Chloroplast genome features of *Moricandia arvensis* (Brassicaceae), a C3-C4 intermediate photosynthetic species

Bin Zhu*, Lijuan Hu*, Fang Qian, Zuomin Gao, Chenchen Gan, Zhaochao Liu, Xuye Du, Hongcheng Wang*

School of Life Sciences, Guizhou Normal University, Guiyang, People’s Republic of China

*These authors contributed equally to this work.
* besthongcheng@163.com

Abstract

*Moricandia arvensis*, a plant species originating from the Mediterranean, has been classified as a rare C3-C4 intermediate species, and it is a possible bridge during the evolutionary process from C3 to C4 plant photosynthesis in the family Brassicaceae. Understanding the genomic structure, gene order, and gene content of chloroplasts (cp) of such species can provide a glimpse into the evolution of photosynthesis. In the present study, we obtained a well-annotated cp genome of *M. arvensis* using long PacBio and short Illumina reads with a *de novo* assembly strategy. The *M. arvensis* cp genome was a quadripartite circular molecule with the length of 153,312 bp, including two inverted repeats (IR) regions of 26,196 bp, divided by a small single copy (SSC) region of 17,786 bp and a large single copy (LSC) region of 83,134 bp. We detected 112 unigenes in this genome, comprising 79 protein-coding genes, 29 tRNAs, and four rRNAs. Forty-nine long repeat sequences and 51 simple sequence repeat (SSR) loci of 15 repeat types were identified. The analysis of Ks (synonymous) and Ka (non-synonymous) substitution rates indicated that the genes associated with “subunits of ATP synthase” (*atpB*), “subunits of NADH-dehydrogenase” (*ndhG* and *ndhE*), and “self-replication” (*rps12* and *rpl16*) showed relatively higher Ka/Ks values than those of the other genes. The gene content, gene order, and LSC/IR/SSC boundaries and adjacent genes of the *M. arvensis* cp genome were highly conserved compared to those in related C3 species. Our phylogenetic analysis demonstrated that *M. arvensis* was clustered into a subclade with cultivated *Brassica* species and *Raphanus sativus*, indicating that *M. arvensis* was not involved in an independent evolutionary origin event. These results will open the way for further studies on the evolutionary process from C3 to C4 photosynthesis and hopefully provide guidance for utilizing *M. arvensis* as a resource for improving photosynthesis efficiency in cultivated *Brassica* species.
Introduction

*Moricandia arvensis* (Brassicaceae) originates from the Mediterranean and is mainly distributed in the Mediterranean Basin, North Africa, and west and southeast Asia [1]. *M. arvensis* is commonly used as an ornamental garden flower because of its vivid violet purple petals. Additionally, *M. arvensis* leaf extracts have strong antioxidant and antigenotoxic effects [2], making this species a suitable nutraceutical research model. Moreover, interspecific hybridization between *M. arvensis* and cultivated *Brassica juncea* demonstrated that *M. arvensis* can be used as an important cytoplasmic male sterility resource for *B. juncea* [3]. Furthermore, somatic hybridization between *M. arvensis* and *B. juncea* has been achieved to develop a novel cytoplasmic male sterility (CMS) system and restore *B. juncea* lines [4, 5].

Intriguingly, despite the fact that the family Brassicaceae does not contain any C4 species, *M. arvensis* has been identified as a C3-C4 intermediate species based on gas exchange parameters, leaf anatomy, metabolome and transcriptome [6–8], making it a desirable resource for introducing an intermediate C3-C4 photosynthetic phenotype into cultivated *Brassica* species. Interspecific hybridization between *M. arvensis* and cultivated *Brassica* crops employing ovary and ovule rescue methods has been extensively conducted for decades to transfer the C3-C4 photosynthetic character and drought tolerance traits [9–11]. The C3-C4 intermediate stage is believed to be a transition state between the evolutionary history of C3 and C4 plants [8, 12]. Therefore, it is of great interest to use *M. arvensis* to study photosynthetic evolution [8, 13]. A recent study employing C3-C4 intermediate, C4, C4-like, and C3 photosynthetic species within the genus *Flaveria* demonstrated that the RLSB (the nuclear-encoded *rbcL* RNA S1 binding domain protein), the only mRNA-binding protein associated with cp *rbcL* gene regulation, is likely involved in the evolution of C3-C4 photosynthesis [14]. Moreover, the cp *rbcL* gene encodes eight large subunits of Rubisco [15], which is a key enzyme involved in the first major step of carbon fixation. Given the indispensable role of chloroplasts in plant photosynthesis, deciphering the cp genome features of a C3-C4 intermediate plant could offer a glimpse into the evolution of photosynthesis.

The cp genome generally shows a typical quadripartite cycle (120–160 kb in size), harboring 110–130 genes [16, 17]. In most angiosperms, this quadripartite circular structure comprises of a large single copy (LSC) region and a small single copy (SSC) region, which are separated by a pair of inverted repeats (IR) regions [18]. The cpDNA evolution rate is much slower than that of nuclear DNA [19, 20] because of fewer recombination events, lower nucleotide replacement rates, and predominantly maternal inheritance of the cp genome. Therefore, cpDNA has been widely employed to decipher the genealogical relationships among plant species [21–23]. Previous, phylogenetic studies that used several nuclear/cp genes (sequences) have demonstrated that C3-C4 intermediate species generally have closer relationships with C4 relatives than with C3 relatives [24]. Studies based on cytological analysis [25, 26] and restriction site analysis [27] have revealed that *M. arvensis* is closely related to cultivated *Brassica* species. However, one study that carried out a genetic analysis of the S-locus reported a contrary result; it indicates that *Moricandia* species had a distant relationship with *Brassica* species [28].

In the present study, we obtained and fully described the complete cp genome of *M. arvensis* through *de novo* assembly based on long PacBio reads and short Illumina reads. To determine whether the C3-C4 intermediate *M. arvensis* evolved in an independent path among Brassicaceae species, we used the cp genomes of 59 other Brassicaceae species downloaded from GenBank to determine the genealogical relationship between *M. arvensis* and these species. Our results will open the way for further studies on the evolutionary path from C3 to C4 photosynthesis and hopefully provide guidance for utilizing *M. arvensis* as a resource to improve photosynthesis in cultivated *Brassica* species.
Materials and methods

Ethical statement

This study did not involve in any human or animal research participant data. The plant sample tested in this study is not endangered species, and the collection of sample didn’t cause any environmental problem.

Plant materials and DNA library preparation

Seeds from a pure *M. arvensis* line were cultivated in a glasshouse at Guizhou Normal University (Guiyang, China). When the plants had seven true leaves, 5 g of fresh leaves were collected for total DNA isolation using a commercial DNA extraction kit (TIANGEN, KG203, Beijing) according to the manufacturer’s instructions. After checking the integrity of the DNA with the Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, USA), ~1 μg DNA was fragmented to ~450 bp to construct a short-insert library for Illumina sequencing (HiSeq X Ten). Approximately 5 μg of DNA were used to construct the library with insert sizes of 20 kb for PacBio sequencing (PacBio, Menlo Park, USA), according to the manufacturer’s instructions. The raw sequence data reported in this paper were deposited in the Genome Sequence Archive of the National Genomics Data Center under the accession number CRA003542 (https://bigd.big.ac.cn/search/?dbId=gsa&q=CRA003542).

