Analysis of codon usage patterns of the chloroplast genome in *Delphinium grandiflorum* L. reveals a preference for AT-ending codons as a result of major selection constraints

Huirong Duan¹, Qian Zhang¹, Chunmei Wang¹, Fang Li², Fuping Tian¹, Yuan Lu¹, Yu Hu¹, Hongshan Yang¹ and Guangxin Cui¹

¹Chinese Academy of Agricultural Sciences Lanzhou Institute of Husbandry and Pharmaceutical Science, Lanzhou, China
²Institute of Grassland Science, Chinese Academy of Agricultural Sciences, Hohhot, China

**ABSTRACT**

**Background.** Codon usage bias analysis is a suitable strategy for identifying the principal evolutionary driving forces in different organisms. *Delphinium grandiflorum* L. is a perennial herb with high economic value and typical biological characteristics. Evolutionary analysis of *D. grandiflorum* can provide a rich resource of genetic information for developing hybridization resources of the genus *Delphinium*.

**Methods.** Synonymous codon usage (SCU) and related indices of 51 coding sequences from the *D. grandiflorum* chloroplast (cp) genome were calculated using Codon W, Cups of EMBOSS, SPSS and Microsoft Excel. Multivariate statistical analysis combined by principal component analysis (PCA), correspondence analysis (COA), PR2-plot mapping analysis and ENC plot analysis was then conducted to explore the factors affecting the usage of synonymous codons.

**Results.** The SCU bias of *D. grandiflorum* was weak and codons preferred A/T ending. A SCU imbalance between A/T and G/C at the third base position was revealed by PR2-plot mapping analysis. A total of eight codons were identified as the optimal codons. The PCA and COA results indicated that base composition (GC content, GC³ content) and gene expression were important for SCU bias. A majority of genes were distributed below the expected curve from the ENC plot analysis and up the standard curve by neutrality plot analysis. Our results showed that with the exception of notable mutation pressure effects, the majority of genetic evolution in the *D. grandiflorum* cp genome might be driven by natural selection.

**Discussions.** Our results provide a theoretical foundation for elucidating the genetic architecture and mechanisms of *D. grandiflorum*, and contribute to enriching *D. grandiflorum* genetic resources.

**Subjects** Bioinformatics, Genomics, Plant Science

**Keywords** *Delphinium grandiflorum* L., Chloroplast genome, Synonymous codon usage bias, Evolutionary forces
INTRODUCTION

The codon is crucial in the process of genetic information transmission, and is the most fundamental step in biological activities (Powell & Moriyama, 1997; Chen et al., 2014). The accurate identification of codons encoding different amino acids is key to ensuring the correct expression of genetic information (Morton, Sorhannus & Fox, 2002; Sau et al., 2006). Most of the amino acids (except methionine (Met) and tryptophan (Trp)) are encoded by two to six synonymous codons (Guan et al., 2018). The choices of synonymous codons in different plant genomes are non-random, which is known as synonymous codon usage (SCU) bias (Wright, 1990). SCU bias reflects a mutation-selection balance, which can be affected by mutation pressure, natural selection, and genetic drift in a population (Bulmer, 1991; Eyre-Walker, 1991). Therefore, understanding the SCU bias can reveal the effects of long-term evolution on plant genomes.

The possible evolutionary forces based on codon usage patterns have been investigated in the genomes of numerous organisms. Generally, codon usage biases in microbes are driven by mutation pressure, such as in Xanthophyllomyces dendrorhous and Escherichia coli (Baeza et al., 2015; Boël et al., 2016). For invertebrate animals, codon usage bias is mainly driven by selection constraints, as exemplified in Bemisia tabaci and Hirudinaria manillensis (Sharma, Chakraborty & Uddin, 2014). Additionally, in plant species, codon usage bias seems to prefer a balance of mutation pressure and selection constraints (Zhang et al., 2018a; Zhang et al., 2018b; Zhang et al., 2018c; Liu et al., 2010). In the rice genome, the heterogeneity of codon usage patterns reflects a balance between a directional mutational bias and negative selection (Wang & Hickey, 2007). The codon usage bias in the Porphyra umbilicalis chloroplast (cp) genome is influenced by natural selection, mutation pressure, and nucleotide composition (Li et al., 2019). Moreover, codon usage bias results from Wang et al. (2018) indicated that translation selection has a more dominant role than mutation pressure in four cotton species. These studies indicate that complex evolutionary factors vary in different organisms, and analyzing codon usage bias can provide suitable strategies for identifying the principal driving forces. Delphinium grandiflorum L. (Ranunculaceae, Delphinium), a perennial herb with a blue flower, is mainly distributed in Mongolia, Siberia, and the Northwest of China (Chen et al., 2017). Owing to its high contents of two novel diterpenoid alkaloids, namely, grandiflodines A and B, D. grandiflorum is cultivated as a medicinal plant for toothache treatment and as a native pesticide (Zhang, Li & He, 2012). Furthermore, ovule culture is applied in D. grandiflorum to avoid hybrid embryos from aborting. For example, new interspecific hybrid plants (D. grandiflorum × D. nudicaule, D. grandiflorum × D. cardinal) are successfully selected with the intermediate flower color between the parents (Honda, Tsutsui & Hosokawa, 1999). Thus, D. grandiflorum is of great biological significance, and evolutionary analysis of D. grandiflorum can provide a rich resource of genetic information for developing hybridization resources for the genus Delphinium.

The chloroplast is a photosynthetic organelle in plant cells that plays crucial roles in photosynthesis and metabolite biosynthesis, for example, the synthesis of amino acids, starch, fatty acids, and pigments (Wicke et al., 2011). Compared to the
mitochondrial genome and nuclear genome, the complete cp genome, which possesses many characteristics, including a small size, simple and highly conserved structure, single parental inheritance, and haploid nature, is widely applied in species identification, phylogenetic analysis, and adaptive evolutionary analysis (Raubeson et al., 2007). Codon usage in many plant species, such as Hemiptelea davidii, Haberlea rhodopensis, Medicago sativa, and so forth, has been investigated extensively based on the cp genome database (Liu et al., 2020; Ivanova et al., 2017; Tao et al., 2017). The cp genome of D. grandiflorum has been assembled and characterized using Illumina sequencing platform, it was 157,339 bp in length, which contained a pair of inverted repeated regions (52,304 bp), a large single copy region (88,098 bp) and a small single copy region (16,937 bp) (Duan et al., 2020). However, the SCU bias of D. grandiflorum cp genome has not been investigated. In this study, we analyzed the codon bias and related indices of D. grandiflorum cp DNA, and then used multivariate statistical analysis to determine the general evolutionary driving factors. These results improve our understanding of the genetic architecture of D. grandiflorum, and also contribute to enriching the genetic resources and conservation of D. grandiflorum species.

