Complete Chloroplast Genome of *Calligonum Mongolicum*: Genome Organization, Codon Usage Pattern, Phylogenetic Relationships, Comparative Structure and Adaptive Evolution Analysis

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Abstract

Background

The perennial shrub of *Calligonum mongolicum* is a dominant native plant in all *Calligonum* species, which has the largest and most widespread geographic distribution in arid deserts of northern China. Understanding the phylogenetic relationship between *C. mongolicum* and closely related plant species will offer guidance on the classification and identification of inter-species and their varieties. The chloroplast (cp) genome is an optimal model to decipher phylogenetic relationships and genome evolution in related plant families. In the present study, the complete cp genome of *C. mongolicum* was sequenced, and the characteristics were described, then the genomic structure was compared to other three Polygonaceae species.

Results

The cp genome of *C. mongolicum* was 162,124 bp in length with a quadripartite structure. A total of 131 functional genes were annotated, 14 different genes of which harbored introns and exons, 50 long repeat sequences and 244 simple sequences repeats were identified. Synonymous codon usage (SCU) analysis exhibited A/T preference, and 7 codons were identified as the optimal codons. Multivariate statistical analysis of parity rule 2, ENC-plot, and neutrality plot were combined conducted to imply natural selection as the crucial constraint in SCU bias in *C. mongolicum* cp genome. The phylogenetic tree showed that *Rumex acetosa* was the most related plant to *C. mongolicum*. From the comparative analysis of genomic structures, the inverted repeat regions (IRa and IRb) were less divergent than other parts and coding regions was relatively conserved than non-coding regions. Compared to other species in the Polygonaceae, the borders of IRb/SSC and SSC/IRa in *C. mongolicum* changed greatly. Furthermore, adaptive evolution analysis of 75 orthologous protein-coding genes indicated that only the *psbK* gene was under positive selection, which might be crucial in the adaptive evolution of *C. mongolicum*.

Conclusions

Our results comprehensively depicts the architecture of *C. mongolicum* cp genome, and will lay a vigorous foundation for further study on molecular marker selection, phylogenetic analysis, and population researches in *Calligonum* species.

Background

Calligonum L. belonging to the family Polygonaceae, contains approximately 35 species of perennial xerophytic shrubs or subshrubs, are successful pioneer plants for desert vegetation succession, and distribute in northern Africa, Southern Europe and Asia [1, 2]. There are 24 *Calligonum* species in China and almost of which possess significant ecological values [3]. The *Calligonum* species are divided into four groups according to the surface appendage of the achene [4, 5]. *Calligonum mongolicum*, belonging to Sect. Medusa Sosk. et Alexandr., has the largest and most widespread geographic distribution in arid

deserts of northern China of all *Calligonum* species [6]. As a perennial shrub, *C. mongolicum* is native dominant in active sand dunes, exhibits strong resistance of drought, salt-alkali, high temperature, barren soil, wind erosion, also propagation strategies through sexual and asexual ways, thus it is often used for vegetation restoration in the desert [7, 8]. In recent years, the population of *C. mongolicum* exhibits a fast expansion tendency in mobile sand dunes of the Minqin Desert [9]. Actually, the surface appendages of the achene from *C. mongolicum* and *C. roborovskii* have similar characteristics, and the geographical distributions of *C. mongolicum* and *C. roborovskii* are overlapped to some certain areas, as a result causing difficulty in identifying *C. mongolicum* [10]. Understanding the phylogenetic relationship between *C. mongolicum* and closely related plant species will offer guidance on the classification and identification of inter-species and their varieties.

Phylogenetic relationships are revolutionarily and frequently analyzed by genome sequencing, including the chloroplast genome (cp genome), the mitochondrion genome, and nucleus genome [11, 12]. The complete cp genome, which possesses many characteristics of small size, simple and highly conserved structure, single parentalin heritage, and haploid nature, is widely applied for species identification, phylogenic analysis, and adaptive evolution analysis [13]. Huang et al. [14] sequenced the complete cp genomes of five *Dilcpiptera* species, and compared the interspecies relationships of the five species. Xue et al. [12] also finished the cp genome comparison of three economic trees from *Prunus*, found the genomic structure difference, revealed the possible molecular markers, and concluded the phylogenetic evolution relationships. Until now, studies on the cp genome sequences have been conducted in many plant species, for example, watercress, yellow mustard, *Echinacanthus attenuatus*, and so on [15-17]. Thus, the cp genome is an optimal model to decipher phylogenetic relationships and genome evolution in related plants families.

Chloroplasts is a photosynthetic organelle in plant cells that plays crucial roles in the photosynthesis and crucial metabolites biosynthesis, for example amino acids, starch, fatty acids and pigments [18]. In general, the length of the cp genome ranges from 120 - 160 kb, the difference of which is mainly due to inverted repeated regions (IR) expansion, contraction or loss [19]. The cp genome encodes 63–209 unique genes, and is conserved with quadripartite organizations including a pair of IR regions (IRa and IRb), a large single copy (LSC) region and a small single copy (SSC) region [20]. For majority of higher plants, chloroplast is highly conservative in genes number, arrangement order and function [21]. However, gene losses and genome rearrangement can be found in some leguminous plants and conifer algae [22, 23]. After the first cp genome data of tobacco published in 1986, more and more plant cp genomes are completed sequencing, yet none of species from *Calligonum* L. has obtained the complete cp genomes data [24].