Cp genome assembly and genome feature analysis

The Illumina platform produced 150 bp of paired-end reads. Then, the Trimmomatic software (version-0.39) with default settings was used to remove low-quality reads and trim out the adapters to obtain clean reads [29]. To identify the cp-related reads, the clean reads were mapped to the published *Arabidopsis thaliana* cp genome (NC_000932) using the BLASR software under basic local alignment with default settings [30]. The cp-related reads were combined into contigs using the SOAPdenovo software (version 2.04) with default parameters [31]. After removing the debased PacBio reads with read quality < 0.80 or read length < 500 bp, long PacBio subreads were used to repair the gaps between contigs using PBjelly [32]. Then, the BWA software (version 0.5.9) was employed to correct possible misassembly and errors throughout these cp-related Illumina reads [33]. Finally, the frameshift errors were manually corrected during gene prediction.

GeSeq (https://chlorobox.mpimp-golm.mpg.de/geseq.html/) was used to annotate *M. arvensis* cp genome using default settings. Basic Local Alignment Search Tool (BLAST) was used to define the start and stop codons of each gene through homology searches [34]. The complete gene map of the *M. arvensis* cp genome was plotted using the OGDRAW software (version 1.2) [35]. Finally, the well-annotated *M. arvensis* cp genome was submitted to public GenBank under the accession number MW279233.

Long repeat and simple sequence repeat (SSR) analysis

REPuter (https://bibiserv.cbi.tuebingen.de/reputer/index.php) [36] was used to detect the long repeat sequences in the *M. arvensis* cp genome with the default settings: minimal repeat size, 30; sequence consistency, > 90%; maximum computed repeats, 50. The MISA (https://webblast.ipk-gatersleben.de/misa/) software was used to detect the SSR loci with the following settings: 10 repeats for mono-unit, five repeats for di-unit, four repeats for tri-units, and three repeats for tetra-, penta-, and hexa-units.
Codon usage bias of the coding sequences

Codon usage bias is believed to affect translational dynamics, including protein folding, translation accuracy, and efficiency [38]. The CodonW 1.4.2 program (http://downloads.fyxm.net/CodonW-76666.html/) with default settings was used to study the translational dynamics of the *M. arvensis* cp genome [39].

Comparison of the *M. arvensis* cp genome with C3 cp genomes of other Brassicaceae species

To detect sequence divergence between the *M. arvensis* cp genome and other related C3 species, the mVISTA web service was used to visualize genome divergence, using the *M. arvensis* cp genome as a reference. The related cp genome sequences included *B. rapa* (NC_040849), *B. oleracea* (NC_041167), *B. juncea* (NC_0282720), *Raphanus sativus* (NC_024469), and *Orychophragmu s diffuses* (NC_033498), and they were downloaded from the NCBI. Additionally, IRscope was used to compare the LSC/IRB/SSC/IRA junction regions among the selected cp genomes.

Ks/Ka substitution rate calculation

Synonymous (Ks) and non-synonymous (Ka) nucleotide substitution rates are valuable markers for evaluating genomic evolution [40, 41]. To calculate the Ks and Ka ratios, total pairwise comparisons of 77 common shared coding genes (S1 Table) among the selected Brassicaceae cp genomes (*B. rapa, B. oleracea, B. juncea, O. diffuses, R. sativus, and M. arvensis*), were determined using the KaKs calculator (version 2.0) with default parameters [42]. Pairwise alignments of these genes were carried out using MAFFT [43] with default settings.

Table 1. The detail characteristics of the complete cp genome of *Moricandia arvensis*.

| Category               | Items                | Descriptions   |
|------------------------|----------------------|----------------|
| Construction of cp genome | LSC region (bp)   | 83134          |
|                        | IRA region (bp)   | 26196          |
|                        | SSC region (bp)   | 17786          |
|                        | IRB region (bp)   | 26196          |
|                        | Genome Size (bp)  | 153,312        |
| Gene content           | Total genes        | 132            |
|                        | Protein-coding genes | 87             |
|                        | tRNAs              | 37             |
|                        | rRNAs              | 4              |
|                        | Two copy genes     | 20             |
|                        | Genes on LSC region | 84             |
|                        | Genes on IRA region | 18             |
|                        | Genes on SSC region | 12             |
|                        | Genes on IRB region | 18             |
|                        | Gene total length (bp) | 78,396      |
|                        | Average of genes length (bp) | 933         |
|                        | Gene length / Genome (%) | 51.13       |
| GC content             | GC content of LSC region (%) | 34.15       |
|                        | GC content of IRA region (%) | 42.34       |
|                        | GC content of SSC region (%) | 29.17       |
|                        | GC content of IRB region (%) | 42.34      |
|                        | Overall GC content (%) | 36.37        |

https://doi.org/10.1371/journal.pone.0254109.t001
Phylogenetic analysis

The maximum likelihood (ML) method with the Tamura-Nei model which was automatically recommended by the MEGA7 was used to determine the genealogical relationship between *M. arvensis* and related Brassicaceae species [44]. The initial tree for the heuristic search was obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated by the Maximum Composite Likelihood (MCL) approach. A total of 60 cp Brassicaceae species genomes (S2 Table) were downloaded from GenBank to...
### Table 2. Summary of assembled gene functions of *Moricandia arvensis* cp genome.

| Category for genes | Groups of genes | Name of genes |
|--------------------|-----------------|----------------|
| Genes involving in photosynthesis | Subunits of photosystem | psaA, psbA, psaC, psaI, psaL, psbA, psbB, psbC, psbD, psbE, psbF, psbH, psbI, psbJ, psbK, psbL, psbM, psbN, psbT, psbZ |
| | Subunits of cytochrome b/f complex | petA, petB, petD, petG, petL, petN |
| | Large subunit of Rubisco | rbcL |
| | Large subunit of Rubisco | rbcL |
| | Subunits of ATP synthase | atpA, atpB, atpE, atpF, atpH, atpI |
| Self-replication | Subunits of NADH-dehydrogenase | ndhA, ndhB, ndhC, ndhD, ndhE, ndhF, ndhG, ndhH, ndhI, ndhK |
| | Ribosomal RNA genes | rrn16, rrn23, rrn4.5, rrn5 |
| Transfer RNA genes | trnA-UGC, trnC-GCA, trnD-GUC, trnE-UUC, trnF-GAA, trnG-GCC, trnH-GUG, trnI-GAU, trnK-UUU, trnL-CAA, trnL-CAA, trnL-UAG, trnM-CAU, trnN-GUU, trnP-UGG, trnQ-UUG, trnR-ACG, trnR-UCU, trnS-GCU, trnS-GGA, trnT-GGU, trnT-UGU, trnV-GAC, trnV-UAC |
| | Small subunit of ribosome | rps11, rps12, rps14, rps16, rpoA, rps18 |
| | Large subunit of ribosome | rpl14, rpl16, rpl20, rpl22, rpl23, rpl32, rpl33, rpl36 |
| | DNA-dependent RNA polymerase | rpoA, rpoB, rpoC1, rpoC2 |
| Other genes | Maturease | matK |
| | Envelope membrane protein | cemB |
| | Subunit of acetyl-CoA synthase | accD |
| Functionally unknown genes | C-type cytochrome synthesis gene | ccsA |
| | Protease | cpP |
| | Conserved Open reading frames | ycf1, ycf2, ycf3, ycf4, ycf15 |

* a, b, c The letters indicate the gene with two copies, harboring one intron and two introns, respectively.

https://doi.org/10.1371/journal.pone.0254109.t002

Construct the phylogenetic trees. To increase the efficiency of the phylogenetic analysis, 66 homologous coding sequences (S3 Table) shared by the studied cp genomes were used. A total of 1,000 bootstrap replications were used to increase confidence.