MATERIALS & METHODS

Sequence data
A total of 117 genes were obtained from the D. grandiflorum cp genome (Genbank accession number: MN556604), and the sequence information is shown in Table S1 (Duan et al., 2020). After filtering the repeated sequences and genes with sequence length <300 bp using an in-house Python script (Sanner, 1999), ORFfinder (http://www.geneinfinity.org/sms/sms_orffinder.html) was used to distinguish and filter out non-coding regions of the remaining genes (Guan et al., 2018). Finally, a total of 51 qualified CDSs (complete coding sequence) were retained for subsequent analysis.

Codon usage bias and related indices analysis
A number of the codon usage indicators were estimated 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 (GC3s), and the base compositions (A3s, T3s, G3s, and C3s) (Zhang et al., 2018a; Zhang et al., 2018b; Zhang et al., 2018c). The RSCU value and ENC value were used together to describe codon usage patterns. 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 determined by the Cusp function from EMBOSS (http://imed.med.ucm.es/cgi-bin/emboss.pl?_action=input&_app=cusp).

Identification of the optimal codon
According to the RSCU values, the synonymous codons with the highest frequencies, accompanied by the largest RSCU values, were identified (Yu et al., 2012). Using ENC analysis as a preference standard, the 51 sequences of D. grandiflorum were ordered, and 5% of the dataset with high bias (ENC value was less than 30) and low bias (ENC value was
larger than 55) were selected (Cui et al., 2020). The sequences with high bias and low bias were recognized as highly and lowly expressed genes, respectively, as a result of codon bias and were positively correlated with gene expression level (Li et al., 2016). Highly expressed codons, were defined as those codons that occurred significantly more often in highly expressed genes relative to their frequency in lowly expressed genes, which was reflected by ΔRSCU. The ΔRSCU of each codon was calculated following the formula of ΔRSCU = RSCU (high bias) - RSCU (low bias) (Wang et al., 2019). Finally, the optimal codon of the gene was speculated as the codon with both the highest RSCU value and the largest ΔRSCU (Sharp & Li, 1986).

**Multivariate statistical analysis**

Principal component analysis (PCA) was used as a dimensionality reduction tool to reduce the data complexity in CodonW, with the principal components used to explore the codon usage variation among genes (Greenacre, 1984). PCA was performed on the RSCU values, the data were plotted in a 59-dimensional space of different axes, and the 59-dimensional space was based on the 59 triplet nucleotide codons (ATG encoding Met and TGG encoding Trp were excluded) (Gupta & Ghosh, 2001). Finally, the most prominent axes with important implications for codon usage variation were revealed (Choudhury, Uddin & Chakraborty, 2017).

Correspondence analysis (COA) was used to compare two or more categories of variable data, and provide visual results for the major changes in the trends of codon usage and genes (Choudhury, Uddin & Chakraborty, 2017). The relationship between prominent axes and codons, prominent axes and GC content, and prominent axes and genes were visualized in scatter plots.

Parity rule 2 (PR2) plot mapping analysis was used to show the relationship of the values \( A_3/(A_3 + T_3) \) and \( G_3/(G_3 + C_3) \) related to codons and four-degenerate synonymous-codon amino acids (alanine, glycine, proline, threonine, valine, arginine [CGA, CGU, CGG, and CGC], leucine [CUA, CUU, CUG, and CUC] and serine [UCA, UCU, UCG, and UCC]), then the data were distributed into four quadrants in a scatter diagram (Sueoka, 1995; Sueoka, 1999).

ENC-plot mapping analysis was employed to analyze and determine the crucial factors influencing the codon usage bias. The ENC plot reflects the relationship of the ENC values against the GC\(_{3s}\) values. The standard curve shows the optimal functional relation between ENC and GC\(_{3s}\) (Gupta, Bhattacharyya & Ghosh, 2004).

Neutrality plot mapping analysis was used to analyze the relationship of the GC\(_{12}\) values and GC\(_{3}\) values of all the genes. In the neutral graph, the value of GC\(_{12}\) was used as a vertical coordinate, and the value of GC\(_{3}\) was used as the horizontal axis (Wei et al., 2014).

**Statistical analysis**

Correlation analysis among many important indices was implemented in SPSS 16.0 software (SPSS Inc., Chicago, US) with the Spearman’s test (two-tailed). The graphs were depicted in Microsoft EXCEL 2016 (Microsoft Corporation, Redmond, WA, US).
RESULTS

Nucleotide composition between codon positions in the *D. grandiflorum* cp genome

We identified 51 CDSs longer than 300 bp, and the average length of the CDSs was 1212.6 bp. In general, the four nucleotides were unevenly represented in the 51 CDSs. Thymine (T) was the most represented (31%), adenine (A) was the second-most represented (30%), cytosine (C) and guanine (G) were less represented (18% and 21%, respectively), and the average GC content of the CDSs was 39%. To better evaluate the nucleotide base composition in *D. grandiflorum*, we summarized the CDS numbers with different GC content levels, and all CDSs contained 30–46% GC content (Fig. 1A). We further divided the GC content range into three parts and analyzed the number of CDSs attributable to each part. The 35–40% part contained the most CDSs (the total number was 28), followed sequentially by the 30–35% and 40–46% intervals.

We also summed the GC content at different codon positions (1st, 2nd, and 3rd) in the CDSs. The composition at the 2nd codon position was similar to that of the overall nucleotide composition. The average GC content and the range between the upper and lower quartiles in the 1st codon position were the highest, and accordingly, the corresponding data in the 3rd codon position were the lowest (Fig. 1B).

The codon usage pattern of the *D. grandiflorum* cp genome

The amino acids number of 51 genes ranged between 101 and 2,138 with an average of 404. We identified a total of 61 synonymous codons (stop codons were excluded), among which 31 were more frequently represented with an RSCU value ≥ 1 (Table 1). The codon TTA encoding Leu exhibited the highest RSCU value of 1.88. The above 31 codons with different end bases were divided into three classes, and the number of codons ending with T, A, and G was 16, 12, and 3, respectively, thus suggesting that the genes from the *D. grandiflorum* cp genome preferred codons with AT-endings, especially those ending with T. Moreover, we focused on the preferred and weak preferred codons, mainly emphasizing codons with extremely high (>1.5) and low RSCUs (<0.5). We found that codons such as ACT, TAT, CAA, and GGA were highly preferred and codons such as CTC, AAC, CTG, and CAG were less preferred in the CDSs. The two distinct patterns deviated from the neutral RSCU value of 1, further indicating that codons preferred an ending with A/T.

The average content of GC, GC$_1$, GC$_2$, and GC$_3$ of the CDSs from the *D. grandiflorum* was calculated (Table 2). The ENC values of the different genes varied from 37.11 to 61.00, the average of which was 48.12, displaying different trends between the genes. Strong and weak SCU biases are typically distinguished by the ENC value with 35, and all of the ENC values of the genes in this study were greater than 35, suggesting a weak codon bias (*Song et al.*, 2018).

