MITOCHONDRIAL DNA PART B: RESOURCES
2025, VOL. 10, NO. 5, 331–336
https://doi.org/10.1080/23802359.2024.2423830

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MITOGENOME REPORT

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Complete mitogenome and phylogenetic analysis of *Brassica oleracea* L. var. *italica* Plenck

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ABSTRACT

In this study, we assembled and annotated the mitochondrial genome (mitogenome) of *B. oleracea* L. var. *italica* Plenck. This mitogenome was found to span 219 964 bp, with a GC content of 45.25%. It comprised 61 genes, including 35 protein-coding, 23 transfer RNA, and three ribosomal RNA genes. Among these, only 11 genes contained introns. Codon preference analysis revealed a bias toward codons ending in A/U bases. A phylogenetic analysis demonstrated a close relationship between *B. oleracea* L. var. *italica* Plenck, *B. oleracea* L. botrytis, and *B. oleracea* var. *capitata*. This reference mitogenome provides a basis for research on genetic conservation, phylogenetic relationships, and molecular breeding strategies among members of the *Brassica* genus.

ARTICLE HISTORY
Received 20 December 2023
Accepted 26 October 2024

KEYWORDS
Brassica oleracea L. var. *italica* Plenck; mitochondrial genome; comparative analysis; phylogeny analysis

Introduction

Broccoli originated on the eastern Mediterranean coast and was classified as *Brassica oleracea* L. var. *italic* Plenck by Switzer in 1829. This cruciferous vegetable is highly nutritious, cultivated globally, and consumed worldwide (Pei et al. 2017; Jo et al. 2022). *B. oleracea* L. var. *italic* Plenck hybridizes readily with other varieties of *Brassica oleracea*, sometimes inducing cytoplasmic DNA replacement across *B. oleracea* crops, complicating the use of the cytoplasmic genome.

The complete mitogenome sequences of several *Brassica oleracea* varieties, including *B. oleracea* var. *capitata* (Tanaka et al. 2014) and *B. oleracea* var. *gongylodes* (Shao et al. 2021), have been reported. However, the mitogenome of *B. oleracea* L. var. *italic* Plenck has yet to be sequenced. Therefore, we performed the inaugural assembly and annotation of the broccoli mitogenome. These findings will contribute to the broader understanding of *Brassica* mitogenomes in general and also provide a reference for genetic conservation studies, phylogenetic analyses, and advancements in molecular breeding strategies.

Materials and methods

A specimen of *B. oleracea* L. var. *italic* Plenck (Figure 1) was obtained from Kunming University in Yunnan province, P.R. China (102.799°E, 24.971°N). The seeds were disinfected with 75% alcohol for 2 min and then rinsed three times with sterilized water before being placed in a petri dish to initiate germination. After one day, the germinated seeds were sown in plastic pots and grown in a controlled environment chamber under a 16-h/8-h light/dark cycle at 25 °C/18 °C, with a light intensity of 100% during the light period. The seedlings were watered weekly with a 1/2 Hoagland nutrient solution.

Once the plants reached the four-leaf stage, they were transplanted to experimental plots located at Kunming University. A representative specimen corresponding to this material was deposited at Kunming University (https://www.kmu.edu.cn/; Contact: Zange Jing, jingzange@aliyun.com) with the voucher number ZF2021003.

Approximately 75 days post-transplantation, fresh leaves were selected for total genomic DNA extraction during the harvest period. DNA extraction was performed using TRIzol reagent (Invitrogen, USA), following the manufacturer's protocol. The qualified genomic DNA was then sequenced using the Illumina NovaSeq 6000 platform. Raw reads were filtered using Fastp software v.0.20.0 (Chen et al. 2018). The complete mitochondrial genome sequence was assembled using NOVAPlasty v.2.4 and annotated using MITOS (Bernt et al. 2013). The encoded proteins and ribosomal RNAs (rRNAs) were compared with published plant mitochondrial sequences using the

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/23802359.2024.2423830.