### Result

#### The features of *M. arvensis* cp genome

A total of 6,834 Mb of raw data (45,560,522 raw reads) were obtained from the Illumina sequencing platform. After filtering, 6,543.7 Mb of clean data (43,846,822 clean reads) were obtained with an average Q20 value of 98.46%. A total of 3,530 PacBio subreads with a mean length of 12,382 bp were obtained (S4 Table). After being mapped to the *Arabidopsis thaliana* cp genome, 482.3 Mb (7.37% of clean reads) cp-related reads was obtained, representing an coverage of 3,306x over the cp genome. And 187 PacBio subreads (5.3% of total PacBio subreads) were demonstrated to cp-related subreads. Because of the relatively low coverage of the cp genome, these cp-related PacBio subreads were only used to fill the gaps between the contigs constructed by the Illumina reads.

The cp genome of *M. arvensis* had a quadripartite structure of 153,312 bp in size, including an SSC region of 17,786 bp and an LSC region of 83,134 bp, which were separated by two IR regions of 26,196 bp (Table 1 and Fig 1). Its average GC content was 36.37%, and the IR region had the highest GC content (42.34%), followed by LSC (34.15%), and SSC (29.17%). The M.
Moricandia arvensis cp genome encoded 112 genes, including four rRNAs, 29 tRNAs, and 79 protein-coding genes. Among these genes, 20 genes (four rRNAs, eight tRNAs, and eight protein-coding genes) were duplicated. The coding sequence region was 78,396 bp in size, accounting for 51.13% of the whole genome. Among these genes, 82 genes, including 59 coding genes and 23 tRNAs, were identified in the LSC region. Twelve genes were observed in the SSC region, whereas 18 genes, including seven tRNAs, four rRNAs, and seven protein coding genes, were duplicated in the IR regions. In the whole genome, 12 genes (four tRNAs and eight coding genes) had one intron, whereas two genes (ycf3 and clpP) had two introns (Table 2). The rps12 was identified to be a trans-spliced gene harboring one intron.

Comparisons of whole cp genomes

In the comparison of the gene contents of cp genomes among the five related species (R. sativus, B. juncea, B. rapa, B. oleracea, and O. diffuses), novel genes were not observed in the M. arvensis cp genome. The mVISTA program was used to determine whether a change in gene...
order has occurred in the \textit{M. arvensis} cp genome compared to the above mentioned cp genomes using the complete cp genome of \textit{M. arvensis} as a reference. The results showed that all selected cp genomes had high similarity (>95%), indicating a high degree of gene synteny, and no gene rearrangement was detected (Fig 2). However, the untranslated regions showed relatively high divergence among the selected cp genomes.

The LSC/IR/SSC junction regions are believed to be changeable and are considered to be the main factor in structural variations in the cp genomes of higher plants [45]. To detect the divergence of the junction regions between the \textit{M. arvensis} cp genome and related species, the LSC/IR/SSC junction regions and adjacent genes in the \textit{M. arvensis} cp genome were compared with those in the five above-mentioned species (\textit{R. sativus}, \textit{B. rapa}, \textit{B. oleracea}, \textit{B. juncea}, and \textit{O. diffuses}). The results showed that the \textit{M. arvensis} cp genome was extremely similar to all tested \textit{Brassica} cp genomes, except for the \textit{B. juncea} and \textit{R. sativus} cp genomes at the junction regions. Differences only occurred in the distance/cover of the \textit{ycf1} gene to the SSC/IRa and IRb/SSC junctions (Fig 3). The \textit{rps19} gene was always located at the LSC/IRB junction in all cp genomes.

**Repeat sequence and SSR loci detection**

We found 49 pairs of long repeat sequences ranging from 30 to 69 bp in the \textit{M. arvensis} cp genome using REPutere, and we found that these sequences were predominantly composed of palindromic repeats (28 pairs), followed by forward repeats (19 pairs), and reverse repeats (two pairs), but no complementary repeats were observed (Table 3). Most long repeat sequence pairs (67.35%) were found in the same gene or in the same intergenic spacer region (IGS) region. Sixteen long repeat sequence pairs, most of which were belonged to the forward type, were identified in different genes or intergenic spacer (IGS) regions.

Additionally, we identified 51 SSR loci in the \textit{M. arvensis} cp genome, comprising 33 mononucleotides, 12 dinucleotides, one trinucleotide, four tetranucleotides, and one hexanucleotide sequence.
Table 3. Repeat sequences in the *Moricandia arvensis* cp genome.