In the current study, the cp genome of *C. mongolicum* was constructed firstly by using Illumina sequencing and integrating a combination of *de novo* sequencing and reference-guided assembly. Then, the whole cp genome characteristics of *C. mongolicum* were described, and the synonymous codon usage (SCU) pattern, simple sequence repeats (SSRs) and long repeats were analyzed. Besides, we compared the cp genome of *C. mongolicum* with the published cp genomes of other three related species,

including genome structure, IR contraction and expansion, and selective pressure events. This study may provide positive clues for adaptive evolution analysis of the *Calligonum* species.

Materials And Methods

Materials

The wild seedlings of *C. mongolicum* were transplanted from the Minqin desert to Lanzhou Scientific Observation and Experiment Field Station of the Ministry of Agriculture for Ecological System in the Loess Plateau Area (36°01'N–103°45'E, altitude 1700 m), Gansu, China in 15 May 2019. The station was belonged to Lanzhou Institute of Husbandry and Pharmaceutical Science, Chinese Academy of Agricultural Sciences, and we were approved to perform plant species planting and cultivating. *C. mongolicum* is a common wild plant in the Minqin desert, thus collecting or transplanting it for scientific research is not restricted in Ganu Province. Fresh leaf samples of *C. mongolicum* were collected in 22 July 2019. The sample collecting procedure was complied with our institutional guidelines. The voucher specimen was identified formally by expert on plant taxonomy from Gansu Grass Variety Committee and kept in Herbarium of Lanzhou Institute of Husbandry and Pharmaceutial Science (CYSLS-CmZhang20190722). Samples were quickly frozen in liquid nitrogen and conserved in -80°C for the subsequent analysis.

DNA Extraction, Illumina Sequencing, Assembly and Annotation

Genomic DNA was isolated by the Plant Genomic DNA Rapid Extraction Kit (Biomed Gene Technology) with the modified CTAB method [25]. 1% Agarose gel electrophoresis and Qubit Fluorometer (Invitrogen) were used to check DNA integrity and quality. One library (350 bp) was constructed by using pure DNA with the NEBNext®Ultra™ DNA Library Prep Kit for Illumina®. The library was sequenced with an Illumina NovaSeq platform (Benganen Tech Solution CO., Ltd, Wuhan, China) and finally 150 bp paired-end reads were generated. The unknown reads and low-quality reads were filtered using SOAPnuke software (version: 1.3.0). Chloroplast-like reads were identified by BLAST (E-value $\leq 1e^{-5}$) with other related species. Finally, the chloroplast-like reads were assembled using NOVOPlasty (version: 32) with the parameter of k-mer 39 to form a circular genome. The sequences were annotated using GeSeq, mainly containing coding gene prediction and non-coding RNAs annotation (rRNA and tRNA) [26]. The circular genome map of *C. mongolicum* was drawn through the OGDRAWv1.2 program [27].

Synonymous Codon Usage Bias Analysis

A number of the codon usage indicators were performed via the program codon W version 1.3 (<https://sourceforge.net/projects/codonw/>), including the relative synonymous codon usage value (RSCU), the effective number of codons (ENC), G + C content of the gene (GC), the frequency of the nucleotides G + C at the 3rd position of synonymous codons (GC_{3s}), and the base compositions (A_{3s}, T_{3s}, G_{3s}, and C_{3s}) [28]. The RSCU value and ENC value were used together to describe codon usage patterns

[29]. The G+C content at the 1st, 2nd, 3rd of codons (GC₁, GC₂, GC₃) and the average GC content of the 1st and 2nd (GC₁₂) were calculated by Cusp function from EMBOSS (<http://imed.med.ucm.es/EMBOSS/>) [30]. Synonymous codons with RSCU values >1.3 were identified as high frequency codons. The optimal codon of the gene was speculated as the codon with both the highest RSCU value and the largest Δ RSCU [31]. Parity rule 2 (PR2) plot mapping analysis was constructed to show the relationship of 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 [32]. ENC-plot mapping analysis was performed to reflect the relationship of the ENC values against the GC_{3S} values [33]. Neutrality plot mapping analysis was used to analyze the relationship of the GC₁₂ values and GC₃ values of all the genes [34].

Long repeat sequences and SSRs analysis

Long repeats including forward repeats and reverse repeats were analyzed by REPuter (<http://bibiserv.techfak.uni-bielefeld.de/reputer>) [30]. The hamming distance was set to 0 and the minimal repeat size was 20 bp. The SSRs were analyzed through the Perl script MISA (version: 1.0), and the SSRs parameters were defined as follows, the threshold of mononucleotide SSRs was ten repeats, the thresholds of dinucleotide and hexanucleotide SSRs were five repeats [35].

Phylogenetic Analysis

Phylogenetic tree with the bootstrap replicates set to 1000 was constructed by neighbor joining (NJ) analysis through TreesBeST (Version: 1.9.2, <http://www.mybiosoftware.com/treebest-1-9-2-software-phylogenetic-trees.html>). The cp genomes of *C. mongolicum* and other 36 species were used to investigate the evolution of *C. mongolicum*. The cp genomes information of the 36 plant species were downloaded from NCBI database.

