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PLASTOME ANNOUNCEMENT

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The complete chloroplast genome of *Ranunculus ternatus* (Ranunculaceae)

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ABSTRACT

Ranunculus ternatus Thunb. 1784 is a plant with important medicinal values. Here we report its chloroplast genome. This chloroplast genome was 156,003 bp in length with a GC content of 37.86%. It is composed of a large single copy (LSC) of 85,397 bp and a small single copy (SSC) of 19,856 bp, which are separated by a pair of inverted repeats (IR) of 25,375 bp each. The chloroplast genome contained 110 unique genes, including 77 protein-coding genes, 4 rRNA genes, and 29 tRNA genes. Phylogenetic analysis indicated that *R. ternatus* was closely related to *R. cassubicifolius*. This chloroplast genome not only enriches the genome information of *Ranunculus* but also will be useful for the evolution study of the family Ranunculaceae.

Background

Ranunculus Linaeus 1753 is the largest genus in the family Ranunculaceae with a high species diversity of about 600 species widely distributed worldwide. Most species are found in the temperate to boreal regions and are less common in the tropics (Hörandl et al. 2005). Species classification studies have shown that *Ranunculus* is monophyletic in origin and is divided into two subgenera and 17 series (Tamura 1993). *Ranunculus ternatus* Thunb. 1784 is an annual herbaceous plant of the genus *Ranunculus* mainly distributed in the Henan and Anhui regions of China. Its roots contain a variety of lactones (Guo et al. 1995; Xiong et al. 2008a), flavonoids (Xiong et al. 2008b), triterpenoids (Zhao et al. 2008), glycosides (Tian et al. 2005) and two alkaloids (Zhang et al. 2007), which are used to treat pharyngitis, pulmonary tuberculosis, cervical scrofula, breast cancer, renal fibrosis and other diseases (Zhan et al. 2013; Feng et al. 2017; Xu et al. 2022).

In recent years, the chloroplast genomes of some *Ranunculus* species have been published, such as *R. austro-oreganus* (Zeng et al. 2021), *R. cassubicifolius* (Karbstein et al. 2022), *R. flammula* (Marcel et al. 2017), and *R. japonicus* (Zeng et al. 2021), *R. macranthus* (Raubeson et al. 2007), *R. occidentalis* (Zeng et al. 2021), *R. pekinensis* (Liu et al. 2022), *R. repens* (Marcel et al. 2017), *R. reptans* (Marcel et al. 2017), and *R. sceleratus* (Kim et al. 2023), *R. yunnanensis* (Rao et al. 2022). However, the chloroplast genome of *R. ternatus* has not been reported yet. Therefore, we assembled the chloroplast genome of *R. ternatus* and performed comparative genomic analyses with other *Ranunculus* species. Our primary objectives were to study the chloroplast genome of *R. ternatus* and to determine its phylogenetic position.

Materials and methods

Plant material, DNA extraction and sequencing

Dry leaves of *R. ternatus* were collected in this study from Gushi County, Xinyang City, Henan Province, China (Figure 1, 115.672500°E, 31.911667°N). Our experimental studies, including the collection of plant material, were in accordance with institutional, national, or international guidelines. The sample was deposited at the herbarium of the College of Pharmaceutical Engineering, Xinyang Agriculture and Forestry University (voucher number: RT001, Min Song, 1797405679@qq.com). Total genomic DNA was extracted using the CTAB method (Doyle and Doyle 1987). The DNA library of next generation sequencing with an insert size of 300 bp was constructed and sequenced using the MGISEQ-2000 platform (Benagen Technology Company, Wuhan, Hubei), yielding ∼4 Gb of raw data, and low-quality sequences were removed to obtain clean data.

