microsatellites in chloroplast genomes of lower and higher plants. Current Genetetics. 2015; 61: 39–48. https://doi.org/10.1007/s00294-015-0495-9 PMID: 25999216

50. Jeong YM, Chung WH, Mun JH, Kim N, Yu HJ. De novo assembly and characterization of the complete chloroplast genome of radish (Raphanus sativus L.). Gene. 2014; 551: 39–48. https://doi.org/10.1016/j.gene.2014.08.038 PMID: 25151309

51. Yamane K, Lü N, Ohnishi O. Multiple origins and high genetic diversity of cultivated radish inferred from polymorphism in chloroplast simple sequence repeats. Breeding Science. 2009; 59: 55–65. https://doi.org/10.1270/jsbbs.59.55

52. Ebert D, Peakall R. Chloroplast simple sequence repeats (cpSSRs): technical resources and recommendations for expanding cp SSR discovery and applications to a wide array of plant species. Molecular Ecology Resources. 2009; 9: 673–690. https://doi.org/10.1111/j.1755-0998.2008.02319.x PMID: 21564725

53. Jiang M, Chen HM, He SB, Wang LQ, Chen A, Liu C. Sequencing, characterization, and comparative analyses of the plastome of Caragana rosea var. rosea. International Journal of Molecular Science. 2018; 19: 1419. https://doi.org/10.3390/ijms19051419 PMID: 29747436

54. Bernhardt N, Brassac J, Kilian B, Blattner FR. Dated tribe-wide whole chloroplast genome phylogeny indicates recurrent hybridizations within Triticeae. BMC Evolutionary Biology. 2017; 17: 141. https://doi.org/10.1186/s12862-017-0989-9 PMID: 28622761

55. Pugh T, Fouet O, Risterucci AM, Brottier P, Abouladze M, Deletrez C et al. A new cacao linkage map based on codominant markers: development and integration of 201 new microsatellite markers.
56. Chumley TW, Palmer JD, Mower JP, Fourcade HM, Calie PJ, Boore JL et al. The complete chloroplast genome sequence of Pelargonium x hortorum: organization and evolution of the largest and most highly rearranged chloroplast genome of land plants. Molecular Biology and Evolution. 2006; 23: 2175–2190. https://doi.org/10.1093/molbev/msl089 PMID: 16916942

57. Wang RJ, Cheng CL, Chang CC, Wu CL, Su TM, Chaw SM. Dynamics and evolution of the inverted repeat-large single copy junctions in the chloroplast genomes of monocots. BMC Evolutionary Biology. 2008; 8: 36. https://doi.org/10.1186/1471-2148-8-36 PMID: 18237435

58. Lu QX, Ye WQ, Lu RS, Xu WQ, Qiu YX. Phylogenomic and comparative analyses of complete plastomes of Croomia and Stemona (stemonaceae). International Journal of Molecular sciences. 2018; 19: 2383. https://doi.org/10.3390/ijms19082383 PMID: 30104517

59. Zhao YM, Yang ZY, Zhao YP, Li XL, Zhao ZX, Zhao GF. Chloroplast genome structural characteristics and phylogenetic relationships of oleaceae. Chinese Bulletin of Botany. 2019; 54: 441–454. https://doi.org/10.11983/CBB1819