Genome Structure Comparison

Based on the above results of the phylogenetic analysis, the complete cp genome of *C. mongolicum* was compared with other three closely related species of *Rumex acetosa* (NC_042390.1), *Rheum palmatum* (NC_027728.1), and *Fagopyrum esculentum* (NC_010776.1) using the mVista program with the shuffle-LAGAN mode [36]. The annotation of *C. mongolicum* was used as reference. The IRscope tool was used to visualize the genes on the boundaries of the junction sites of these four closely related species [37].

Selective pressure analysis

The orthologous genes of the Polygonaceae family were identified by OrthoMCL [38]. The sequences alignment of each orthologous gene was conducted using MAFFT [39]. The non-synonymous substitution rate (K_A) and synonymous substitution rate (K_S) were calculated by PAML [40]. The ω value was the ratio of K_A / K_S .

Results

Features of *C. mongolicum* Cp Genome

The cp genome of *C. mongolicum* was 162,124 bp in length, was comprised by a pair of IR regions (IRa and IRb) (30,512 bp), a large single copy (LSC) region of 87,718 bp and a small single copy (SSC) region of 13,382 bp (Fig. 1). The nucleotide composition of *C. mongolicum* cp genome was enriched in A/T nucleotides. The A + T content of the cp genome were 62.5%, which was significantly higher than the overall G + C content. The A + T content of the IR regions were 58.66%, obviously lower than LSC and SSC regions (64.42% and 67.51%, respectively) (Table 1). Weak base composition asymmetry (A-T, C-G) was found in *C. mongolicum* cp genome.

The positions of the 131 functional genes annotated in *C. mongolicum* cp genome were shown in Fig. 1. 78 genes were protein-coding genes, accounting for the half portion (59.5%) of the total genes. The remaining genes included 45 tRNA genes and 8 rRNA genes. According to the different functions, all the annotated genes were classified into four classes, including photosynthesis, self-replication, biosynthesis, and unknown functions (Table S1). Seventeen genes were duplicated in the IR regions, harboring 6 protein coding genes, 4 rRNA genes, and 6 tRNA genes.

In *C. mongolicum* cp genome, 12 different genes possessed a single intron and two exons, containing 5 tRNA genes and 7 protein coding genes, whereas the protein coding gene of *ycf3* and *clpP* had two introns and three exons (Table 2). Of the total intron-containing genes, the gene of *trnK-UUU* had the largest intron (2511 bp), and the *trnL-UAA* had the smallest intron (520 bp).

Synonymous Codon Usage Analysis

A total of 51 coding sequences (CDSs) with length longer than 300 bp were screened for synonymous codon usage (SCU) bias analysis. In general, the four nucleotides were unevenly represented in the 51 CDSs. Adenine (A) and thymine (T) were the most represented (43.3% and 46.4%, respectively), cytosine (C) and guanine (G) were the least represented (16.7% and 16.9%, respectively). The average GC content of the CDSs was 38.7%. We identified the total of 61 synonymous codons except for stop codons, among which, the total of 18 codons with RSCU value more than 1.3 was identified as high frequency synonymous codons, 29 codons with Δ RSCU value more than 0.08 were identified as the high expressed codons (Table 3 and Table S2). 7 codons with high frequency as well as high expression including TTT, GGA, CAT, AAA, TTA, AAT and CCT were identified as the optimal codons.

To further analyze the SCU pattern in *C. mongolicum* cp genome, multivariate statistical analysis of PR2, ENC-plot analysis, and neutrality plot were combined conducted. PR2 plot mapping showed that the genes distributed unevenly in the four quadrants centered on 0.5, most points located under the horizontal centered line of 0.5 (the ratio of $A_3/(A_3 + T_3) < 0.5$) (Fig. 2a). ENC plot was used to analyze the codon usage variation of the 51 CDSs (Fig. 2b). A majority of the points were lying away from the expected curve, accompanied with a relative concentrate distribution, and except for some points (*rp116*, *ycf2*, *ycf3*, and so on) located on the curve. Besides, we performed neutrality plot analysis to reveal the

relationship of GC₁₂ and GC₃ (Fig. 2c). Only one gene of *ycf2* located on the effected curve, the remaining genes were up the standard curve.

Long-Repeat Sequences and Simple Sequences Repeats (SSRs) Analysis

The long repeat sequences in *C. mongolicum* cp genome were searched by REPuter software. A total of 50 long repeats were detected, 44 were forward and 6 were reverse repeats (Table 4). A majority long repeat sequences were only located in intergenic spaces (IGSs) (47%), 39% long repeat sequences were distributed in different genes, and the remaining long repeats (14%) were detected both in IGSs and genes. It was worth noting that the six reverse repeats were all located in IGSs. Besides, a total of 17, 1, 12, and 10 repeats harbored only one region of LSC, SSC, IRa and IRb regions, respectively. Another 10 repeats were detected simultaneously in two regions. *Ycf1* CDS possessed the highest number of long repeats (14) and the longest repeats at 45 bp.