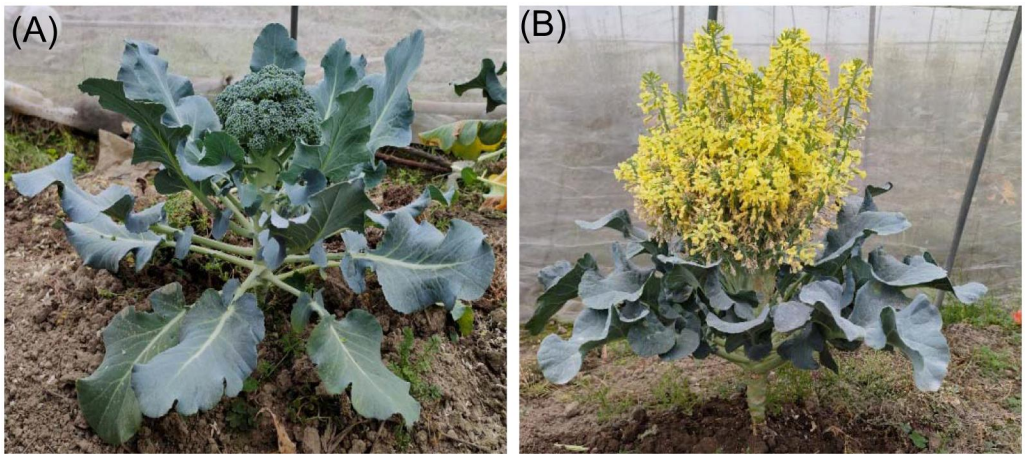

Figure 1. Plant images of *Brassica oleracea* L. var. *Italic* Plenck. These photos were taken by Zheng Tang. (A) *Brassica oleracea* L. var. *Italic* Plenck at the time of harvesting. (B) *Brassica oleracea* L. var. *Italic* Plenck at flowering time.

BLAST tool. The results were manually reviewed and corrected to obtain the final annotation. A circular map of the mitogenome and a schematic map of the cis- and trans-splicing genes were visualized using CPGView.

For synteny comparison, the mitogenome sequences of seven proximal species were selected and, together with the obtained *B. oleracea* L. var. *italica* Plenck mitogenome sequence, parsed through the pairwise BLAST results using Mauve software with default parameters (Darling et al. 2004). BLASTn was used to identify homologous sequences between chloroplasts and mitochondria in *B. oleracea* L. var. *italica* Plenck, with similarity set to 70% and the E-value set to 10E-5. Homologous sequences were visualized using Circos v.0.69.5.

To explore the phylogenomic relationships among members of the Cruciferae family, 14 mitogenome sequences (JF920285.1, AP017997.1, AP018473.1, NC_049892.1, KJ820683.1, KU831325.1, KF442616.1, NC_037070.1, MT675104.1, AP018472, AP018041.1, NC_031896.1, JF920287.1, NC_029182.1) were retrieved from GenBank. Moreover, *Arabidopsis thaliana* (LUHQ01000021.1) was used as an outgroup. The protein-coding genes from these mitochondrial genomes were extracted and concatenated using Phylosuite (Zhang et al. 2020). The sequences were then aligned using MAFFT v.7.427 (Rozewicki et al. 2019). Subsequently, a maximum likelihood phylogenetic tree was constructed by employing RAxML v.8.2.10 under the GTR GAMMA model, incorporating a bootstrap analysis with 1000 replicates (Stamatakis 2006).

Results

Sequencing statistics

The mitochondrial genome of *B. oleracea* L. var. *italica* Plenck was assembled using a combination of second- and third-generation sequencing technology. From the second-generation sequencing results, we obtained 19.70 M clean reads, yielding a total of 5.91 GB of clean data. The third-generation sequencing analysis provided 0.57 M clean reads, resulting in 7.05 GB of clean data, with an average read length of 12 374 bp. These reads were subsequently used for mitogenome assembly.

The average read mapping depths of the assembled genome, obtained from the second- and third-generation sequencing datasets, were 394.8 and 253.7, respectively (Figure S1). The circular map of the mitochondrial genome and schematic maps of the cis- and trans-splicing genes are provided in Figure 2, Figure S2, and Figure S3, respectively. The mitogenome of *B. oleracea* L. var. *italica* Plenck spanned 219 964 bp in length, with a GC content of 45.25%. This mitogenome was cataloged meticulously and is available in the GenBank repository under the accession number OL351256.