A total of 18 codons with the largest RSCU value based on each amino acid were identified as high frequency synonymous codons (Table 1). Twenty-three codons were identified as highly expressed codons (Table 3). Eight codons with a high frequency as well as high expression, including GCT, GAT, TTT, ATT, AAA, TCT, ACT, and TAT, were identified as optimal codons, of which, seven ended with T and only one ended with A,
Figure 1  **Base composition of *D. grandiflorum* cp genome.** (A) Distribution of genes with different GC contents; (B) Box plot of GC contents variation in different codon positions (1st, 2nd, 3rd and all (overall cp genome)). The numbers on the box plot from top to bottom represent GC content of the maximum, upper quartile (75%), middle quartile (50%), lower quartile (25%), and minimum, respectively.

Further confirming that the codons ending with C and G were lacking preference in the *D. grandiflorum* cp genome.

**PCA analysis**

The 51 CDSs of the *D. grandiflorum* cp genome were analyzed using PCA analysis, and were distributed in 50 dimensional axes. The contribution of 40 axes was calculated, and
Table 1  Codon usage and high frequency used codons in *D. grandiflorum* cp genome. The highest frequency used codons (the largest RSCU value) are in bold. RSCU: the relative synonymous codon usage value.

| Amino acid | Condon | Number | RSCU | Amino acid | Condon | Number | RSCU |
|------------|--------|--------|------|------------|--------|--------|------|
| Ala (A)    | GCT    | 482    | 1.71 | Asn (N)    | AAT    | 775    | 1.57 |
|            | GCC    | 186    | 0.66 |            | AAC    | 214    | 0.43 |
|            | GCA    | 326    | 1.15 | Pro (P)    | CCT    | 331    | 1.51 |
|            | GCG    | 136    | 0.48 |            | CCC    | 166    | 0.76 |
| Cys (C)    | TGT    | 170    | 1.47 |            | CCA    | 257    | 1.18 |
|            | TGC    | 61     | 0.53 |            | CCG    | 120    | 0.55 |
| Asp (D)    | GAT    | 677    | 1.57 | Gln (Q)    | CAA    | 550    | 1.52 |
|            | GAC    | 183    | 0.43 |            | CAG    | 173    | 0.48 |
|            | GAA    | 820    | 1.48 | Arg (R)    | CGT    | 280    | 1.38 |
|            | GAG    | 290    | 0.52 |            | CGC    | 76     | 0.37 |
| Phe (F)    | TTT    | 749    | 1.32 |            | CGA    | 276    | 1.36 |
|            | TTC    | 386    | 0.68 |            | CGG    | 88     | 0.43 |
| Gly (G)    | GGT    | 482    | 1.34 |            | AGA    | 360    | 1.77 |
|            | GGG    | 241    | 0.67 |            | AGG    | 139    | 0.68 |
|            | GGA    | 552    | 1.54 | Ser (S)    | TCT    | 423    | 1.65 |
| His (H)    | CAT    | 386    | 1.48 |            | TCA    | 311    | 1.22 |
|            | CAC    | 135    | 0.52 |            | TCG    | 160    | 0.63 |
| Ile (I)    | ATT    | 874    | 1.48 |            | AGT    | 299    | 1.17 |
|            | ATC    | 344    | 0.58 |            | AGC    | 90     | 0.35 |
|            | ATA    | 558    | 0.94 | Thr (T)    | ACT    | 421    | 1.59 |
| Lys (K)    | AAA    | 763    | 1.47 |            | ACC    | 194    | 0.73 |
|            | AAG    | 274    | 0.53 |            | ACA    | 329    | 1.24 |
|            | TTA    | 673    | 1.88 |            | ACG    | 116    | 0.44 |
|            | TTG    | 458    | 1.28 | Val (V)    | GTT    | 421    | 1.51 |
|            | CTT    | 456    | 1.28 |            | GTC    | 114    | 0.41 |
|            | CTC    | 135    | 0.38 |            | GTA    | 430    | 1.54 |
|            | CTA    | 288    | 0.81 |            | GTG    | 152    | 0.54 |
|            | CTG    | 135    | 0.38 | Tyr (Y)    | TAT    | 617    | 1.59 |
| Met (M)    | ATG    | 500    | 1    |            | TAC    | 157    | 0.41 |
| Trp (W)    | TGG    | 386    | 1    |            |        |        |      |

the gene variations from the four major axes (Axis 1 to Axis 4) accounted for 35.5% of the total axis variation. Axis 1 and Axis 2 explained 10.71% and 8.96% of the total variation, while Axis 3 and Axis 4 explained 8.36% and 7.47% of that the variation, respectively.

**COA analysis**

To determine how the codons ending with different bases were contributing toward codon usage variation in the major axes of Axis 1 and Axis 2, the location of codons ending with different bases was drawn using different color points between Axis 1 and Axis 2 by COA analysis (Fig. 2A). The codons with A/T ends were closer to Axis 1 and were more tightly clustered than the codons with G/C ends, suggesting that the base composition probably
Table 2  Indices of codon usage of 51 genes from the cp genome of *D. grandiflorum*.

| Gene   | GC  | GC3 | GC3s | ENC  | Gene   | GC  | GC3 | GC3s | ENC  |
|--------|-----|-----|------|------|--------|-----|-----|------|------|
| accD   | 0.36| 0.31| 0.28| 50.99| psbA   | 0.43| 0.34| 0.30| 40.89|
| atpA   | 0.42| 0.31| 0.29| 49.59| psbB   | 0.43| 0.30| 0.25| 47.36|
| atpB   | 0.42| 0.29| 0.27| 47.81| psbC   | 0.45| 0.33| 0.30| 46.17|
| atpE   | 0.39| 0.27| 0.25| 47.65| psbD   | 0.44| 0.35| 0.30| 46.28|
| atpF   | 0.38| 0.34| 0.32| 44.90| rbcL   | 0.44| 0.31| 0.29| 49.45|
| atpI   | 0.38| 0.27| 0.24| 46.31| rpl14  | 0.39| 0.26| 0.24| 49.88|
| ccsA   | 0.32| 0.25| 0.20| 46.36| rpl16  | 0.44| 0.27| 0.21| 41.65|
| cemA   | 0.32| 0.31| 0.27| 56.82| rpl20  | 0.37| 0.29| 0.26| 43.10|
| clpP   | 0.45| 0.34| 0.31| 55.82| rpl22  | 0.36| 0.30| 0.24| 47.84|
| matK   | 0.31| 0.26| 0.23| 47.53| rpoA   | 0.36| 0.29| 0.26| 48.30|
| ndhA   | 0.36| 0.25| 0.22| 42.71| rpoB   | 0.40| 0.30| 0.28| 50.41|
| ndhB   | 0.37| 0.32| 0.27| 47.37| rpoC1  | 0.38| 0.28| 0.25| 48.97|
| ndhC   | 0.36| 0.26| 0.20| 41.24| rps11  | 0.45| 0.26| 0.22| 50.83|
| ndhD   | 0.36| 0.29| 0.24| 48.99| rps12  | 0.38| 0.29| 0.27| 50.39|
| ndhE   | 0.34| 0.23| 0.20| 52.21| rps14  | 0.41| 0.32| 0.29| 40.28|
| ndhF   | 0.33| 0.24| 0.20| 44.71| rps18  | 0.35| 0.24| 0.21| 37.11|
| ndhG   | 0.36| 0.26| 0.22| 43.72| rps2   | 0.38| 0.26| 0.23| 45.79|
| ndhH   | 0.39| 0.30| 0.25| 51.44| rps3   | 0.36| 0.26| 0.24| 48.96|
| ndhI   | 0.37| 0.28| 0.26| 50.38| rps4   | 0.39| 0.27| 0.26| 51.15|
| ndhJ   | 0.41| 0.35| 0.30| 57.32| rps7   | 0.41| 0.24| 0.21| 46.08|
| ndhK   | 0.40| 0.30| 0.27| 51.77| rps8   | 0.37| 0.25| 0.22| 42.77|
| petA   | 0.39| 0.32| 0.30| 50.42| ycf1   | 0.32| 0.29| 0.25| 49.67|
| petB   | 0.43| 0.36| 0.30| 45.84| ycf2   | 0.38| 0.37| 0.34| 53.44|
| petD   | 0.38| 0.24| 0.21| 40.60| ycf3   | 0.40| 0.49| 0.46| 61.00|
| psaA   | 0.44| 0.35| 0.30| 50.76| ycf4   | 0.40| 0.34| 0.31| 51.36|
| psaB   | 0.42| 0.34| 0.29| 51.35| Average| 0.39| 0.30| 0.26| 48.12|