A total of 244 SSRs were found in *C. mongolicum* cp genome using MISA perl script. Among the identified SSRs, 67.2% was located in the LSC regions, 23.0% and 9.8% were found in the IR and SSC regions, respectively (Fig. 3a). 158 SSRs were located in IGSs, 80 SSRs were found in the coding regions and only 6 were found in introns (Fig. 3b). The numbers of mono-, di-, tri-, and tetranucleotides were 147, 43, 4, and 7, respectively (Fig. 3c). Mononucleotide repeats were the most frequented, accounting for 60.2% of the total repeats, while dinucleotides repeats accounted for 17.6%, and other SSRs were less common. Among all the identified SSRs, 20 SSRs belonged to G/C types, and the remaining SSRs belonged to the A/T types.

Phylogenetic Analysis

The phylogenetic tree was constructed based on a multiple alignment of nucleotide sequences of complete cp genomes from 37 plant species (Fig. 4). *Drosera rotundifolia* was used as the outgroup. The results showed that the species in the Polygonaceae family formed a clade, *C. mongolicum* was clustered closely to *R. acetosa*, *R. palmatum*, *Oxyria sinensis*, and *F. esculentum*. Furthermore, *R. acetosa* was the most related plant to *C. mongolicum*.

Comparative Analysis of Genomic Structure

The cp genome of *C. mongolicum* was compared to its closely related species including *R. acetosa*, *R. palmatum*, and *F. esculentum* (Table 5). *C. mongolicum* had the largest cp genome size, the largest SSC region and the most tRNA genes. *F. esculentum* had the smallest cp genome size and the largest LSC region. To further verify the genome divergence among these four species, sequence identity was compared using mVISTA with *C. mongolicum* as a reference (Fig. 5). Generally, IR regions were relatively conserved, while LSC and SSC regions were more divergent. Higher divergence of conserved non-coding regions were found than coding regions, for example, the IGS regions of *rps16* and *tmQ-UUG*, *ycf3* and *tmS-GGA*. Besides, significant differences were found in the regions of coding genes (*petD* and *ndhA*) and non-coding RNAs (*tml-GAU*).

IR Contraction and Expansion

The LSC/IR and SSC/IR boundaries of the cp genomes of *C. mongolicum* and other three related plant species were compared (Fig. 6). Six different genes were located at the juncture of the LSC/IRb (*rps19* and *rp12*), IRb/SSC (*ndhF*), SSC/IRa (*rps15* and *ycf1*), and IRa/LSC borders (*rp12* and *trnH*), respectively. The *ndhF* gene crossed the IRb/SSC border, with 62-95 bp lengths within IRb region. Compared to other species in the Polygonaceae, the borders of the IRb/SSC and SSC/IRa in *C. mongolicum* changed greatly. The LSC/IRb and IRa/LSC borders were relatively conserved in *C. mongolicum*, *R. palmatum*, and *F. esculentum*, however the *rps19* gene at the LSC/IRb border and the *trnH* gene at the IRa/LSC border in *R. acetosa* varied from the other three species.

Selective pressure events

A total of 75 orthologous protein-coding genes were found in the family of Polygonaceae. The ω values of most genes were lower than 1, except for the *psbK* gene found in the LSC region, which had a ω value of 1.0556 (Figure 7). The ω values of some genes were 0, such as *psbI*, *petN*, *ycf3*, *psbE*, *petG*, *rps12*, and *ndhE*.

Discussion

Features of the *C. mongolicum* Cp Genome

Cp genomes of land plants are mostly conserved in structures, gene content, and organization of content [41]. Generally, the cp genome is a typical quadripartite circular structure and composed by two IR regions, large (LSC) and small single-copy (SSC) regions [42]. However, Tao et al. [21] reported alfalfa had a special cp genome structure with only one IR region. Besides, the linear cp genome is existed which is different from the typical plant cp genomes with a single circular molecule [43]. In this study, the complete cp genome of *C. mongolicum* revealed a typical circular and quadripartite structure, implying the relatively conserved cp genome in land plants. Despite the structures of cp genomes in different plant species are overall conserved, the size of which varies from 107 kb to 218 kb [19]. The cp genome of *C. mongolicum* was ~162 kb, longer than the closely related plant species of *R. acetosa* (~160 kb), *R. palmatum* (~161 kb), and *F. esculentum* (~159 kb) to a certain extent. We also found that the A + T contents of the IR regions were significantly lower than other regions, which was similar to the observations in *Prunus* species, *Quercus acutissima*, and *Phleum pratense* and so on [14, 42, 44].

In land plant cp genomes, gene and intron content are highly conserved, although losses of them have been found in many angiosperms [11]. Funk et al. [45] found the losses of *ndh* genes in *Cuscuta reflexa*, they speculated the genes might be transferred to nuclear or the genes did not part in the critical life development. Here, we analyzed the genes and intron contents in *C. mongolicum* cp genome. The cp genome of *C. mongolicum* exhibited a complete set of genes (131), suggesting these genes might be critical to its development. Our results were similar to the finding of 130 genes in *Nelumbo nucifera* cp genome [46]. The cp genomes of the earliest diverging angiosperms contain the complete repertoire of 18

genes with introns [11]. Additionally, in many plants, the loss phenomenon of introns within protein-coding genes is often occurred, for example, *Cicer arietinum*, *Mahonia bealei*, and *Hordeum vulgare* [47-49]. The proteins encoded by genes with intron loss possess diverse functions. In *C. mongolicum* cp genome, we searched a total of 14 different genes with intron, and 4 intron losses were found, including *ycf1*, *rpoC2*, *rps19*, and *ndhF*. The genes with intron losses might endow *C. mongolicum* diverse functions on RNA polymerase, ribosomal proteins, and NADH dehydrogenase.