| No. | Repeat | Type | Repeat 1 Start (Location) | Repeat 2 Start (Location) | Region |
|-----|--------|------|---------------------------|---------------------------|--------|
| 1   | 30     | F    | 106671 (rrn4.5s)          | 106703 (rrn4.5s)          | IRA    |
| 2   | 34     | F    | 129713 (rrn4.5s)          | 129745 (rrn4.5s)          | IRB    |
| 3   | 34     | F    | 106667 (rrn4.5s)          | 106699 (rrn4.5s)          | IRA    |
| 4   | 37     | F    | 97748 (rps7-trnV-GAC)     | 119335 (ndhA)            | IRA;SSC|
| 5   | 39     | F    | 43158 (ycf3)              | 97745 (rps7-trnV-GAC)     | SSC;IRA|
| 6   | 40     | F    | 148328 (ycf2)             | 148349 (ycf2)             | IRB    |
| 7   | 42     | F    | 97743 (rps7-trnV-GAC)     | 119330 (ndhA)            | IRA;SSC|
| 8   | 47     | F    | 148321 (ycf2)             | 148342 (ycf2)             | IRB    |
| 9   | 47     | F    | 88057 (ycf2)              | 88078 (ycf2)              | IRA    |
| 10  | 52     | F    | 38049 (psaB)              | 40273 (psaA)              | LSC    |
| 11  | 55     | F    | 38046 (psaB)              | 40270 (psaA)              | LSC    |
| 12  | 58     | F    | 38043 (psaB)              | 40267 (psaA)              | LSC    |
| 13  | 64     | F    | 38007 (psaB)              | 40231 (psaA)              | LSC    |
| 14  | 71     | F    | 38013 (psaB)              | 40237 (psaA)              | LSC    |
| 15  | 73     | F    | 38028 (psaB)              | 40252 (psaA)              | LSC    |
| 16  | 74     | F    | 38010 (psaB)              | 40234 (psaA)              | LSC    |
| 17  | 76     | F    | 38025 (psaB)              | 40249 (psaA)              | LSC    |
| 18  | 79     | F    | 38016 (psaB)              | 40240 (psaA)              | LSC    |
| 19  | 81     | F    | 38020 (psaB)              | 40244 (psaA)              | LSC    |
| 20  | 30     | P    | 7725 (trnS-GCU)           | 44261 (trnS-GGA)          | LSC    |
| 21  | 30     | P    | 61749 (petA-psbJ)         | 61749 (petA-psbJ)         | LSC    |
| 22  | 30     | P    | 106671 (rrn4.5s)          | 129713 (rrn4.5s)          | IRA;IRB|
| 23  | 30     | P    | 106703 (rrn4.5s)          | 129745 (rrn4.5s)          | IRA;IRB|
| 24  | 34     | P    | 106667 (rrn4.5s)          | 129713 (rrn4.5s)          | IRA;IRB|
| 25  | 34     | P    | 106699 (rrn4.5s)          | 129745 (rrn4.5s)          | IRA;IRB|
| 26  | 37     | P    | 119335 (ndhA)             | 138661 (trnV-GAC-rps7)    | SSC;IRB|
| 27  | 38     | P    | 79499 (petA-psbJ)         | 79499 (petA-psbJ)         | LSC    |
| 28  | 39     | P    | 51159 (trnM-CAU-atpE)     | 51159 (trnM-CAU-atpE)     | LSC    |
| 29  | 39     | P    | 43158 (ycf3)              | 138662 (trnV-GAC-rps7)    | SSC;IRB|
| 30  | 40     | P    | 28339 (petN-psbM)         | 28339 (petN-psbM)         | LSC    |
| 31  | 42     | P    | 296 (trnH-GUG-psbA)       | 296 (trnH-GUG-psbA)       | LSC    |
| 32  | 42     | P    | 119330 (ndhA)             | 138661 (trnV-GAC-rps7)    | SSC;IRB|
| 33  | 44     | P    | 73294 (psbT-psbN)         | 73294 (psbT-psbN)         | LSC    |
| 34  | 44     | P    | 61981 (petA-psbJ)         | 61981 (petA-psbJ)         | LSC    |
| 35  | 44     | P    | 76808 (petD-rpoA)         | 76808 (petD-rpoA)         | LSC    |
| 36  | 45     | P    | 112700 (rpl32-trnL-UAG)   | 112700 (rpl32-trnL-UAG)   | SSC    |
| 37  | 46     | P    | 9315 (trnG-UCC-trnR-UCU)  | 9315 (trnG-UCC-trnR-UCU)  | IRA;LSC|
| 38  | 46     | P    | 209 (trnH-GUG-psbA)       | 209 (trnH-GUG-psbA)       | LSC    |
| 39  | 46     | P    | 4866 (matK-rps16)         | 4866 (matK-rps16)         | LSC    |
| 40  | 47     | P    | 64216 (psbE-petL)         | 64216 (psbE-petL)         | LSC    |
| 41  | 47     | P    | 88057 (ycf2)              | 148321 (ycf2)             | IRA;IRB|
| 42  | 47     | P    | 88078 (ycf2)              | 148342 (ycf2)             | IRA;IRB|
| 43  | 50     | P    | 55457 (rbcL-accD)         | 55457 (rbcL-accD)         | LSC    |
| 44  | 50     | P    | 288 (trnH-GUG-psbA)       | 296 (trnH-GUG-psbA)       | LSC    |
| 45  | 53     | P    | 66075 (psa1-rp33)         | 66075 (psa1-rp33)         | SSC    |
| 46  | 53     | P    | 112692 (rpl32-trnL-UAG)   | 112700 (rpl32-trnL-UAG)   | SSC    |
| 47  | 58     | P    | 64205 (psbE-petL)         | 64216 (psbE-petL)         | LSC    |
| 48  | 41     | R    | 30823 (trnE-UCU-trnT-GGU)  | 30823 (trnE-UCU-trnT-GGU)  | LSC    |
| 49  | 47     | R    | 80843 (rpl16)             | 80843 (rpl16)             | LSC    |

F: forward repeats; R: reverse repeats; P: palindrome repeats.

https://doi.org/10.1371/journal.pone.0254109.t003
| Type | Unit | Length | No. | Position on Genome | Location |
|------|------|--------|-----|-------------------|----------|
| P1   | A    | 10     | 4   | 13019–13028       | atpF-atpH |
|      |      |        |     | 29879–29888       | trnD-GUC-trnY-GUA |
|      |      |        |     | 35524–35533       | psbZ-trnG-GCC |
|      |      |        |     | 109311–109320     | ycf1      |
|      | 11   | 2      |     | 13753–13763       | atpH-ycf1 |
|      |      |        |     | 27132–27143       | rpoB-trnC-GCA |
|      | 12   | 2      |     | 122601–122612     | rps15-ycf1 |
|      |      |        |     | 137357–137368     | trnV-GAC-rps7 |
|      | 13   | 3      |     | 41894–41906       | psaA-ycf3 |
|      |      |        |     | 64295–64307       | psbE-petL |
|      |      |        |     | 80068–80080       | rpl14-rpl16 |
|      |      | 14     |     | 113672–113685     | ccsA      |
|      | T    | 10     | 9   | 25406–25415       | rpoB      |
|      |      |        |     | 49134–49143       | ndhK-ndhC |
|      |      |        |     | 50320–50329       | trnV-UAC  |
|      |      |        |     | 51211–51220       | trnM-CAU-atpE |
|      |      |        |     | 70416–70425       | clpP      |
|      |      |        |     | 78955–78964       | rpl36-rps8 |
|      |      |        |     | 80675–80684       | rpl16     |
|      |      |        |     | 112043–112052     | ndhF-rpl32 |
|      |      |        |     | 127127–127136     | ycf1      |
|      | 11   | 4      |     | 17669–17679       | rpoC2     |
|      |      |        |     | 29564–29574       | psbM-trnD-GUC |
|      |      |        |     | 45504–45514       | rps4-trnT-UGU |
|      |      |        |     | 123187–123197     | ycf1      |
|      | 12   | 4      |     | 74235–74246       | petB      |
|      |      |        |     | 99079–99090       | rps7-trnV-GAC |
|      |      |        |     | 124947–124958     | ycf1      |
|      |      |        |     | 125265–125276     | ycf1      |
|      | 13   | 3      |     | 65602–65614       | trnP-UGG-psaA |
|      |      |        |     | 77163–77175       | rpoA      |
|      |      |        |     | 81549–81561       | rpl16     |
|      |      |        |     | 124755–124776     | ycf1      |
| P2   | AT   | 10     | 6   | 26644–26653       | rpoB-trnC-GCA |
|      |      |        |     | 35661–35670       | psbZ-trnG-GCC |
|      |      |        |     | 107443–107452     | trnR-ACG-trnN-GUU |
|      |      |        |     | 120298–120307     | ndhA      |
|      |      |        |     | 128995–129004     | trnN-GUU-trnR-ACG |
|      |      |        |     | 142979–142988     | ndhB-trnL-CAA |
|      | 14   | 1      |     | 13471–13484       | atpH-ycf1 |
|      |      |        |     | 20     | 30833–30832       | trnE-UUC-trnT-GGU |
|      | TA   | 10     | 4   | 19041–19050       | rpoC2     |
|      |      |        |     | 62092–62101       | petA-psbJ |
|      |      |        |     | 93458–93467       | trnL-CAA-ndhB |
|      |      |        |     | 111594–111603     | ndhF-rpl32 |
|      | P3   | ATT    | 12  | 1      | 45957–45968       | trnT-UGU-trnL-UAA |
|      | P4   | CAAA   | 12  | 1      | 28186–28197       | petN-psbM |

(Continued)
The longest SSR was a 22 bp long mononucleotide repeat located within ycf1. Only A-type (12 SSRs) and T-type (21 SSRs) mononucleotide repeats were observed. AT/TA was present in all 12 dinucleotide repeats, and the longest type of dinucleotide repeat was AT (20 bp). The unique trinucleotide repeat was ATT (12 bp), which was found in the intergenic spacer IGS region between trnT-UGU and trnL-UAA. Three tetranucleotide repeat types (CAAA, TAAA, and ATAG) and one hexanucleotide repeat (GAAAGT) were also detected in the M. arvensis cp genome. Among the SSR loci, 32 were found in the intergenic spacer IGS regions, accounting for 62.75% of the total SSRs. The remaining SSR loci were distributed in 11 genes, and the pseudogene ycf1 harbored most SSRs (six SSR loci).