Genome assembly and annotation

De novo genome assembly from the clean data was performed using GetOrganelle v.1.7.5 (Jin et al. 2020). The parameters applied for the plastome were "-R 15 -k 21,45,65,85,105 -F embplant_pt." Samtools v.1.7 (Li et al. 2009) and bedtools v.2.28 (Quinlan and Hall 2010) were used for depth detection. The chloroplast genome was annotated using CPGAVAS2 (Shi et al. 2019), and PGA (Qu et al. 2019) with a reference genome (*R. membranaceus*, GenBank: NC_065303). GB2sequin (https://chlorobox.mpimp-golm.mpg.de/GenBank2Sequin.html) was then used to confirm the

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Figure 1. Species reference map of *R. ternatus*. This picture was taken by Min Song from the Xinyang Agriculture and Forestry University, Xinyang City, Henan Province, China. (voucher number: RT001; 113.82350°E, 31.96808°N). core features: *R. ternatus* is an annual herb. Root tubers clustered, tips stiff, resembling cat's claws. Leaf blade glabrous. Flowers yellow or late white. Achenes ovoid. The tuberous roots are used medicinally for lymphatic tuberculosis.

annotation results (Tillich et al. 2017). CPGView (Liu et al. 2023) was used to visualize the maps of the chloroplast genome, cis- and trans-splicing genes. In addition, we also performed genomic hotspot analysis using mVISTA (Frazer et al. 2004).

Repeat and IR boundary analysis

Simple sequence repeats (SSRs) were identified using the MISA software (Beier et al. 2017), including mono-, di-, tri-, tetra-, penta-, and hexa-nucleotides with minimum numbers of 10, 5, 4, 3, 3, and 3, respectively. Additionally, REPuter (https://bibiserv.cebitec.uni-bielefeld.de/reputer/) was used to calculate palindromic, forward, reverse, and complementary repeats with the following settings: minimum repeat size of 30 bp and hamming of 3 (Kurtz et al. 2001). In addition, comparisons between the boundaries of three regions (large single-copy (LSC), small single-copy (SSC), and inverted repeats (IRs)) were generated using IRscope (Amiryousefi et al. 2018).

Phylogenetic analysis

We performed a phylogenetic analysis of the chloroplast genome of *R. ternatus* along with 15 other *Ranunculus* species. We extracted 77 common protein-coding genes (PCGs) from the genome annotation files using PhyloSuite v. 1.2.3 (Zhang et al. 2020). 77 genes were aligned using MAFFT v. 7.4 (Katoh and Standley 2013), and these aligned genes were concatenated. Based on the concatenation matrix, a phylogenetic tree was constructed using the maximum likelihood (ML) method implemented in IQ-TREE v. 2.1.2 (Nguyen et al. 2015), and the best model (GTR + F+R2) was inferred using ModleFinder (Kalyaanamoorthy et al. 2017). The bootstrap replicates were set to 1000. Tree visualization was performed in Figtree v. 1.4.3 (https://github.com/rambaut/figtree/releases/tag/v1.4.3).

Results

General features of the chloroplast genome

We analyzed the coverage depth of the chloroplast genome and the annotation of some difficult genes, the results indicated that the chloroplast genome of *R. ternatus* was similar to that of other *Ranunculus* species with some confidence (Figure S1; Figure S2). This chloroplast genome had a circular quadripartite structure of 156,003 bp in length (Figure 2, Table S1), which consisted of a large single-copy (LSC) region (85,397 bp), a small single-copy (SSC) region (19,856 bp), and a pair of inverted repeats (IR) (25,375 bp). This chloroplast genome had a total GC content of 37.86%, the GC content in the IR region (43.46%) was significantly higher than that in the LSC region (36.03%) and the SSC region (31.30%). In addition, the annotation results showed that there were 127 genes in the chloroplast genome, leaving 110 unique genes after removing 17 duplicated genes. These unique genes included 77 PCGs, 4 ribosomal RNA genes and 29 transfer RNA genes (Table S1, Table S2). Similar to other *Ranunculus* species, these gene with two copies in *R. ternatus* contained six PCGs (*ndh*B, *rpl*2, *rpl*23, *rps*7, *rps*12, *ycf*2), seven tRNAs (*trn*A-UGC, *trn*I-CAU, *trn*I-GAU, *trn*L-CAA, *trn*N-GUU, *trn*R-ACG, *trn*V-GAC) and four rRNAs (rrn4.5, rrn5, rrn16, rrn23). The mVISTA analysis showed that the IR region was more conserved than the LSC and SSC regions (Figure S3).