Synonymous Codon Usage Pattern

SCU bias reflects uneven usage of synonymous codons with the same amino acid, which is different among different species and genes [50]. The possible causes of SCU bias have been investigated in the genomes of numerous living organisms, for example, *Zea mays*, cotton, *Arabidopsis* and so on [51-53]. In this study, 51 CDSs of *C. mongolicum* cp genome were selected to analyze the SCU bias. AT/GC nucleotide usage differed among the three positions of codon, and the genes showed a preference for AT-ending codons, thus revealing a SCU imbalance of A/T and G/C at the third base position. This speculation was further confirmed by PR2 analysis. The similar observations were also found in *Elaeagnus angustifolia*, *Porphyra umbilicalis* and so on [17, 54].

In the case of random mutation or mutation pressure in a certain direction, there should be no change in the three different positions of each codon and the base content should be similar [31]. Thus, the preference for A/T ending bases would drive the observation of natural selection competing against mutation pressure. In order to analyze the two major evolutionary factors on codon usage in *C. mongolicum*, we constructed ENC plot analysis and neutrality plot analysis. ENC plot analysis reflects the relationship of ENC value and GC_{3s}, thus detecting the SCU variation among the genes [33]. The distribution comparison of genes and the standard curve could be indicative for some other factors except mutation pressure [33]. In our study, it was observed that a few genes were lying on the curve, which definitely originated from the extreme mutation pressure. However, a majority of the points were lying well below the curve, suggesting that a majority of genes in *C. mongolicum* cp genome had other SCU bias, for example natural selection. This hypothesis was largely supported by neutrality plot mapping analysis. Neutrality plot analysis is useful to compare the impacts of selection constraints and mutation on SCU [55]. The low correlation between GC₁₂ and GC₃ shows that the base composition of the 3 positions are different, and the GC content of the cp genome is highly conserved, indicating that natural selection is the most important determinant of codon usage patterns [56]. In the present neutral graph, no correlation was found between GC₃ and GC₁₂, indicating strong difference appeared, and natural selection would be crucial for SCU bias in *C. mongolicum* cp genome.

Molecular Markers

Long repeat sequences are associated with the sequence divergence and rearrangement of the cp genomes for illogical recombination and slipped-strand mismatch [57]. In *C. mongolicum* cp genome, more long repeats were found in LSC region than in IRs and SSC regions, verifying an uneven distribution

phenomenon of long repeats in cp genomes. Many studies indicate that the sequence divergences are higher in the LSC and SSC regions than IR regions, also higher in IGSs than coding regions [58, 59]. Thus, the long repeats appeared in LSC and SSC regions, such as *ycf3*, *ndhA*, *psaA* and *psaB*, might help reducing sequence mutations in these regions. For the abundant variable sites, the gene of *ycf1* was been reported to be used in DNA barcodes [60]. Huang et al. [14] recommended *ycf1* could be used as a candidate molecular marker for the Dicotyledon species. Similar to the finding in Dicotyledon species, *ycf1* harbored the highest number and the longest length of long repeats in *C. mongolicum* cp genome, thus we speculated *ycf1* might be used as an important molecular marker for phylogenetic elucidation in *Calligonum* species.

SSRs, known as microsatellites, have many forms in the cp genome, and the variations in SSRs copy numbers are different between plant species [61, 62]. SSRs are usually used as potential genetic markers for plant population genetics, polymorphism investigations, and evolutionary research [16, 63]. Different to the nuclear genome, SSR variations in the cp genome exhibit outstanding characteristics in evolutionary study since it is sensitive to population genetic effects and exploration the maternal gene flow of populations [64]. In the present study, the SSRs identified in *C. mongolicum* cp genome were inclined to A/T types, which were similar to the observations in three *Prunus* species and *Nasturtium officinale* [12, 15], supporting the supposition that cp SSRs are generally composed by poly-adenine/-thymine (polyA/T) repeats [42]. Similar to long repeats, more numbers of SSRs in LSC region than IRs and SSC region also implied an imbalanced repeats distribution in the cp genomes. Besides, the presence of the SSRs is indicators of important hotspots for genome recombination [14]. SSRs in *C. mongolicum* cp genome mainly located in IGSs which possessed high variable feature in the cp genome, suggesting that these regions could be treated as mutational hotspots. All the long repeats and SSRs identified in this study might furnish more venues for potential genetic markers in species identification and phylogenetic researches of *Calligonum* species.

Phylogenetic Analysis and Genomic Structure Comparison

Cp genome displays vast phylogenetic information, is usually used for phylogenetic reconstruction and population studies [65, 66]. In order to explore the phylogeny location of *C. mongolicum* and clearly elucidate the genetic evolutionary relationships within Polygonaceae, we performed phylogenetic analysis based on the cp genome of *C. mongolicum* and other 36 cp genomes data of plant species. From the phylogenetic tree, all the 5 studied Polygonaceae species were clustered together, and *C. mongolicum* was accommodated as the neighbouring clade to the branch of *R. acetosa* accompanied with a 100% bootstrap value, fully confirming the genus *Calligonum* as a member of Polygonaceae. Thus, the constructed phylogenetic tree could be useful to confirm the phylogenetic position of *C. mongolicum* and further understanding the phylogeny relationships among more Polygonaceae species in the future.