Analysis of codon usage bias

The sequences of 79 protein-coding genes generated 25,447 codons. Among them, the leucine (Leu) codon was the most common, accounting for 10.46% of total codons, followed by those encoding isoleucine (8.67), whereas the encoding cysteine (Cys) showed the lowest frequency of only 1.21% (Table 5). Additionally, the relative synonymous codon usage (RSCU) value was calculated to assess the codon usage bias of the M. arvensis cp genome. The RSCU values of 30 codons were > 1, indicating that they are preferentially used in the M. arvensis cp genome. The UUA codon, encoding leucine, showed the highest usage bias, with an RSCU value of 2.04. Among the preferentially used codons, all codons ended with U (16 of 30) or A (13 of 30), except for UUG, which encodes leucine.

Analysis of Ka and Ks substitution rate

The Ka/Ks ratio has been extensively used to assess how genomic evolution and selection pressure affect genes [41, 46]. The ratio of Ka/Ks < 1, Ka/Ks = 1, and Ka/Ks > 1 indicate genes that underwent purifying, neutral, and positive selections, respectively [40]. To determine whether the Ka/Ks ratio provides clues for the evolution of photosynthesis, we calculated the Ka/Ks ratios of 77 homologous coding genes (S3 Table) between the cp genome of M. arvensis and cp genome of the five related species (R. sativus, B. juncea, B. rapa, B. oleracea, and O. diffuses). The results showed that almost all selected genes had Ka/Ks values < 1 (except petD in the comparison of B. juncea vs. M. arvensis and ycf2 in the comparison of R. sativus vs. M. arvensis). Some genes associated with “Subunits of ATP synthase” (atpB), “Subunit of acetyl-CoA” (accD), “Subunits of NADH-dehydrogenase” (ndhG and ndhE), and “Self-replication” (rps12 and rpl16), generally showed relatively higher Ka/Ks values (> 0.5) than those of other genes, indicating that these genes have likely undergone relatively higher purifying selection pressure.

Phylogenetic analysis

To determine the genealogical position of M. arvensis within Brassicaceae and to determine whether M. arvensis with C3-C4 characters underwent a unique evolutionary origin event, we downloaded cp genomes of 60 Brassicaceae species from the NCBI to construct a phylogenetic tree. The phylogenetic tree (Fig 4) demonstrated that M. arvensis was not clustered into an
| Codon | Number | Amino acids | Ratio of Codon | RSCU | No.   | Ratio |
|-------|--------|-------------|----------------|------|-------|-------|
| GCA   | 366    | Ala         | 1.44%          | 1.11 | 1319  | 5.18% |
| GCC   | 200    |             | 0.79%          | 0.61 |       |       |
| GCG   | 144    |             | 0.57%          | 0.44 |       |       |
| GCU   | 609    |             | 2.39%          | 1.85 |       |       |
| AGA   | 463    | Arg         | 1.82%          | 1.82 | 1528  | 6.00% |
| AGG   | 153    |             | 0.60%          | 0.60 |       |       |
| CGA   | 349    |             | 1.37%          | 1.37 |       |       |
| CGC   | 104    |             | 0.41%          | 0.41 |       |       |
| CGG   | 123    |             | 0.48%          | 0.48 |       |       |
| CGU   | 336    |             | 1.32%          | 1.32 |       |       |
| AAC   | 284    | Asn         | 1.12%          | 0.46 | 1245  | 4.89% |
| AAU   | 961    |             | 3.78%          | 1.54 |       |       |
| GAC   | 195    | Asp         | 0.77%          | 0.38 | 1015  | 3.99% |
| GAU   | 820    |             | 3.22%          | 1.62 |       |       |
| UGC   | 75     | Cys         | 0.29%          | 0.49 | 309   | 1.21% |
| UGU   | 234    |             | 0.92%          | 1.51 |       |       |
| CAA   | 707    | Gln         | 2.78%          | 1.55 | 911   | 3.58% |
| CAG   | 204    |             | 0.80%          | 0.45 |       |       |
| GAA   | 1029   | Glu         | 4.04%          | 1.51 | 1359  | 5.34% |
| GAG   | 330    |             | 1.30%          | 0.49 |       |       |
| GGA   | 706    | Gly         | 2.77%          | 1.66 | 1705  | 6.70% |
| GCC   | 167    |             | 0.66%          | 0.39 |       |       |
| GGG   | 276    |             | 1.08%          | 0.65 |       |       |
| GGU   | 556    |             | 2.18%          | 1.30 |       |       |
| CAC   | 147    | His         | 0.58%          | 0.50 | 593   | 2.33% |
| CAU   | 446    |             | 1.75%          | 1.50 |       |       |
| AUA   | 710    | Ile         | 2.79%          | 0.97 | 2206  | 8.67% |
| AUC   | 398    |             | 1.56%          | 0.54 |       |       |
| AUU   | 1098   |             | 4.31%          | 1.49 |       |       |
| CUA   | 365    | Leu         | 1.43%          | 0.82 | 2662  | 10.46%|
| CUC   | 162    |             | 0.64%          | 0.37 |       |       |
| CUG   | 162    |             | 0.64%          | 0.37 |       |       |
| CUU   | 558    |             | 2.19%          | 1.26 |       |       |
| UUA   | 905    |             | 3.56%          | 2.04 |       |       |
| UUG   | 510    |             | 2.00%          | 1.15 |       |       |
| AAA   | 1107   | Lys         | 4.35%          | 1.53 | 1451  | 5.70% |
| AAG   | 344    |             | 1.35%          | 0.47 |       |       |
| AUG   | 555    | Met         | 2.18%          | 1.00 | 555   | 2.18% |
| UUC   | 490    | Phe         | 1.93%          | 0.64 | 1521  | 5.98% |
| UUU   | 1031   |             | 4.05%          | 1.36 |       |       |
| CCA   | 285    | Pro         | 1.12%          | 1.12 | 1022  | 4.02% |
| CCC   | 190    |             | 0.75%          | 0.74 |       |       |
| CCG   | 136    |             | 0.53%          | 0.53 |       |       |
| CCU   | 411    |             | 1.62%          | 1.61 |       |       |
| AGC   | 121    | Ser         | 0.48%          | 0.37 | 1956  | 7.69% |
| AGU   | 394    |             | 1.55%          | 1.21 |       |       |
| UCA   | 393    |             | 1.54%          | 1.21 |       |       |

(Continued)
independent branch, but constituted a subclade with cultivated Brassica species (B. oleracea, B. rapa, and B. juncea) and R. sativus, indicating that it is unlikely that M. arvensis was involved in independent evolutionary origin events. Sixty branches were generated in the phylogenetic tree. Among them, 55 branches were supported by node values > 50%, and 48 branches had node values > 95%. Cochlearia species formed an outgroup; however, the four tested Cochlearia species did not constitute a single subclade, suggesting that Cochlearia species radiated in different directions.