affected the SCU bias. In contrast, the genes with lower GC contents (30%–35%) were distributed along the side of Axis 2, and the genes with a relatively lower GC content were more concentrated than the genes with a higher GC content (Fig. 2B), implying that GC content might influence the SCU bias. In addition, considering the positions of different functional gene groups, and following the direction along Axis 1 and Axis 2, we also found that the different groups were distributed discretely, indicating that many other factors (i.e., natural selection) might play a role in SCU bias (Fig. 2C).

In order to analyze the relationship of the important indices to the four main axes, correlation analysis was conducted to determine the central factors influencing codon usage bias (Table 4). The GC content showed an extremely negative correlation with Axis 2 (*P* < 0.01), and the GC3s and GC3 contents also exhibited a significant negative correlation with Axis 2 and Axis 4.

**PR2-plot mapping analysis**

Using PR2 plot mapping analysis, the points in our plot fell among 0.39 to 0.59 on $A_3/(A_3 + T_3)$, and 0.26 to 0.82 $G_3/(G_3 + C_3)$ (Fig. 3A). The genes were clearly distributed unevenly...
## Table 3 The codons statistics with high and low expression genes of the *D. grandiflorum* cp genome.

| Amino acid | Codon | High expressed gene | Low expressed gene | ΔRSCU | Amino acid | Codon | High expressed gene | Low expressed gene | ΔRSCU |
|------------|-------|---------------------|-------------------|-------|------------|-------|---------------------|-------------------|-------|
|            | Frequency | RSCU | Frequency | RSCU | Asn (N) | AAT | Frequency | RSCU | Frequency | RSCU |
| Ala (A)    | GCT    | 4.00 | 1.45 | 4.00 | 1.00 | 0.45 | Asn (N) | AAT | 4.00 | 1.33 | 2.00 | 1.33 | 0.00 |
|            | GCC    | 1.00 | 0.36 | 3.00 | 0.75 | −0.39 |            |     |       |     |       |     |       |     |
|            | GCA    | 4.00 | 1.45 | 6.00 | 1.50 | −0.05 | Pro (P) | CTT | 4.00 | 1.60 | 5.00 | 1.54 | 0.06 |
|            | GCC    | 2.00 | 0.73 | 3.00 | 0.75 | −0.02 |            |     |       |     |       |     |       |     |
|            | GCG    | 2.00 | 0.73 | 3.00 | 0.75 | −0.02 |            |     |       |     |       |     |       |     |
| Cys (C)    | TGT    | 0.00 | 0.00 | 3.00 | 2.00 | −2.00 |            |     |       |     |       |     |       |     |
|            | TGC    | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |            |     |       |     |       |     |       |     |
|            | Asn (N) | AAT | 4.00 | 1.33 | 2.00 | 1.33 | 0.00 |      |       |     |       |     |       |     |
| Asp (D)    | GAT    | 7.00 | 2.00 | 0.00 | 0.00 | 2.00 | Gln (Q) | CAA | 6.00 | 2.00 | 4.00 | 1.00 | 1.00 |
|            | GCC    | 2.00 | 0.73 | 3.00 | 0.75 | −0.02 |            |     |       |     |       |     |       |     |
|            | GCA    | 4.00 | 1.45 | 6.00 | 1.50 | −0.05 | Pro (P) | CTT | 4.00 | 1.60 | 5.00 | 1.54 | 0.06 |
|            | GCC    | 2.00 | 0.73 | 3.00 | 0.75 | −0.02 |            |     |       |     |       |     |       |     |
|            | GCG    | 2.00 | 0.73 | 3.00 | 0.75 | −0.02 |            |     |       |     |       |     |       |     |
|            | GCG    | 2.00 | 0.73 | 3.00 | 0.75 | −0.02 |            |     |       |     |       |     |       |     |
| Cys (C)    | TGT    | 0.00 | 0.00 | 3.00 | 2.00 | −2.00 |            |     |       |     |       |     |       |     |
|            | TGC    | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |            |     |       |     |       |     |       |     |
|            | Asn (N) | AAT | 4.00 | 1.33 | 2.00 | 1.33 | 0.00 |      |       |     |       |     |       |     |
| Asp (D)    | GAT    | 7.00 | 2.00 | 0.00 | 0.00 | 2.00 | Gln (Q) | CAA | 6.00 | 2.00 | 4.00 | 1.00 | 1.00 |
|            | GCC    | 2.00 | 0.73 | 3.00 | 0.75 | −0.02 |            |     |       |     |       |     |       |     |
|            | GCA    | 4.00 | 1.45 | 6.00 | 1.50 | −0.05 | Pro (P) | CTT | 4.00 | 1.60 | 5.00 | 1.54 | 0.06 |
|            | GCC    | 2.00 | 0.73 | 3.00 | 0.75 | −0.02 |            |     |       |     |       |     |       |     |
|            | GCG    | 2.00 | 0.73 | 3.00 | 0.75 | −0.02 |            |     |       |     |       |     |       |     |
| Cys (C)    | TGT    | 0.00 | 0.00 | 3.00 | 2.00 | −2.00 |            |     |       |     |       |     |       |     |
|            | TGC    | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |            |     |       |     |       |     |       |     |
|            | Asn (N) | AAT | 4.00 | 1.33 | 2.00 | 1.33 | 0.00 |      |       |     |       |     |       |     |
| Asp (D)    | GAT    | 7.00 | 2.00 | 0.00 | 0.00 | 2.00 | Gln (Q) | CAA | 6.00 | 2.00 | 4.00 | 1.00 | 1.00 |
|            | GCC    | 2.00 | 0.73 | 3.00 | 0.75 | −0.02 |            |     |       |     |       |     |       |     |
|            | GCA    | 4.00 | 1.45 | 6.00 | 1.50 | −0.05 | Pro (P) | CTT | 4.00 | 1.60 | 5.00 | 1.54 | 0.06 |
|            | GCC    | 2.00 | 0.73 | 3.00 | 0.75 | −0.02 |            |     |       |     |       |     |       |     |
|            | GCG    | 2.00 | 0.73 | 3.00 | 0.75 | −0.02 |            |     |       |     |       |     |       |     |