Although cp genomes have highly conserved structures of most plants, four regions have varied genome sizes [49, 67]. The genomic structure comparison of four Polygonaceae species including *C. mongolicum* showed that they possessed different sizes in the four regions, mainly due to their different genus

classifications. Among the four cp genomes of Polygonaceae species, more divergences emerged in LSC region, and higher divergence of conserved non-coding regions were found than coding regions. Our results were similar to the reports in other angiosperms, for example, *Kaempferia galangal* [68] and four *Echinacanthus* species [17], implying that less divergence in the IR regions and coding regions possibly might cause copy corrections in the process of gene conversion. Besides, as the most conserved regions, frequent expansions and contractions at the boundaries of SSC/IR and LSC/IR can reflect the taxa relationships, thereby recognizing as evolutionary signals [69]. Compared to *C. mongolicum* cp genome, the IR regions of *R. acetosa* cp genome exhibited a slight contraction, and to the contrary, in *F. esculentum* and *R. palmatum* exhibited a slight expansion. Thus, the contractions and expansions at the two boundaries would contribute to the size variations of the four Polygonaceae species cp genomes.

Adaptive evolution analysis

Adaptability improvement of a species during evolutionary progress is reflected by adaptive evolution [14]. Adaptive evolution, driven by evolutionary factors (for instance natural selection), leads to pressures and diversity at different biological organization processes [70]. Makalowski and Boguski [71] reported that the ω value had been widely applied to identify the evolutionary dynamics and explore adaptive characteristics among species. The gene is under positive selection when the ω value is more than 1. Otherwise, the gene is negatively selected. The positively selected genes play important roles in diverse environment adaptation [17]. In the present study, the ω value of *psbK* gene was more than 1, suggesting *psbK* was under positive selection. Similar to our result, *psbK* was also detected under positive selection in karst topography [17]. Thus, we speculated the gene *psbK* could play an important role in the adaptation evolutionary process of Polygonaceae species to the diverse environment, and its unique function needs to be validated in further study.

Conclusions

In the study, the complete cp genome of *C. mongolicum* was firstly depicted comprehensively, including genome features, SCU bias, identification of long-repeat sequence and SSRs, and adaptation evolution analysis. The cp genome of *C. mongolicum* was a typical quadripartite structure and 131 functional genes were annotated, while 4 intron losses including *ycf1*, *rpoC2*, *rps19*, and *ndhF* were found. Codons in *C. mongolicum* cp genome presented A/T ending preference, possibly caused by major natural selection constraints. The phylogenetic analysis among 37 species revealed *C. mongolicum* was closely related to *R. acetosa*. Besides, comparative analysis of genomic structure of *C. mongolicum* and other three Polygonaceae species was conducted, revealing more divergence in LSC and SSC regions than IR regions, also more divergence in IGSs than coding regions, and these divergent regions could be treated as mutational hotspots. Furthermore, expansions and contractions at SSC/IRs and LSC/IRs junctions were also analyzed. A total of 50 long repeats and 244 SSRs were identified. The adaptation evolution analysis showed that only *psbK* gene was positively selected, thus *psbK* might play a crucial role in the adaptation of Polygonaceae species. In summary, our results would lay a vigorous foundation for further

study on molecular marker exploration, phylogenetic signature, and population studies in *Calligonum* species.

Abbreviations

CDS: Coding sequences; LSC: Large single copy; SSC: Small single copy; IR: Inverted repeat; Cp: Chloroplast; A: Adenine; T: Thymine; C: Cytosine; G: Guanine; SCU: Synonymous codon usage; RSCU: Relative synonymous codon usage; ENC: Effective number of codons; IGS: Intergenic spaces; SSR: Simple sequence repeat; A₃: Adenine content at the third position of synonymous codons; T₃: Thymine content at the third position of synonymous codons; C₃: Cytosine content at the third position of synonymous codons; G₃: Guanine content at the third position of synonymous codons; GC₁₂: Average G + C content at the first and second positions of synonymous codons; GC₃: G + C content at the third positions of synonymous codons; GC_{3S}: G + C frequencies of at the third positions of synonymous codons; PR2: Parity rule 2.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publish

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author Contributions

D. H. R. and C. G. X. designed the experiments. Z. Q. and Y. H. S. performed the experiments. T. F. P., H. Y. and W. C. M. analyzed the sequencing data. D. H. R. wrote the paper. L. Y., Y. H. J. and C. G. X. revised this

paper. All authors have read and approved the manuscript.

Availability of data and materials

The raw sequence data of *C. mongolicum* cp genome have been submitted to GenBank under accession number MT568767. The address is as follows: <https://www.ncbi.nlm.nih.gov/genbank>.

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Tables

Table 1. Bases composition of the *C. mongolicum* cp genome.

Region	A (%)	T (U) (%)	C (%)	G (%)	A+T (%)	G+C (%)
LSC	31.74	32.68	18.24	17.35	64.42	35.58
SSC	35.78	31.73	17.08	15.41	67.51	32.49
IR	29.33	29.33	20.67	20.67	58.66	41.34
Total	31.16	31.34	19.06	18.44	62.5	37.5

Table 2. The statistics of exons and introns in genes from *C. mongolicum* cp genome.