Discussion

C4 plants have been studied extensively because of their effective photosynthetic carbon fixation efficiency, accounting for up to 25% of the Earth’s primary productivity [14, 47]. These C3-C4 intermediate plants are believed to be a bridge during C3–C4 evolution [48, 49]. Understanding the cp genome of such C3-C4 intermediates will likely provide essential information on the evolution of photosynthesis. The present study, which reports the complete plastome sequence of M. arvensis confirmed, that joint application of two sequencing platforms, PacBio with long reads and Illumina with short reads, provide efficient and reliable approach for assembling and annotation of chloroplast genomes [17, 50].

The M. arvensis cp genome had a typical quadripartite structure of 153,312 bp, which is comparable with the size of other Brassicaceae cp genomes [17, 51–53]. The gene content, gene order, and LSC/IR/SSC junction regions of the M. arvensis cp genome were highly conserved compared to those of related C3 species, indicating that no significant change has occurred in the cp genome during C3 to C3-C4 intermediate evolution. A recent study aimed at deciphering the cp genomes of C3, Kranz type C4 and single cell C4 photosynthetic members in Chenopodiaceae also showed that the cp genomes of C3, C4, and single cell C4 species had similar organizations, gene orders, and contents [54].

RLSB binding of chloroplastic rbcL mRNA is associated with C3 to C4 evolution in Flaveria [14]. However, rbcL did not reveal high Ka or Ks ratios in the M. arvensis cp genome (S3 Table) compared to those in related C3 species. In contrast, the genes associated with

Table 5. (Continued)

| Codon | Number | Amino acids | Ratio of Codon | RSCU | No. | Ratio |
|-------|--------|-------------|----------------|------|-----|-------|
| UCC   | 286    |             | 1.12%          | 0.88 |     |       |
| UCG   | 192    |             | 0.75%          | 0.59 |     |       |
| UCU   | 570    |             | 2.24%          | 1.75 |     |       |
| UAA   | 41     | TER         | 0.16%          | 1.86 | 66  | 0.26% |
| UAG   | 17     |             | 0.07%          | 0.77 |     |       |
| UGA   | 8      |             | 0.03%          | 0.36 |     |       |
| ACA   | 406    | Thr         | 1.60%          | 1.25 | 1300| 5.11% |
| ACC   | 228    |             | 0.90%          | 0.70 |     |       |
| ACG   | 143    |             | 0.56%          | 0.44 |     |       |
| ACU   | 523    |             | 2.06%          | 1.61 |     |       |
| UGG   | 430    | Trp         | 1.69%          | 1.00 | 430 | 1.69% |
| UAC   | 176    | Tyr         | 0.69%          | 0.37 | 951 | 3.74% |
| UAU   | 775    |             | 3.05%          | 1.63 |     |       |
| GUA   | 489    | Val         | 1.92%          | 1.46 | 1343| 5.28% |
| GUC   | 165    |             | 0.73%          | 0.49 |     |       |
| GUG   | 190    |             | 0.75%          | 0.57 |     |       |
| GUU   | 499    | Ala         | 1.96%          | 1.49 |     |       |

https://doi.org/10.1371/journal.pone.0254109.t005
"Subunits of ATP synthase" ($atpB$), "Subunits of NADH-dehydrogenase" ($ndhG$ and $ndhE$), and "Self-replication" ($rps12$ and $rpl16$) had relatively higher Ka/Ks values than those of other genes, indicating that these genes have likely accompanied the evolution of C3 to C3-C4 intermediates.

Phylogenetic studies have demonstrated that C3-C4 intermediates are more closely related to their C4 relatives than to their C3 relatives in families comprising C3, C3-C4 intermediate, and C4 (C4-like) species [24]. However, C4 plants are not distributed equally in the plant kingdom [55], and they have not been identified in Brassicaceae. To determine whether $M$. arvensis underwent a special evolutionary origin event compared to its C3 relatives, a phylogenetic tree was constructed based on common coding sequences. $M$. arvensis was not clustered into an independent branch, but it constituted a subclade with the cultivated $Brassica$ species and $R$. sativus. This result is in accordance with the results of the phylogenetic analysis carried out by Warwick and Sauder [56] based on cp $trnL$ intron sequences. A recent phylogenetic study of different Moricandia lines based on ITS data revealed a close relationship between C3-C4 intermediates and their C3 siblings [8]. These results indicate that the C3-C4 intermediate $M$. arvensis did not evolve through an independent evolutionary origin event.

**Conclusion**

In the present study, we obtained a well-annotated cp genome of $M$. arvensis with a C3-C4 intermediate character. We did not detect conspicuous genomic divergence in the $M$. arvensis cp genome compared to that of other related C3 species. However, the Ka/Ks analysis showed that some genes associated with photosynthesis had high Ka/Ks values compared with the genes of several related C3 species, indicating that these genes have likely accompanied the evolution of C3 to C3-C4 intermediates. Our phylogenetic analysis demonstrated that $M$. arvensis was clustered into a subclade with cultivated $Brassica$ species and $R$. sativus, indicating that $M$. arvensis was not involved in an independent evolutionary origin event. These results will provide guidance for utilizing $M$. arvensis as a resource for improving photosynthesis in cultivated $Brassica$ species and enable the collection of more data regarding the evolutionary path from C3 to C4. In future studies, deciphering nuclear genomic information will be vital to reveal the key steps in the evolution from C3 to C3-C4 intermediates.

**Supporting information**

S1 Table. Synonymous (Ks) and non synonymous (Ka) substitution rate of pairwise comparisons of 77 protein coding genes between Moricandia arvensis and other five closed related species. (XLSX)

S2 Table. List of the cp genome of 60 Brassicaceae species used for phylogenetic analysis. (XLSX)

S3 Table. List of the 66 shared genes used to construct the phylogenetic tree. (XLSX)
S4 Table. Summary of de novo sequencing of cp genome of Moricandia arvensis. (XLSX)

Author Contributions

Data curation: Lijuan Hu.
Formal analysis: Lijuan Hu.
Funding acquisition: Bin Zhu.
Investigation: Xuye Du.
Methodology: Chenchen Gan, Zhaochao Liu, Xuye Du.
Project administration: Hongcheng Wang.
Resources: Fang Qian.
Software: Zuomin Gao.
Writing – original draft: Bin Zhu.
Writing – review & editing: Hongcheng Wang.

References

1. Tahir M, Watts R. Moricandia. Wild Crop Relatives: Genomic and Breeding Resources. Springer, Berlin, Heidelberg. 2011; pp: 191–198. https://doi.org/10.1007/978-3-642-14871-2_12.