Mitogenome sequence feature analysis

Overall, 1 735 bp of the mitogenome comprised transfer RNA (tRNA) genes, which harbored a GC content of 51.53%. Individual tRNA genes ranged in length from 68 bp (trnY-GTA) to 88 bp (trnS-GCT). Two rRNA genes (rrn26 and rrn18) were 3 148 bp and 1 848 bp in length, with GC contents of 50.41% and 53.14%, respectively. Another rRNA gene (rrn5) was shorter, spanning only 119 bp (Supplementary Tables S1, S2).

Altogether, 61 genes were identified, encompassing 35 protein-coding, 23 tRNA, and three rRNA genes. Notably, all protein-coding genes were initiated with ATG codons, while the termination codons were more diverse. Seven genes (*atp1*, *matR*, *mttB*, *nad7*, *rpl10*, *rps14*, and *rps3*) terminated with a TAG codon, while 10 genes (*atp8*, *atp9*, *ccmC*, *ccmFC*, *ccmFN1*, *cob*, *cox3*, *nad4*, *rpl2*, and *rps12*) terminated with TGA. The remaining genes utilized TAA as the stop codon (Supplementary Table S2).

Annotation of these genes revealed their classification into different groups, including ATP synthase, Cytochrome c biogenesis, Ubiquinol cytochrome c reductase, Cytochrome c oxidase, Maturases, Transport membrane protein, NADH dehydrogenase, Ribosomal proteins, Ribosomal RNAs and Transfer RNAs (Supplementary Table S3).

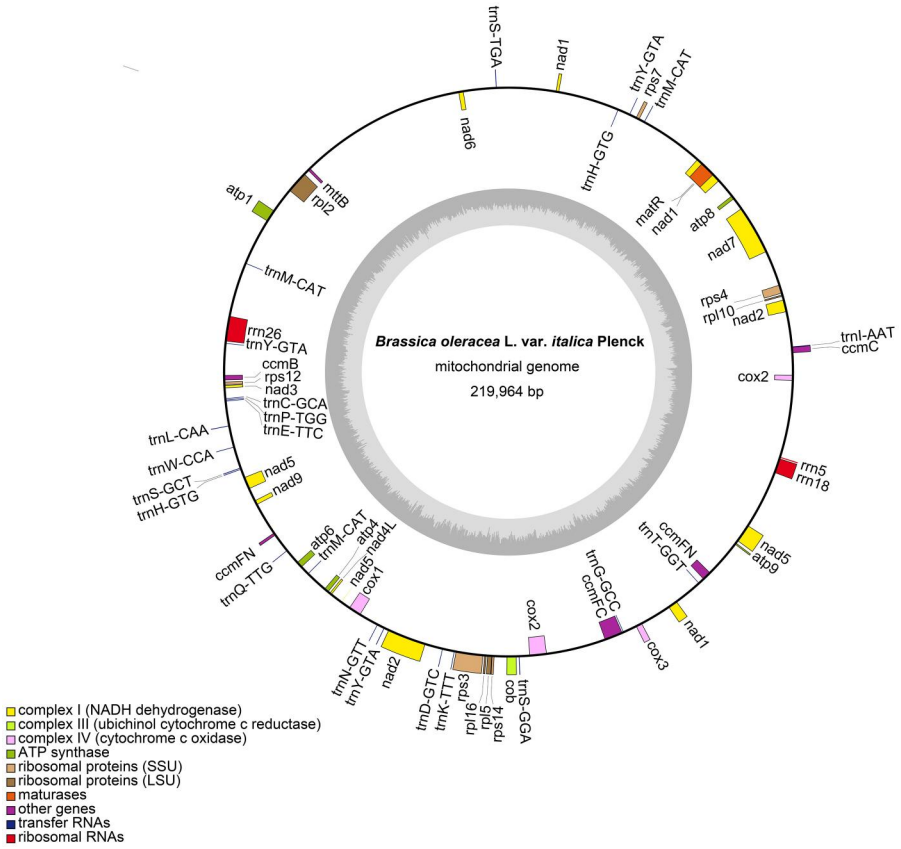

Figure 2. Schematic map of the mitochondrial genome of *Brassica oleracea* L. var. *italica* Plenck. GC contents are shown inside the inner circle. The outer circle shows transcription directions for the inner and outer genes are anticlockwise and clockwise, respectively.