Notes.
RSCU: the relative synonymous codon usage value.
*indicates the high expression codons (ΔRSCU > 0.08).
Figure 2  Correspondence analysis (COA) of SCU in *D. grandiflorum* cp genome. (A) COA analysis of SCU toward the codons, codons with different ending bases are represented by different colors. (B) COA analysis of SCU toward different GC contents, codons with different GC contents are represented by different colors. (C) COA analysis of SCU toward the coding genes, different gene types are represented by different colors and symbols.

Table 4  Correlation coefficients of the indices influencing codon bias in *D. grandiflorum* cp genome.

| Indices | GC   | ENC  | GC3s | GC3 | Axis 1 | Axis 2 | Axis 3 | Axis 4 |
|---------|------|------|------|-----|--------|--------|--------|--------|
| GC      | 1    |      |      |     |        |        |        |        |
| ENC     | 0.089| 1    |      |     |        |        |        |        |
| GC3s    | 0.424* | 0.548** | 1    |     |        |        |        |        |
| GC3     | 0.437** | 0.521** | 0.964** | 1   |        |        |        |        |
| Axis 1  | 0.123| 0.275| 0.198| 0.166| 1      |        |        |        |
| Axis 2  | −0.393** | −0.498** | −0.623* | −0.664** | −0.004| 1      |        |        |
| Axis 3  | 0.226| 0.045| 0.134| 0.104| −0.002| 0.005| 1      |        |
| Axis 4  | 0.111| −0.331* | −0.385** | −0.292** | 0.007| −0.015| −0.006| 1    |

Notes.

*Positive correlation (P < 0.05).

**Significant positive correlation (P < 0.01).

GC, G + C content of the gene; ENC, the effective number of codons; GC3s, the frequency of the nucleotides G + C at the 3rd of synonymous codons; GC3, The G + C content at the 3rd of codons.
Figure 3  Multivariate statistical analysis for genes in \textit{D. grandiflorum} cp genome. (A) PR2 analysis. \(A_3/(A_3 + T_3)\): the ratio of A against A + T at the third position of codons. \(G_3/(G_3 + C_3)\): the ratio of G against G + C at the third position of codons. The curves show the center line on 0.5. (B) ENC-plot analysis. ENC: effective number of codons. \(GC_3\): the frequencies of nucleotide G + C at the third position of synonymous codons. The curve shows the expected relationship between ENC values and \(GC_3\)s under random codon usage assumption. (C) Neutrality plot analysis. \(GC_{12}\): the average frequencies of nucleotide G + C at the first and second positions of synonymous codons. \(GC_3\): the frequencies of nucleotide G + C at the third position of synonymous codons. The curve shows that \(GC_{12}\) is equal to \(GC_3\).

in the four quadrants centered on 0.5, with most points located under the horizontal centered line of 0.5 (in which the ratio of \(A_3/(A_3 + T_3) < 0.5\)) and a slightly greater number of points distributed on the right side of the vertical centered line of 0.5 (in which the ratio of \(G_3/(G_3 + C_3) > 0.5\)). These results indicated that the genes in \textit{D. grandiflorum} preferred T and G, especially T at the third codon position.

Furthermore, we performed PR2 plot analysis of four-codon amino acids, including alanine, glycine, proline, threonine, valine, arginine (CGA, CGU, CGG, and CGC), leucine (CUA, CUU, CUG, and CUC), and serine (UCA, UCU, UCG, and UCC) (Fig. 4). It was
clear that PR2 violation was the rule rather than the exception, and the distribution pattern was unique for each of the eight amino acids. The average value of $A_3/(A_3 + T_3)$ and $G_3/(G_3 + C_3)$ from the eight amino acids weighted with codon numbers for each gene was 0.44 and 0.38, respectively, suggesting that the eight amino acids had a preference for T and C when the eight amino acids were combined. Therefore, the balance between A/T and G/C was disrupted in *D. grandiflorum*.

**ENC plot analysis**

An ENC plot was used to analyze the codon usage variation of the 51 CDSs in *D. grandiflorum* (Fig. 3B). Some genes were located on the standard curve toward the lower GC content region, for example, *rps3* and *rps4* from ribosomal proteins (SSU), *ndhI* and *ndhK* from NADH dehydrogenase, and so forth, which definitely originated from the extreme compositional constraints. However, a majority of the points were distributed away...
from the expected curve and were accompanied by a relatively concentrated distribution, suggesting that these genes should have additional codon usage biases, that are independent of compositional constraints. In addition, correlation analysis of the ENC and GC₃s values showed an extreme positive correlation ($r = 0.548$, $P<0.01$), suggesting that the base composition on the third position of the codons might play an important role in determining codon usage patterns.

**Neutrality plot analysis**

From the neutrality plot, the relationship of GC₁₂ and GC₃ was analyzed, and the degree of change in natural selection and mutation pressure was estimated (Fig. 3C). The *ycf2* and *cemA* genes were located around the effected curve, while the remaining genes were above the standard curve. Using Pearson’s correlation analysis, a weak correlation of all coding genes between GC₁₂ and GC₃ was found ($r = 0.261$).

**DISCUSSION**

The transition of genetic information from mRNA to protein relies on the formation of codons (Chakraborty et al., 2017). The basic characteristic of a genetic code is that an amino acid is often encoded by different codon combinations, known as synonymous codons (Baeza et al., 2015). The uneven usage of synonymous codons with the same amino acid is reflected by SCU bias, and the SCU bias differs among various species and genes (Karumathil et al., 2018). The possible causes of SCU bias have been investigated in the genomes of numerous living organisms, for example, in *Zea mays*, *Arabidopsis thaliana*, cotton, and so others (Liu et al., 2010; Wang et al., 2018; Qiu et al., 2011). In this study, 51 CDSs of the *D. grandiflorum* cp genome were selected to analyze the SCU bias, and the possible factors influencing SCU bias were inferred.

**Unique codon usage pattern in the *D. grandiflorum* cp genome**

The RSCU values reflect the codon usage pattern of different genes. The codon lacks bias when the RSCU value is less than 1 (Karumathil et al., 2018). In the *D. grandiflorum* cp genome, codons with the largest RSCU value based on each amino acid were suggested as high frequency codons. ENC reflects the degree of codon deviation from random selection and is an important index for reflecting the preference degree of the unequal use of synonymous codons (Gupta, Bhattacharyya & Ghosh, 2004). The range of ENC values is from 20 to 61, and the boundary value of ENC is 35. A value less than 35 represents strong codon preference, otherwise weak codon preference will occur (Song et al., 2018). In our study, the average ENC value of the codon genes was 48.12, implying a weak preference for SCU bias.