Gene	Location	Length				
		Exon I	Intron I	Exon II	Intron II	Exon III
<i>trnK-UUU</i>	LSC	35	2511	37		
<i>rps16</i>	LSC	231	858	36		
<i>atpF</i>	LSC	411	758	144		
<i>rpoC1</i>	LSC	1607	776	430		
<i>ycf3</i>	LSC	147	738	122	840	124
<i>trnL-UAA</i>	LSC	35	520	50		
<i>trnV-UAC</i>	LSC	37	593	36		
<i>clpP</i>	LSC	270	633	292	1143	71
<i>rpl2_1</i>	IRa	393	662	435		
<i>ndhB_1</i>	IRa	777	679	756		
<i>rps12_1</i>	IRa	25	533	232		
<i>trnI-GAU_1</i>	IRa	37	944	35		
<i>trnA-UGC_1</i>	IRa	38	803	36		
<i>ndhA</i>	SSC	537	1200	561		
<i>trnA-UGC_2</i>	IRb	36	803	38		
<i>trnI-GAU_2</i>	IRb	35	944	37		
<i>rps12_2</i>	IRb	232	533	25		
<i>ndhB_2</i>	IRb	756	679	777		
<i>rpl2_2</i>	IRb	435	662	393		

Table 3. Codon usage and high frequency used codons in *C. mongolicum* cp genome. The highest frequency used codons (RSCU values >1.3) are in bold. RSCU: relative synonymous codon usage.

Amino acid	Condon	Number	RSCU	Amino acid	Condon	Number	RSCU
Ala(A)	GCT	497	1.72	Asn(N)	AAT	722	1.56
	GCC	208	0.72		AAC	202	0.44
	GCA	324	1.12	Pro(P)	CCT	350	1.6
	GCG	130	0.45		CCC	167	0.76
Cys(C)	TGT	160	1.45		CCA	239	1.09
	TGC	61	0.55		CCG	120	0.55
Asp(D)	GAT	654	1.57	Gln(Q)	CAA	552	1.49
	GAC	181	0.43		CAG	187	0.51
Glu(E)	GAA	832	1.48	Arg(R)	CGT	298	1.43
	GAG	290	0.52		CGC	68	0.33
Phe(F)	TTT	765	1.34		CGA	310	1.48
	TTC	374	0.66		CGG	85	0.41
Gly(G)	GGT	441	1.23		AGA	338	1.62
	GGC	182	0.51		AGG	155	0.74
	GGA	561	1.57	Ser(S)	TCT	415	1.68
	GGG	248	0.69		TCC	232	0.94
His(H)	CAT	380	1.53		TCA	290	1.17
	CAC	118	0.47		TCG	134	0.54
Ile(I)	ATT	875	1.52		AGT	305	1.23
	ATC	323	0.56		AGC	106	0.43
	ATA	525	0.91	Thr(T)	ACT	397	1.56
Lys(K)	AAA	818	1.51		ACC	191	0.75
	AAG	263	0.49		ACA	316	1.24
Leu(L)	TTA	696	1.92		ACG	115	0.45
	TTG	453	1.25	Val(V)	GTT	422	1.49
	CTT	455	1.26		GTC	147	0.52
	CTC	133	0.37		GTA	414	1.46
	CTA	303	0.84		GTG	150	0.53

	CTG	135	0.37	Tyr(Y)	TAT	609	1.6
Met(M)	ATG	474	1		TAC	151	0.4
Trp(W)	TGG	383	1				

Table 4. Long repeat sequences in the *C. mongolicum* cp genome. F, forward; R, reverse; IGS, intergenic space.

ID	Repeat start 1	Type	Size (bp)	Repeat start 2	Mismatch(bp)	E- Value	Gene	Region
1	101960	F	42	128315	0	3.82E-16	IGS, <i>ndhA</i>	IRa, SSC,
2	114286	F	45	114298	-3	2.29E-12	<i>ycf1</i>	IRa
3	135499	F	45	135511	-3	2.29E-12	<i>ycf1</i>	IRb
4	70628	F	38	115967	-1	1.12E-11	IGS, <i>ycf1</i>	LSC, IRa
5	45870	F	39	101962	-2	1.63E-10	<i>ycf3</i> , IGS	SSC, IRa
6	45870	F	39	128317	-2	1.63E-10	<i>ycf3</i> , <i>ndhA</i>	LSC, SSC
7	89639	F	39	89682	-2	1.63E-10	IGS	IRa
8	89646	F	32	89689	0	4.01E-10	IGS	IRa
9	160121	F	32	160164	0	4.01E-10	IGS	IRb
10	114296	F	35	114308	-2	3.35E-08	<i>ycf1</i>	IRa
11	128532	F	28	128561	0	1.03E-07	<i>ndhA</i>	SSC
12	28584	R	34	28584	-2	1.26E-07	IGS	LSC
13	110672	F	34	110704	-2	1.26E-07	IGS	IRa
14	139104	F	34	139136	-2	1.26E-07	IGS	IRb
15	112364	F	31	112392	-1	1.49E-07	IGS	IRa
16	137419	F	31	137447	-1	1.49E-07	IGS	IRb
17	52182	F	27	52196	0	4.10E-07	IGS	LSC
18	29564	F	30	29580	-1	5.77E-07	IGS	LSC