Introns were present in 11 genes, with *ccmFc*, *cox2-1*, *rpl2*, *rps3*, *trnI-AAT*, and *trnT-GGT* containing one intron each; *nad4* harboring three introns, and *nad1*, *nad2*, *nad5*, and *nad7* containing four introns each (Supplementary Table S3).

A codon preference analysis revealed 29 codons with RSCU value >1, 27 of which ended with an A/U base, indicating a preference for codons ending in A/U (Supplementary Table S4). A total of 9 209 codons (excluding stop codons) were detected, with 10.8% encoding leucine (995 codons) and 8.96% encoding serine (825 codons). The least abundant amino acid was cysteine, for which there were only 127 codons (1.38% of the total codons) (Supplementary Figure S4).

The mitogenome contained 345 repeats, including 170 forward and 175 palindromic repeats. Most (130 forward and 121 palindromic repeats) were 30–39 bp long. Fewer repeats were 70–79 bp, 80–89 bp, 90–99 bp, and >200 bp in size.

Homology and collinearity analysis of mitogenome sequences

Sequence homology alignment and collinearity analysis were performed on the mitochondrial genomes of *B. oleracea* L. var. *italica* Plenck, *B. oleracea* L. *botrytis*, *B. oleracea* var. *capitata*, *Brassica napus*, *Brassica carinata*, *Brassica nigra*, *Brassica rapa,* and *Arabidopsis thaliana*. The results showed that *B. oleracea* L. var. *Italica* Plenck was revealed to be most similar to *B. oleracea* L. *botrytis*, and both were genetically close to *B. oleracea* var. *capitata*. The mitochondrial genomes of *B. oleracea* L. var. *italica* Plenck and *B. oleracea* L. *botrytis* showed good collinearity and consistency in their gene locations.

Homologous analysis of the mitochondrial and chloroplast genomes of B. oleracea L. var. italica Plenck

Twenty-two sequence fragments were homologous between the mitochondrial and chloroplast genomes of *B. oleracea* L. var. *italica* Plenck (Supplementary Figure S5). Overall, the homologous fragments totaled 13 355 bp in length, with an average fragment length of 605 bp. The longest homologous sequence was 2 186 bp in size, and the shortest was 42 bp. Six homologous fragments were >1 000 bp, seven were between 100 and 1 000 bp, and nine were <100 bp long.

Phylogenetic analysis

To determine the phylogenetic position of *B. oleracea* L. var. *italica* Plenck, the phylogenies of 16 selected Cruciferae species and 35 mitochondrial protein-coding genes totaling 30,113 bp were compared. The phylogenetic analysis revealed that *B. oleracea* L. var. *italica* Plenck was monophyletic. The outgroup *Arabidopsis thaliana* occurred as a single branch, and *B. oleracea* L. var. *italica* Plenck was positioned close to the clade containing *B. oleracea* L. *botrytis* and *B. oleracea* var. *capitata*, with high bootstrap support. Simultaneously, three selected *B. oleracea* varieties exhibited noteworthy