Our results indicated that the AT/GC nucleotide usage differed among the three positions of the codon, and these differences in base compositions might affect the total SCU bias in the *D. grandiflorum* cp genome. However, the overall SCU bias that we detected was low, which might be because the majority of codons were used during translation, and extreme SCU bias might only develop under particular conditions (Guan et al., 2018). In addition, we found that the genes from the *D. grandiflorum* cp genome showed a
preference for AT-ending codons, particularly T-ending codons. Eight optimal codons further exhibited the similar patterns, seven of which ended with T, and one of which ended with A. PR2 is a rule of DNA base composition that endows A = T and G = C within a single strand when there is no any preference in mutation pressure and natural selection in both strands of DNA (Sueoka, 2001; Sueoka, 1995; Lobry, 1995). The present results showed that the distribution of genes with different ending bases was asymmetric and exhibited a preference for T-ending codons, and an apparent PR2 violation of the eight amino acids was further detected, thus revealing an SCU imbalance between A/T and G/C at the third base position. Our results were similar to those in other plant species. In the cotton genome, codons ending with T/A are preferred (Wang et al., 2018). A similar pattern was found in the codon usage of Elaeagnus angustifolia and Porphyra umbilicalis (Li et al., 2019; Wang et al., 2019). However, this phenomenon has not been observed in monocot species, for example, Z. mays, Oryza sativa, and Hordeum vulgare (Liu et al., 2010; Wang & Hickey, 2007; Kawabe & Miyashita, 2003). The opposing patterns of codon ending bases might reflect the differences in differentiation between monocot and dicot plant species (Camiolo, Melito & Porceddu, 2015).

Base composition affects the SCU bias of the D. grandiflorum cp genome

PCA analysis is usually used to analyze genes located in a 59-dimensional space and relies on the RSCU values. PCA can extract considerable variations and concentrate them together, thus helping to determine the major factors influencing SCU bias (Wei et al., 2014). In the present study, four main axes reflecting variation were determined, and the major indices versus the four axes were analyzed by correlation analysis. The codons with A/T endings plotted on Axis 1 and Axis 2 and showed a more tightly clustered distribution, indicating that this base composition could explain the variation in codon use. The significant correlations of GC content, GC$_3$s and GC$_3$ content against the Axis 2 suggested that the base compositions as GC contents of the total and the third position of codons were valuable for SCU bias in the D. grandiflorum cp genome. However, Axis 1 and Axis 2 only explained 19.67% amount of the variation, and it appeared that the base composition had at most a partial influence on codon usage.

Natural selection plays a major role in the SCU bias of D. grandiflorum cp genome

Synonymous codons are uneven by their nature, the mutations of which often occur at the 3rd base of a codon (Comeron & Aguadé, 1998). If there is no external pressure, as 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 (Guan et al., 2018). Thus, the preference for AT ends caused by directional substitution implied that evolutionary factors of SCU bias from D. grandiflorum cp genome were indeed existed. Generally, mutation pressure acts on nucleotide composition bias through shuffling A/T and G/C pairs, selection constraints lead to codon bias through maximizing protein production efficiency in high expressed genes (Guan et al., 2018). In our study, A/T and G/C at the third base position were asymmetric by PR2 analysis, and the significant
correlations of GC content, GC$_{3s}$ and GC$_3$ content against the Axis 2 were found, which of them indicated that mutation pressure of base composition influenced SCU bias in the D. grandiflorum cp genome. However, Axis 2 only explained 8.96% amount of the variation, thus mutation pressure was not the determining factor shaping codon usage, other factors as well as natural selection might be more important than mutation pressure. ENC plot analysis and neutrality plot analysis are commonly combined to explore the two major evolutionary factors influencing codon usage in plant species (Wang & Hickey, 2007; Raubeson et al., 2007; Li et al., 2016). In order to determine whether natural selection was the main driving force affecting codon usage bias in the D. grandiflorum cp genome, we performed ENC plot analysis and neutrality plot analysis. ENC plot analysis is an important indicator that reflects the relationship of the two different indices (ENC value and GC$_{3s}$), thus detecting the SCU variation among the genes (Wright, 1990). Wright concluded that the distribution comparison of genes and the standard curve could be indicative of some other factors, with the exception of mutation pressure. If the codon usage of a particular gene is under no selection, it should fall on the expected curve. In our study, it was observed that a few genes were positioned on the curve, which likely originated from the extreme mutation pressure. However, a majority of the points were lying well below the expected curve. This result suggested that a majority of genes in the D. grandiflorum cp genome had other SCU biases that were independent of mutation pressure, for example, natural selection. This hypothesis was largely supported by the neutrality plot mapping analysis. Neutrality plot analysis can effectively compare the effects of natural selection and mutation on codon usage bias (Sueoka, 1988). The low correlation between GC$_{12}$ and GC$_3$, that is, the smaller regression coefficient of approximately 0, showed that the base composition of the three positions differ, and the GC content of the cp genome is highly conserved, indicating that natural selection was the most important determinant of codon usage patterns. Conversely it shows that codon usage patterns are evidently reliant on mutation pressure (Zhang et al., 2018a; Zhang et al., 2018b; Zhang et al., 2018c). In the neutral graph, no correlation was found between GC$_3$ and GC$_{12}$, indicating a strong difference and that natural selection should be crucial for SCU bias in the D. grandiflorum cp genome. However, the signatures of selection constraints (positive, neutral, and negative) in D. grandiflorum cp genome could not be inferred for the lack of a reference sequence that is unaffected by selection, which need to be further detected in the following work.

CONCLUSIONS

This study systematically analyzed the codon usage pattern in the D. grandiflorum cp genome, and the factors affecting SCU bias were comprehensively explored. The SCU bias in the D. grandiflorum cp genome is weak, preferring A/T ending bases. Excepting the notable mutation pressure effects, the majority of genetic evolution in the D. grandiflorum cp genome may be driven by natural selection. These results are the first to provide a clear set of SCU patterns and explore the possible evolutionary forces acting on the D. grandiflorum cp genome.
ADDITIONAL INFORMATION AND DECLARATIONS

Funding
This work was supported by the National Natural Science Foundation of China (No. 31700338), the Central Public-interest Scientific Institution Basal Research Fund (No. 1610322019012), the Science and Technology Innovation Program of Lanzhou Institute of Husbandry and Pharmaceutical Science, Chinese Academy of Agricultural Sciences (No. CAAS-LMY-04), the Innovation Project of Chinese Academy of Agricultural Sciences (No. CAAS-XTCX2016011-02) and Gansu Provincial Science and Technology Major Projects (No. 19ZD2NA002). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures
The following grant information was disclosed by the authors:
National Natural Science Foundation of China: 31700338.
Central Public-interest Scientific Institution Basal Research Fund: 1610322019012.
Science and Technology Innovation Program of Lanzhou Institute of Husbandry and Pharmaceutical Science.
Chinese Academy of Agricultural Sciences: CAAS-LMY-04.
Innovation Project of Chinese Academy of Agricultural Sciences: CAAS-XTCX2016011-02.
Gansu Provincial Science and Technology Major Projects: 19ZD2NA002.