Repeat analysis

We identified 108 simple sequence repeats (SSRs) in the chloroplast genome of *R. ternatus*, including 31 mononucleotides, 37 dinucleotides, 17 trinucleotides, 21 tetranucleotides, one pentanucleotide, and one hexanucleotide (Table S1). Mononucleotides and dinucleotides accounted for most of the SSRs, accounting for 62.96% of the total. The SSRs were highest in the LSC region and lowest in the IR region, and were mainly concentrated in the non-coding regions (Table S3). In addition, we identified 33 long repeats, including
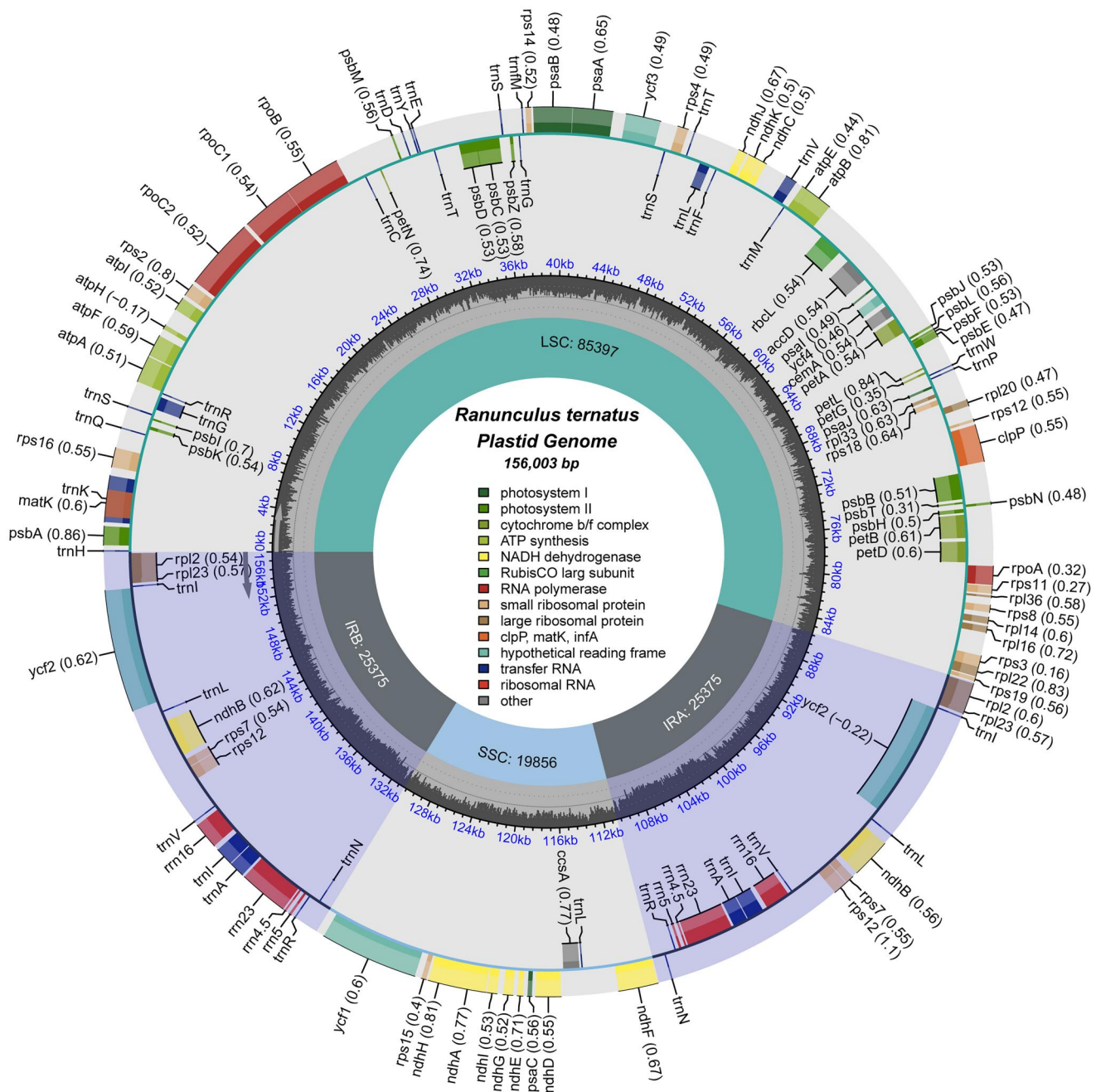
Figure 2. The chloroplast genome map of *R. ternatus*. Genes on the inside of the circle are transcribed in a clockwise direction and genes on the outside of the circle are transcribed in a counter-clockwise direction. The optional codon usage bias is displayed in the parenthesis after the gene name. The small grey bar graphs inner circle shows the GC contents. Genes are color-coded by their functional classification. The functional classification of the genes is shown in the center.