2. Skandrani I, Limem I, Neffati A, Boubaker J, Sghaier MB, Bhouri W, et al. Assessment of phenolic content, free-radical-scavenging capacity genotoxic and anti-genotoxic effect of aqueous extract prepared from Moricandia arvensis leaves. Food Chem Toxicol. 2010; 48: 710–715. https://doi.org/10.1016/j.fct.2009.11.053 PMID: 19951736

3. Kirti PB, Prakash S, Gaikwad K, Kumar VD, Bhat SR, Chopra VL. Chloroplast substitution overcomes leaf chlorosis in a Moricandia arvensis-based cytoplasmic male sterile Brassica juncea. Theor Appl Genet. 1998; 97: 1179–1182. https://doi.org/10.1007/s001220051007.

4. Prakash S, Kirti PB, Bhat SR, Gaikwad K, Kumar VD, Chopra VL. A Moricandia arvensis-based cytoplasmic male sterility and fertility restoration system in Brassica juncea. Theor Appl Genet. 1998; 97: 488–492. https://doi.org/10.1007/s001220050921.

5. Bhat SR, Vijayan P, Ashutosh, Dwivedi KK, Prakash S. Diplotaxis erucoides induced cytoplasmic male sterility in Brassica juncea is rescued by the Moricandia arvensis restorer: genetic and molecular analyses. Plant Breeding. 2006; 125: 150–155. https://doi.org/10.1111/j.1439-0523.2006.01184.x.

6. Krenzer EG, Moss DN, Crookston PK. Carbon dioxide compensation points of flowering plants. Plant Physiol. 1975: 56: 194–206. https://doi.org/10.1104/pp.56.2.194 PMID: 16659272

7. Holaday AS, Shieh YJ, Lee KW, Chollet R. Anatomical, ultrastructural and enzymic studies of leaves of Moricandia arvensis, a C3-C4 intermediate species. BBA-Bioenergetics. 1981; 637: 334–341. https://doi.org/10.1016/0005-2728(81)90172-9.

8. Schlüter U, Bräutigam A, Gowik U, Melzer M, Christin PA, Kurz S, et al. Photosynthesis in C3–C4 intermediate Moricandia species. J Exp Bot. 2017; 68: 191–206. https://doi.org/10.1093/jxb/erw391 PMID: 28110276

9. Warwick SI, Francis A, Gugel RK. Guide to wild germplasm of Brassica and allied crops (tribe Brassicaceae, Brassicaceae). Canada: Agriculture and Agri-Food Canada. 2009: 1–6.

10. Tsuttsui K, Jeong BH, Ito Y, Bang SW, Kaneko Y. Production and characterization of an alloplasmic and monosomic addition line of Brassica rapa carrying the cytoplasm and one chromosome of Moricandia arvensis. Breeding Sci. 2011; 61: 373–379. https://doi.org/10.1270/jsbbs.61.373 PMID: 2136474

11. Katche E, Quezada-Martinez D, Katche EI, Vasquez-Teuber P, Mason AS. Interspecific hybridization for Brassica crop improvement. Crop Breed Genet Genom. 2019; 1: e190007. https://doi.org/10.20900/cbgg20190007.

12. Kennedy RA, Laetsch WM. Plant species intermediate for C3, C4 photosynthesis. Science. 1974; 184: 1087–1089. https://doi.org/10.1126/science.184.4141.1087 PMID: 17736195
13. Holaday AS, Harrison AT, Chollet R. Photosynthetic/photorespiratory CO₂ exchange characteristics of the C3-C4 intermediate species, *Moricandia arvensis*. Plant Sci Let. 1982; 27: 181–189. https://doi.org/10.1016/0304-4211(82)90147-X.

14. Yerramsetty P, Agar EM, Yim WC, Cushman JC, Berry JO. An *rbcL* mRNA-binding protein is associated with C3 to C4 evolution and light-induced production of Ribulose in *Flaveria*. J Exp Bot. 2017; 68: 4635–4649. https://doi.org/10.1093/jxb/erx264 PMID: 28981775.

15. Andersson I. Catalysis and regulation in Ribulose. J Exp Bot. 2008; 59: 1555–1568. https://doi.org/10.1093/jxb/ern091 PMID: 18417482.

16. Rodríguez-Ezepeleta N, Brinkmann H, Burey SC, Roure B, Burger G, Löffelhardt W, et al. Monophyly of primary photosynthetic eukaryotes: green plants, red algae, and glaucophytes. Curr Biol. 2005; 15: 1325–1330. https://doi.org/10.1016/j.cub.2005.06.040 PMID: 16051178.

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

18. Shetty SM, MdShah MU, Makale K, Mohd-Yusuf Y, Khalid N, Othman RY. Complete chloroplast genome sequence of *corrobrates* structural heterogeneity of inverted repeats in wild progenitors of cultivated bananas and plantains. Plant Genome. 2016; 9: 1–14. https://doi.org/10.3835/plantgenome2015.09.0089 PMID: 27898825.

19. Wolfe KH, Li WH, Sharp PM. Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. P Natl Acad Sci USA. 1987; 84: 9054–9058. https://doi.org/10.1073/pnas.84.24.9054.

20. Smith DR. Mutation rates in plastid genomes: they are lower than you might think. Genom Biol Evol. 2015; 7: 1227–1234. https://doi.org/10.1093/gbe/evv069 PMID: 25869380.

21. Shaw J, Lickey EB, Beck JT, Farmer SB, Liu W, Miller J, et al. The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. Am J Bot. 2005; 92: 142–166. https://www.jstor.org/stable/4123961. https://doi.org/10.3732/ajb.92.1.142 PMID: 21652394.

22. Moore MJ, Soltis PS, Bell CD, Burleigh JG, Soltis DE. Phylogenetic analysis of 83 plastid genes further resolves the early diversification of eudicots. P Natl Acad Sci USA. 2010; 107: 4623–4628. https://doi.org/10.1073/pnas.0907801107 PMID: 21079554.

23. Walker JF, Zanis MJ, Emery NC. Comparative analysis of complete chloroplast genome sequence and inversion variation in *Lasthenia burkei* (Madieae, Asteraceae). Am J Bot. 2014; 101: 722–729. https://doi.org/10.3732/ajb.1400049 PMID: 24699541.

24. Fisher AE, McDade LA, Kiel CA, Koshravesh R, Johnson MA, Stata M, et al. Evolutionary history of *Blepharis* (Acanthaceae) and the origin of C4 photosynthesis in section Acanthodium. Int J Plant Sci. 2015; 176: 770–790. https://doi.org/10.1086/683011.

25. Takahata Y, Takeda T. Intergeneric (intersubtribe) hybridization between *Moricandia arvensis* and *Brassica* A and B genome species by ovary culture. Theor Appl Genet. 1990; 80: 38–42. https://doi.org/10.1007/BF00224013 PMID: 24220808.

26. Takahata Y, Takeda T, Kaizuma N. Wide hybridization between *Moricandia arvensis* and *Brassica* amphidiploid species (B. napus and B. juncea). Euphytica. 1993; 69: 155–160. https://doi.org/10.1007/BF00021740.

27. Warwick SI, Black LD. Phylogenetic implications of chloroplast DNA restriction site variation in subtribes Raphaninae and Cakilinae (Brassicaceae, tribe Brassiceae). Can J Bot. 1997; 75: 960–973. https://doi.org/10.1139/b97-107.