Competing Interests
The authors declare there are no competing interests.

Author Contributions
- Huirong Duan conceived and designed the experiments, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Qian Zhang and Hongshan Yang performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Chunmei Wang performed the experiments, prepared figures and/or tables, and approved the final draft.
- Fang Li analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Fuping Tian, Yuan Lu and Yu Hu analyzed the data, prepared figures and/or tables, and approved the final draft.
- Guangxin Cui conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.

Data Availability
The following information was supplied regarding data availability:
The sequence information of genes are available as a Supplemental File. The complete chloroplast of Delphinium grandiflorum is available at Genbank: MN556604.
Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.10787#supplemental-information.

REFERENCES

Baeza M, Alcaíno J, Barahona S, Sepúlveda D, Cifuentes V. 2015. Codon usage and codon context bias in Xanthophyllomyces dendrorhous. BMC Genomics 16:293 DOI 10.1186/s12864-015-1493-5.

Boël G, Letso R, Neely H, Price WN, Wong K, Su M, Luff JD, Valecha M, Everett JK, Acton TB, Xiao R, Montelione GT, Aalberts DP, Hunt JF. 2016. Codon influence on protein expression in E. coli correlates with mRNA levels. Nature 529:358–368 DOI 10.1038/nature16509.

Bulmer M. 1991. The selection–mutation–drift theory of synonymous codon usage. Genetics 129:897–907.

Camiolo S, Melito S, Porceddu A. 2015. New insights into the interplay between codon bias determinants in plants. DNA Research 22(6):461–470 DOI 10.1093/dnares/dsv027.

Chakraborty S, Nag D, Mazumder TH, Uddin A. 2017. Codon usage pattern and prediction of gene expression level in Bungarus species. Gene 604:48–60 DOI 10.1016/j.gene.2016.11.023.

Chen NH, Zhang YB, Li W, Li P, Chen LF, Li YL, Li GQ, Wang GC. 2017. Grandiflodines A and B, two novel diterpenoid alkaloids from Delphinium grandiflorum. RSC Advances 7:24129–24132 DOI 10.1039/C7RA02869E.

Chen Y, Shi YZ, Deng HJ, Gu T, Xu J, Ou JX, Jiang ZG, Jiao YR, Zou T, Wang C. 2014. Characterization of the porcine epidemic diarrhea virus codon usage bias. Infection, Genetics and Evolution 28:95–100 DOI 10.1016/j.meegid.2014.09.004.

Choudhury MN, Uddin A, Chakraborty S. 2017. Codon usage bias and its influencing factors for Y-linked genes in human. Computational Biology and Chemistry 69:77–86 DOI 10.1016/j.compbiolchem.2017.05.005.

Comeron JM, Aguadé M. 1998. An evaluation of measures of synonymous codon usage bias. Journal of Molecular Evolution 47:268–274 DOI 10.1007/pl00006384.

Cui GX, Wang CM, Wei XX, Yang HS, Lu Y, Wang XL, Zhu XQ, Zhang Q, Gao YQ, Duan HR. 2020. Analysis of synonymous codon usage of the complete chloroplast genome in Phleum pratense cv. Minshan. International Journal of Agriculture & Biology 24(2):352–358 DOI 10.17957/IJAB/15.1444.

Duan XR, Lu Y, Duan XR, Zhou XK, Wang CM, Tian FP, Wang XL, Yang HS, Cui GX. 2020. Characterization of the complete chloroplast genome of Delphinium grandiflorum L. Mitochondrial DNA Part B 5:35–36 DOI 10.1080/23802359.2019.1692707.

Eyre-Walker AC. 1991. An analysis of codon usage in mammals, selection or mutation bias? Journal of Molecular Evolution 33:442–449 DOI 10.1007/BF02103136.

Greenacre MJ. 1984. Theory and application of correspondence analysis. Orlando FL: Academic Press, 364.
Guan DL, Ma LB, Khan MS, Zhang XX, Xu SQ, Xie JY. 2018. Analysis of codon usage patterns in Hirudinaria manillensis reveals a preference for GC-ending codons caused by dominant selection constraints. *BMC Genomics* 19:542 DOI 10.1186/s12864-018-4937-x.

Gupta SK, Bhattacharyya TK, Ghosh TC. 2004. Synonymous codon usage in Lactococcus lactis, mutational bias versus translational selection. *Journal of Biomolecular Structure and Dynamics* 21:527–536 DOI 10.1080/07391102.2004.10506946.

Gupta SK, Ghosh TC. 2001. Gene expressivity is the main factor in dictating the codon usage variation among the genes in Pseudomonas aeruginosa. *Gene* 273:63–70 DOI 10.1016/s0378-1119(01)00576-5.

Honda K, Tsutsui K, Hosokawa K. 1999. Analysis of the flower pigments of some Delphinium species and their interspecific hybrids produced via ovule culture. *Scientia Horticulturae* 82:125–134 DOI 10.1016/S0304-4238(99)00039-4.

Ivanova Z, Sablok G, Daskalova E, Zahmanova G, Apostolova E, Yahubyan G, Baev V. 2017. Chloroplast genome analysis of resurrection tertiary relict Haberlea rhodopensis highlights genes important for desiccation stress response. *Frontiers in Plant Science* 8:204 DOI 10.3389/fpls.2017.00204.

Karumathil S, Raveendran NT, Ganesh D, Kumar NS, Nair RR, Dirisala VR. 2018. Evolution of SCU bias in West African and Central African strains of monkeypox virus. *Evolutionary Bioinformatics* 14:1–22 DOI 10.1177/1176943318761368.

Kawabe A, Miyashita NT. 2003. Patterns of codon usage bias in three dicot and four monocot plant species. *Genes and Genetic Systems* 78:343–352 DOI 10.1266/ggs.78.343.

Li GL, Pan ZL, Gao SC, He YY, Xia QY, Yan J, Yao HP. 2019. Analysis of SCU bias in West African and Central African strains of monkeypox virus. *Genes Genomics* 41:1173–1181 DOI 10.1007/s13258-019-00847-1.

Li N, Li YY, Zheng CC, Huang JG, Zhang SZ. 2016. Genome-wide comparative analysis of the codon usage patterns in plants. *Genes Genomics* 38:723–731 DOI 10.1007/s13258-016-0417-3.

Liu HM, He R, Zhang HY, Huang YB, Tian ML, Zhang JJ. 2010. Analysis of SCU in Zea mays. *Molecular Biology Report* 37:677–684 DOI 10.1007/s11033-009-9521-7.