19	113225	F	35	113246	-3	1.11E-06	<i>ycf1</i>	IRa
20	136561	F	35	136582	-3	1.11E-06	<i>ycf1</i>	IRb
21	40309	F	26	42524	0	1.64E-06	<i>psaB, psaA</i>	LSC
22	113217	F	34	113238	-3	4.05E-06	<i>ycf1</i>	IRa
23	136570	F	34	136591	-3	4.05E-06	<i>ycf1</i>	IRb
24	40007	F	28	42243	-1	8.62E-06	<i>psaB, psaA</i>	LSC
25	45882	F	27	101974	-1	3.32E-05	<i>ycf3</i> , IGS	LSC, IRa
26	45882	F	27	128329	-1	3.32E-05	<i>ycf3, ndhA</i>	LSC, SSC
27	4793	F	32	121628	-3	5.37E-05	IGS	LSC, SSC
28	8935	F	29	37672	-2	9.37E-05	<i>trnS-GCU, trnS-UGA</i>	LSC
29	114310	F	29	114322	-2	9.37E-05	<i>ycf1</i>	IRa
30	135491	F	29	135503	-2	9.37E-05	<i>ycf1</i>	IRb
31	29571	F	23	29587	0	1.05E-04	IGS	LSC
32	52185	F	26	52215	-1	1.28E-04	IGS	LSC
33	40858	F	31	43082	-3	1.95E-04	<i>psaB, psaA</i>	LSC
34	10845	F	25	38665	-1	4.92E-04	IGS, <i>trnG-UCC</i>	LSC
35	28587	F	25	34468	-1	4.92E-04	IGS	LSC
36	112307	F	25	112457	-1	4.92E-04	IGS	IRa
37	137360	F	25	137510	-1	4.92E-04	IGS	IRb
38	113157	F	30	136649	-3	7.03E-04	<i>ycf1</i>	IRa, IRb

39	113163	F	30	136655	-3	7.03E-04	<i>ycf1</i>	IRa, IRb
40	30761	R	27	30761	-2	1.30E-03	IGS	LSC
41	136577	F	27	136598	-2	1.30E-03	<i>ycf1</i>	IRb
42	38853	F	21	69758	0	1.68E-03	IGS, <i>trnP-UGG</i>	LSC
43	44430	R	21	50104	0	1.68E-03	IGS	LSC
44	59741	R	21	59741	0	1.68E-03	IGS	LSC
45	112374	F	21	112402	0	1.68E-03	IGS	IRa
46	139117	F	21	139149	0	1.68E-03	IGS	IRb
47	52199	F	24	52215	-1	1.89E-03	IGS	LSC
48	7567	R	29	7572	-3	2.53E-03	IGS	LSC
49	15150	R	29	121615	-3	2.53E-03	IGS	LSC, SSC
50	114278	F	29	114314	-3	2.53E-03	<i>ycf1</i>	IRa

Table 5. Summary of cp genome features of *C. mongolicum* and other three related plants species.

Genome features	<i>Calligonum mongolicum</i>	<i>Fagopyrum esculentum</i>	<i>Rheum palmatum</i>	<i>Rumex acetosa</i>
Genome Size (bp)	162,124	159,599	161,541	160,269
LSC length (bp)	87,718	84,884	86,518	86,135
SSC length (bp)	13,382	13,344	13,111	13,128
IR length (bp)	30,512	30,685	30,956	30,503
Number of genes	131	131	131	129
Number of protein coding genes	78	72	81	67
Number of tRNA genes	45	37	37	36
Number of rRNA genes	8	8	8	8

Figures

Figure 1

Cp genome map of *C. mongolicum*. Genes inside the circle and those outside are transcribed clockwise and counter-clockwise, respectively. Different colored flags show genes with different functions. The GC and AT content are represented by the darker gray and the lighter gray, respectively.

Figure 2

Multivariate statistical analysis for genes in *C. mongolicum* cp genome. a, PR2 analysis, A_3 , T_3 , C_3 and G_3 represents nucleotide A, T, C and G content at the third position of synonymous codons, respectively; b, ENC-plot analysis, ENC: effective number of codons. GC_{3s} : the frequencies of nucleotide G + C at the third position of codons. The expected relationship between ENC values and GC_{3s} was reflected on the curve with random codon usage assumption; c, Neutrality plot analysis. GC_{12} : the average content of nucleotide G + C at the first and second positions of synonymous codons. GC_3 : the content of nucleotide

Figure 4

Phylogenetic relationship analysis of 37 species based on complete cp genomes. The phylogenetic tree was constructed by the neighbor-joining method. The bootstrap values were shown next to the branches based on 1000 replicates. The cp genome accession numbers from Genbank were labeled near to the names of plant species. *C. mongolicum* cp genome was in bold.

Figure 5

Complete cp genome comparison of *C. mongolicum* and other three related plants species. The cp genome of *C. mongolicum* was the reference and shown on the X-axis. The gene's orientations were shown by the grey arrows combined thick black lines on the top of the alignment. The Y-axis denotes identity ranging from 50% to 100%. UTR, untranslated regions. CNS, conserved non-coding sequences.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Additionalfile1.docx](#)
- [Additionalfile2.docx](#)