28. Inaba R, Nishio T. Phylogenetic analysis of Brassicaceae based on the nucleotide sequences of the S-locus related gene, *SLR1*. Theor Appl Genet. 2002; 105: 1159–1165. https://doi.org/10.1007/s00122-002-0968-3 PMID: 12582694.

29. Bolger AM, Lohse M, Usadel B. Trimomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014; 30: 2114–2120. https://doi.org/10.1093/bioinformatics/btu170 PMID: 24695404.

30. Chaisson MJ, Tesler G. Mapping single molecule sequencing reads using basic local alignment with successive refinement (BLASR): application and theory. BMC Bioinformatics. 2012; 13: 1–18. https://doi.org/10.1186/1471-2105-13-238 PMID: 22214541.

31. Xie Y, Wu G, Tang J, Luo R, Patterson J, Liu S, et al. SOAPdenovo-Trans: de novo transcriptome assembly with short RNA-Seq reads. Bioinformatics. 2014; 30: 1660–1666. https://doi.org/10.1093/bioinformatics/btu077 PMID: 24532719.

32. English AC, Richards S, Han Y, Wang M, Vee V, Qu J, et al. Mind the gap: upgrading genomes with Pacific Biosciences RS long-read sequencing technology. PloS One. 2012; 7: e47768. https://doi.org/10.1371/journal.pone.0047768 PMID: 23185243.
33. Li H, Durbin R. Fast and accurate short read alignment with Burrows–Wheeler transform. Bioinformatics. 2009; 25: 1754–1760. https://doi.org/10.1093/bioinformatics/btp324 PMID: 19451168

34. Chen Y, Ye W, Zhang Y, Xu Y. High speed BLASTN: an accelerated Mega BLAST search tool. Nucleic Acids Res. 2015; 43: 7762–7768. https://doi.org/10.1093/nar/gkv784 PMID: 26205111

35. Lohse M, Drechsel O, Bock R. OrganelarGenomeDRAW (OGDRAW): a tool for the easy generation of high-quality custom graphical maps of plastid and mitochondrial genomes. Curr Genet. 2007; 52: 267–274. https://doi.org/10.1007/s00294-007-0161-y PMID: 17957369

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

37. Beier S, Thiel T, Münch T. MISA-web: a web server for microsatellite prediction. Bioinformatics. 2017; 33: 2583–2585. https://doi.org/10.1093/bioinformatics/btx198 PMID: 28398459

38. Yu CH, Dang Y, Zhou Z, Wu C, Zhao F, Sachs MS, et al. Codon usage influences the local rate of translation elongation to regulate co-translational protein folding. Mol Cell. 2015; 59: 744–754. https://doi.org/10.1016/j.molcel.2015.07.018 PMID: 26321254

39. Mower JP. The PREP suite: predictive RNA editors for plant mitochondrial genes, chloroplast genes and user-defined alignments. Nucleic Acids Res. 2009; 37: W253–W259. https://doi.org/10.1093/nar/gkp337 PMID: 19433507

40. Yang Z, Nielsen R. Estimating synonymous and nonsynonymous substitution rates under realistic evolutionary models. Mol Biol Evol. 2000; 17: 32–43. https://doi.org/10.1093/molbev/10.1093/molbev/30.2.772 PMID: 23802359

41. Guo Y, Liu J, Zhang J, Liu S, Du J. Selective modes determine evolutionary rates, gene compactness and expression patterns in Brassica. Plant J. 2017; 91: 34–44. https://doi.org/10.1111/tpj.13541 PMID: 28332757

42. Lang D, Zhang Y, Zhang Z, Zhu J, Yu J. KaKs_Calculator 2.0: a toolkit incorporating gamma-series methods and sliding window strategies. Genom Proteom Bioinf. 2010; 8: 77–80. https://doi.org/10.1016/S1672-0229(10)60008-3 PMID: 20451164

43. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol. 2013; 30: 772–780. https://doi.org/10.1093/molbev/msw054 PMID: 23329690

44. Kumar S, Stecher G, Tamura K. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016; 33: 1870–1874. https://doi.org/10.1093/molbev/msw054 PMID: 27004904

45. Kim KJ, Lee HL. Complete chloroplast genome sequences from Korean ginseng (Panax schinseng Nees) and comparative analysis of sequence evolution among 17 vascular plants. DNA Res. 2004; 11: 247–261. https://doi.org/10.1093/dnares/11.4.247 PMID: 15500250

46. Hurst LD. The Ka/Ks ratio: diagnosing the form of sequence evolution. Trends Genet. 2002; 18: 486–486. https://doi.org/10.1016/s1076-9892(02)02722-1 PMID: 12175810

47. Sage RF, Sage TL, Kocacinar F. Photorespiration and the evolution of C4 photosynthesis. Annu Rev Genet. 2012; 63: 19–47. https://doi.org/10.1146/annurev-arplant-042811-105511 PMID: 22404472

48. Mallmann J, Heckmann D, Bräutigam A, Lercher MJ, Weber AP, Westhoff P, et al. The role of photorespiration during the evolution of C4 photosynthesis in the genus Flaveria. Elife. 2014; 3: e02478. https://doi.org/10.7554/eLife.02478.001 PMID: 24935935

49. Bräutigam A, Gowik U. Photorespiration connects C3 and C4 photosynthesis. J Exp Bot. 2016; 67: 2953–2962. https://doi.org/10.1093/jxb/erw056 PMID: 26912798

50. Zhu B, Feng Q, Yu J, Yu Y, Zhu X, Wang Y, et al. Chloroplast genome features of an important medicinal and edible plant: Houttuynia cordata (Saururaceae). Plos One. 2020; 15: e0239823. https://doi.org/10.1371/journal.pone.0239823 PMID: 32986773

51. Zhou T, Yang Y, Hu Y, Zhang X, Bai G, Zhao G. Characterization of the complete chloroplast genome sequence of Lepidium meyenii (Brassicaceae). Conserv Genet Resour. 2017; 9: 405–408. https://doi.org/10.1007/s12686-017-0695-3

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

53. Zhu B, Gao Z, Luo X, Feng Q, Du X, Wang Q, et al. The complete chloroplast genome sequence of garden cress (Lepidium sativum L.) and its phylogenetic analysis in Brassicaceae family. Mitochondrial DNA B. 2019; 4: 3601–3602. https://doi.org/10.1080/23802359.2019.1677527.
54. Sharpe RM, Williamson-Benavides B, Edwards GE, Dhingra A. Methods of analysis of chloroplast genomes of C3, Kranz type C4 and Single Cell C4 photosynthetic members of Chenopodiaceae. Plant Methods. 2020; 16: 1–14. https://doi.org/10.1186/s13007-020-00662-w PMID: 31911810

55. Sage RF, Sultmann S. Why are there no C4 forests? J Plant Physiol. 2016; 203: 55–68. https://doi.org/10.1016/j.jplph.2016.06.009 PMID: 27481816

56. Warwick SI, Sauder CA. Phylogeny of tribe Brassiceae (Brassicaceae) based on chloroplast restriction site polymorphisms and nuclear ribosomal internal transcribed spacer and chloroplast trn L intron sequences. Canadian Journal of Botany, 2005, 83(5): 467–483. https://doi.org/10.1139/b05-021.