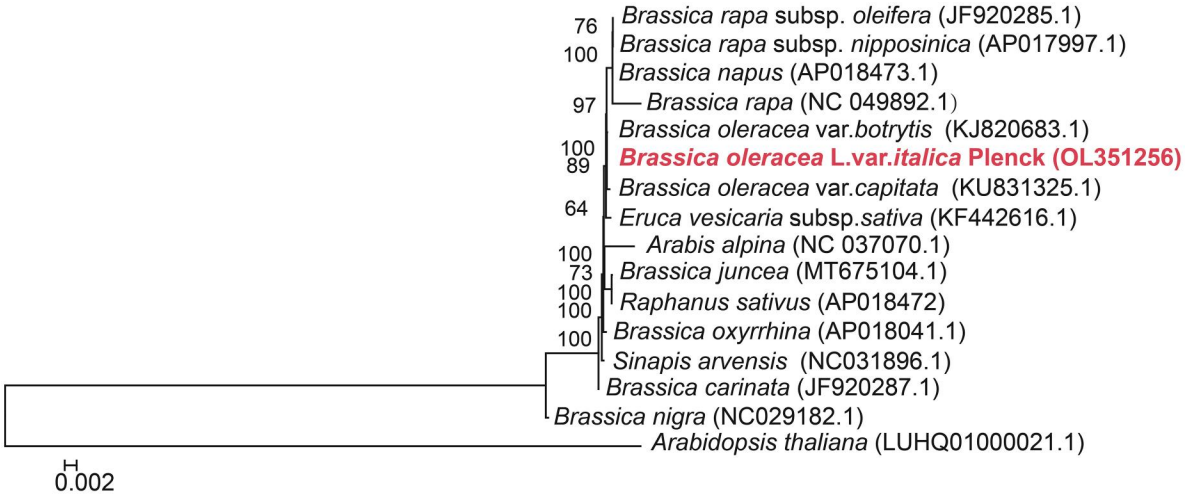

Figure 3. The phylogenetic tree of *Brassica oleracea* L. var. *italica* Plenck and 15 relative species. *Arabidopsis thaliana* was used as outgroups. The following sequences were used: *Brassica rapa* subsp. *oleifera* JF920285.1 (Chang et al. 2011), *Brassica rapa* subsp. *nipposinica* AP017997.1 (Hatono et al. 2017), *Brassica napus* AP018473.1 (Arimura et al. 2018), *Brassica rapa* NC_049892.1 (Ren 2021), *Brassica oleracea* var. *botrytis* KJ820683.1 (Grewe et al. 2014), *Brassica oleracea* var. *capitata* KU831325.1 (Yang et al. 2018), *eruca vesicaria* subsp. *sativa* KF442616.1 (Wang et al. 2014), *arabis alpina* (NC_037070.1), *Brassica juncea* MT675104.1 (Vasupalli et al. 2021), *Raphanus sativus* AP018472 (Arimura et al. 2018), *Brassica oxyrrhina* AP018041.1 (Mukai et al. 2019), *sinapis arvensis* NC_031896.1 (Sang et al. 2020), *Brassica carinata* JF920287.1(Chang et al. 2011), *Brassica nigra* NC_029182.1 (Yang et al. 2016), *Arabidopsis thaliana* LUHQ01000021.1 (Zapata et al. 2016).

affinity with *Brassica rapa* and *Brassica napus* (Figure 3). These findings contribute to a deeper understanding of the genetic relationships between species of the *Brassica* genus and shed light on their phylogenetic dynamics.

Discussion and conclusion

Morphological method for classification and phylogenetic studies often yields inconsistent results due to its subjectivity and environmental influences. Comparing with second-generation sequencing technology, third-generation sequencing technology effectively addresses the challenges of short read lengths and systematic biases by providing longer reads. In this study, we used both second- and third-generation sequencing technology to assemble and annotate the mitogenome of *Brassica oleracea* L. var. *italica* Plenck.

The mitogenome of *B. oleracea* L. var. *italica* Plenck spanned 219 964 bp in length. When compared to the published mitochondrial genomes of B. *oleracea* L. var. botrytis (219 962 bp) and *B. oleracea* var. capitata (219 975 bp), the sizes of mitochondrial genomes were generally consistent among species with close genetic relationships in *B. oleracea*. In contrast, some species with relatively distant genetic relationships, such as *B. napus* (227 181 bp), *B. carinata* (232 241 bp), *A. thaliana* (213 235 bp), and *B. nigra* (232 407 bp), showed slightly larger mitochondrial genomes. The minimal variation among these selected species could likely reflect their genetic relationships and mitochondrial characteristics.