10 forward repeats, 5 reverse repeats, 17 palindromic repeats, and one complement repeat (Table S1), which were mainly located in the IR and LSC regions (Table S4).

IR boundaries analysis

The chloroplast genome structures of the *Ranunculus* species were relatively similar, with the main differences being in the location of genes within the SSC region and at the IR boundary. The *ndh*F genes were located within the SSC region, and the *ycf*1 genes were predominantly present either within the SSC region or at the IR boundary (Figure S4). In clade A, *ycf*1 genes were predominantly located on the IR boundary,

whereas in clade B they were located within the SSC region. Compared to *R. cassubicifolius*, the IR region of the chloroplast genome of *R. ternatus* showed a small expansion, but the difference was not significant.

Phylogenetic analysis

To clarify the phylogenetic position of *R. ternatus* in the genus *Ranunculus*, we performed a phylogenetic analysis. The phylogenetic tree showed that our phylogenetic results were generally consistent with previous studies, where most nodes have high bootstrap values (Figure 3). In section *Auricomus*, the *R. ternatus* was more closely related to *R. cassubicifolius*.
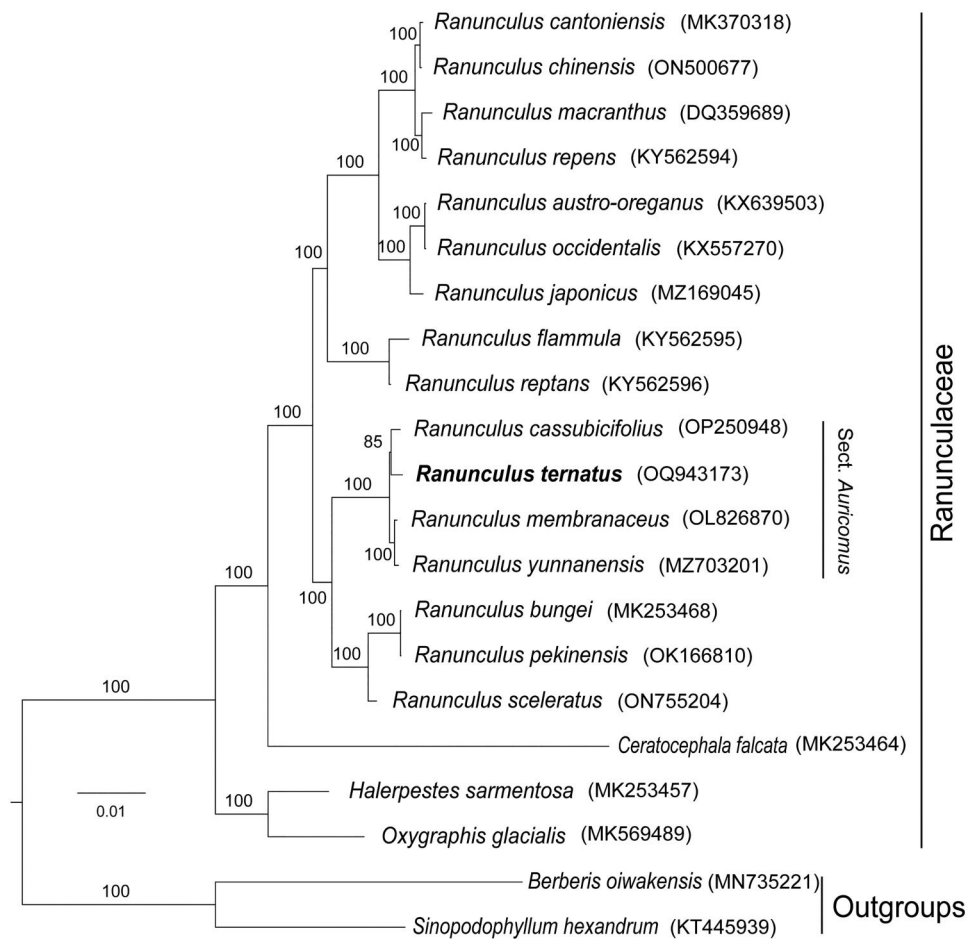
Figure 3. Phylogenetic tree based on the concatenated sequences of 77 protein-coding genes in 21 species by maximum-likelihood (ML). Values split by back-slashes above branches represent ML bootstraps. The best-fit model was GTR + F+R2. Branch supports were tested using ultrafast bootstrap (UFBoot) with 1000 replicates. *Ranunculus ternatus* (OQ943173) was marked in bold. The following sequences were used: *Ranunculus cantoniensis* MK370318 (Li et al. 2019), *Ranunculus chinensis* ON500677, *Ranunculus macranthus* DQ359689 (Raubeson et al. 2007), *Ranunculus repens* KY562594 (Marcel et al. 2017), *Ranunculus austro-oreganus* KX639503, *Ranunculus occidentalis* KX557270, *Ranunculus japonicus* MZ169045 (Zeng et al. 2021), *Ranunculus flammula* KY562595 (Marcel et al. 2017), *Ranunculus reptans* KY562596 (Marcel et al. 2017), *Ranunculus cassubicifolius* OP250948 (Karbstein et al. 2022), *Ranunculus membranaceus* OL826870, *Ranunculus yunnanensis* MZ703201 (Rao et al. 2022), *Ranunculus bungei* MK253468 (He et al. 2019), *Ranunculus pekinensis* OK166810 (Liu et al. 2022), *Ranunculus sceleratus* ON755204 (Kim et al. 2023), *Ceratocephala falcata* MK253464 (He et al. 2019), *Halerpestes sarmentosa* MK253457 (He et al. 2019), *Oxygraphis glacialis* MK569489 (Zhai et al. 2019), *Berberis oiwakensis* MN735221 (Xiao et al. 2020), *Sinopodophyllum hexandrum* KT445939.