Liu HB, Lu YZ, Lan BL, Xu JC. 2020. Codon usage by chloroplast gene is bias in Hemiptera davidi. *Journal of Genetics* 99:8 DOI 10.1007/s12041-019-1167-1.

Lobry JR. 1995. Properties of a general model of DNA evolution under no-strand–bias condition. *Journal of Molecular Evolution* 40:326–330 DOI 10.1007/BF00163237.

Morton BR, Sorhannus U, Fox M. 2002. Codon adaptation and synonymous substitution rate in diatom plastid genes. *Molecular Phylogenetics and Evolution* 24:1–9 DOI 10.1016/s1055-7903(02)00263-4.

Powell JR, Moriyama EN. 1997. Evolution of codon usage bias in Drosophila. *Proceedings of the National Academy Sciences of the United States of America* 94:7784–7790 DOI 10.1073/pnas.94.15.7784.
Qiu S, Zeng K, Slotte T, Wright S, Charlesworth D. 2011. Reduced efficacy of natural selection on codon usage bias in selfing Arabidopsis and Capsella species. *Genome Biology and Evolution* 3:868–880 DOI 10.1093/gbe/evr085.

Raubeson LA, Peery R, Chumley TW, Dziubek C, Fourcade HM, Boore JL, Jansen RK. 2007. Comparative chloroplast genomics, analyses including new sequences from the angiosperms *Nuphar advena* and *Ranunculus macranthus*. *BMC Genomics* 8:174 DOI 10.1186/1471-2164-8-174.

Sanner MF. 1999. Python: a programming language for software integration and development. *Journal of Molecular Graphics & Modelling* 17:57–61 DOI 10.1016/S1093-3263(99)00019-4.

Sau K, Gupta SK, Sau S, Mandal SC, Ghosh TC. 2006. Factors influencing synonymous codon and amino acid usage biases in Mimivirus. *Biosystems* 85:107–113 DOI 10.1016/j.biosystems.2005.12.004.

Sharma J, Chakraborty S, Uddin A. 2014. Codon usage bias in two hemipteran insect species, *Bemisia tabaci* and *Homalodisca coagulata*. *Advances in Biology* 2014:Article 145465 DOI 10.1155/2014/145465.

Sharp PM, Li WH. 1986. An evolutionary perspective on synonymous codon usage in unicellular organisms. *Journal of Molecular Evolution* 24:28–38 DOI 10.1007/bf02099948.

Song H, Liu J, Chen T, Nan ZB. 2018. Synonymous codon usage pattern in model legume *Medicago truncatula*. *Journal of Integrative Agriculture* 17:2074–2081 DOI 10.1007/s13210-017-0436-8.

Sueoka N. 1988. Directional mutation pressure and neutral molecular evolution. *Proceedings of the National Academy Sciences of the United States of America* 85:2653–2657 DOI 10.1073/pnas.85.8.2653.

Sueoka N. 1999. Directional mutation pressure and neutral molecular evolution. *Proceedings of the National Academy Sciences of the United States of America* 85:2653–2657 DOI 10.1073/pnas.85.8.2653.

Sueoka N. 1995. Intra-strand parity rules of DNA base composition and usage biases of synonymous codons. *Journal of Molecular Evolution* 40:318–325 DOI 10.1007/BF00163236.

Sueoka N. 1999. Translation-coupled violation of Parity Rule 2 in human genes is not the cause of heterogeneity of the DNA G+C content of third codon position. *Gene* 238:53–58 DOI 10.1016/S0378-1119(99)00320-0.

Sueoka N. 2001. Near homogeneity of PR2-bias fingerprints in the human genome and their implications in phylogenetic analyses. *Journal of Molecular Evolution* 53:469–476 DOI 10.1007/s002390010237.

Tao XL, Ma LC, Zhang ZS, Liu WX, Liu ZP. 2017. Characterization of the complete chloroplast genome of alfalfa (*Medicago sativa*) (Leguminosae). *Gene Reports* 6:67–73 DOI 10.1016/j.grep.2016.12.006.

Wang HC, Hickey DA. 2007. Rapid divergence of codon usage patterns within the rice genome. *BMC Evolutionary Biology* 7:56 DOI 10.1186/1471-2148-7-S1-S6.

Wang J, Wang TY, Wang LY, Zhang JG, Zeng YF. 2019. Assembling and analysis of the whole chloroplast genome sequence of *Elaeagnus angustifolia* and its codon usage bias. *Acta Botanica Boreali-Occidentalia Sinica* 39:1559–1572 (in Chinese).
Wang LY, Xing HX, Yuan YC, Wang XL, Saeed M, Tao JC, Feng W, Zhang GH, Song XL, Sun XZ. 2018. Genome-wide analysis of codon usage bias in four sequenced cotton species. *PLOS ONE* 13:e0194372 DOI 10.1371/journal.pone.0194372.

Wei L, He J, Jia X, Qi Q, Liang ZS, Zheng H, Ping Y, Liu SY, Sun JC. 2014. Analysis of codon usage bias of mitochondrial genome in *Bombyx mori* and its relation to evolution. *BMC Evolutionary Biology* 14:262 DOI 10.1186/s12862-014-0262-4.

Wicke S, Schneeweiss GM, DePamphilis CW, Müller KF, Quandt D. 2011. The evolution of the plastid chromosome in land plants: gene content, gene order, gene function. *Plant Molecular Biology* 76:273–297 DOI 10.1007/s11103-011-9762-4.

Wright F. 1990. The ‘effective number of codons’ used in a gene. *Gene* 87:23–29 DOI 10.1016/0378-1119(90)90491-9.

Yu T, Li J, Yang Y, Qi L. 2012. Codon usage patterns and adaptive evolution of marine unicellular cyanobacteria *Synechococcus* and *Prochlorococcus*. *Molecular Phylogenetics and Evolution* 62:206–213 DOI 10.1016/j.ympev.2011.09.013.

Zhang DS, Hu P, Liu TG, Wang J, Jiang SW, Xu QH, Chen LB. 2018a. GC bias lead to increased small amino acids and random coils of proteins in coldwater fishes. *BMC Genomics* 19:315 DOI 10.1186/s12864-018-4684.

Zhang YN, Li B, He M. 2012. Effects of saline and alkali stress on seed germination of *Delphinium grandiflorum*. *Pratacultural Science* 29:1235–1239 (In Chinese).

Zhang YY, Shi E, Yang ZP, Geng QF, Qiu YX, Wang ZS. 2018b. Development and application of genomic resources in an endangered palaeoendemic tree, *Parrotia subaequalis* (Hamamelidaceae) from eastern China. *Frontiers in Plant Science* 9:246 DOI 10.3389/fpls.2018.00246.

Zhang RZ, Zhang L, Wang W, Zhang Z, Du HH, Qu Z, Li XQ, Xiang H. 2018c. Differences in codon usage bias between photosynthesis-related genes and genetic system-related genes of chloroplast genomes in cultivated and wild solanum species. *International Journal Molecular Science* 19:3142 DOI 10.3390/ijms19103142.