The GC content of plant mitochondrial genomes tends to stabilize through evolution. In *B. oleracea* L. var. *italica* Plenck, the mitogenome displayed a GC content of 45.25%, consistent with other *B. oleracea* species and similar to the general pattern observed in angiosperms. The genetic composition of *B. oleracea* L. var. *italica* Plenck was similar to that of *B. oleracea* species. This similarity suggested that the mitochondrial genes remained essentially consistent among related species with close genetic relationships, as previously reported (Clifton et al. 2004; Yasunari et al. 2005).

The accumulation of repetitive sequences was closely linked to mitochondrial gene rearrangement (Aguileta et al. 2014). Species with closer genetic relationships exhibited more similar characteristics in their repetitive sequences. In dicotyledonous plants, coding genes tend to favor codons ending in A/U. The mitogenome of *B. oleracea* L. var. *italica* Plenck also showed a preference for these codons, aligning closely with the codon usage patterns of other *B. oleracea* species. This consistency suggested a stable evolutionary trend in the base composition of mitochondrial genomes within *B. oleracea*

Gene arrangement offers insights into mitochondrial evolution (Tan et al. 2018). In this study, selected Brassica genus species displayed gene loss and rearrangement. Notably, the mitochondrial genome of *B. oleracea* L. var. *italica* Plenck exhibited high collinearity with *B. oleracea* L. var. *botrytis* and *B. oleracea* var. *capitate*. However, a significant inversion was observed in *B. oleracea* var. *capitate*. The results indicated that *B. oleracea* L. var. *italica* Plenck and *B. oleracea* L. var. *botrytis* had a closer genetic relationship and shared more similar evolutionary characteristics (Chen et al. 2024).

The Brassica genus is characterized by a high diversity of species. Phylogenetic analysis revealed that *B. oleracea* L. var. *italica* Plenck and *B. oleracea* L. var. *botrytis* formed a distinct clade with strong bootstrap support, indicating a close evolutionary relationship. Comprehensive analyses of genome size, base composition, gene annotation, codon preference, collinearity, and phylogeny further confirmed that *B. oleracea* L. var. *italica* Plenck and *B. oleracea* L. var. *botrytis* had a closer genetic relationship and belong to the *B. oleracea* species group. This finding also aligned with the speciation history of these two varieties. Initially, the wild species *Brassica cretica* was domesticated into *B. oleracea* L. var. *italica* Plenck, evolving from non-flowering buds to green flower heads, and later

diverging into white flower heads (Chen et al. 2024). Our findings provide a reference mitogenome of *B. oleracea* L. var. *italica* Plenck, and the information also is helpful for further research on genetic conservation, phylogenetic relationships, and molecular breeding strategies in *B. oleracea*

Ethical approval statement

This study did not involve humans or animals. The samples of *Brassica oleracea* L. var. *italica* Plenck can be collected without ethical approval or permission.

Author contributions

Xuli Pei, Zange Jing, Zheng Tang and Meiqi Tao conceived and designed the experiments. Xuli Pei, Zange Jing, Qian Xu, Meiqi Tao and Liling Mo performed the experiments. Xuli Pei, Zange Jing, Zhenchao Zhang, Muneeb Ahmad Wani and Peng Jiao analyzed the data. Xuli Pei, Zange Jing, Muneeb Ahmad Wani, Meiqi Tao, Liling Mo and Peng Jiao drafted the manuscript. All authors gave final approval of the version to be published. All authors agree to be accountable for all aspects of the work.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Project for Fundamental Research of Local Undergraduate Universities(Partial)in Yunnan Province under Grant number [202301BA070001-021] and [2018FH001-044]; Major Science and Technology Project for Breeding New Agricultural(Vegetable) Varieties in Zhejiang under Grant number [2021C02065-4-2]; Science and technology project of Wenzhou City under Grant number [2019ZX007-2]; Jiangsu Seed Industry Revitalization Project under Grant number [JBGS[2021]077]; and Science and Technology Innovation Fund Project of Jurong City under Grant number [ZB22105].

Data availability statement

The data that support the findings of this study are openly available in the Genbank database at https://www.ncbi.nlm.nih.gov/, under accession number [OL351256]. The associated BioProject, SRA, and BioSample numbers are PRJNA777727, SRR16767412 and SAMN22871633, respectively.

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