Discussion

Previous phylogenetic studies on the genus *Ranunculus* have not included *R. ternatus* in the sampling range. However, some researchers have suggested that *R. ternatus* should be included in the section *Auricomus* (Hörandl and Emadzade 2012). In this study, we observed that *R. cassubicifolius* belonged to the section *Auricomus* and was closely related to *R. ternatus*. Based on this phylogenetic tree, we inferred that *R. ternatus* was closely related to section *Auricomus*. Chloroplast genomes from *Ranunculus* species are still scarce, and accurate phylogenetic relationships still require comprehensive analyses of the nuclear and organelle genomes of more species (Górniak et al. 2010). Future studies on the nuclear phylogeny of this section are needed to determine the phylogenetic relationships of *Ranunculus* and species within the genus. Therefore, extensive sampling of more *Ranunculus* species with genome sequencing and analysis will be needed in the future to resolve the complex phylogenetic relationships within *Ranunculus*.

Conclusion

In this study, the chloroplast genome of *R. ternatus* was assembled using short-read data. This chloroplast genome had a typical tetrameric structure and is similar to the chloroplast genomes of other *Ranunculus* plants. The phylogenetic tree supported the phylogenetic position of *R. ternatus*. These results clearly show that *R. ternatus* was located in the section *Auricomus* and was closely related to *R. cassubicifolius*. Thus, the chloroplast genome of *R. ternatus* not only enriches the genomic information of *Ranunculus*, but also lays the foundation for understanding the evolution of the genus *Ranunculus*.

Ethical approval

Ranunculus ternatus is widely distributed in fields and wastelands in Henan Province, and is not protected by law. Experimental research do not involve the genetic transformation, preservation of the genetic

background of the species used, or any other processes requiring ethical approval. Therefore, no special permission was required.

Authors' contributions

Xinrong Qiao and Min Song were mainly responsible for the experimental design; Xinrong Qiao, Zexia Wang, and Wei Sun participated in the genome assembly and annotation work. Xinrong Qiao and Nailiang Zhu analyzed and interpreted the data. All authors have read and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability statement

The genome sequence data that support the findings of this study are available in GenBank of NCBI (http://www.ncbi.nlm.nih.gov/) under the accession no. OQ943173. The associated BioProject, BioSample, and SRA numbers are PRJNA995920, SAMN36510160, and SRR25317840, respectively.

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