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Molecular Markers from Chloroplast Genomes in *Eucommia ulmoides*

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Abstract The chloroplast genome is very helpful for understanding the genetics and evolution of *Eucommia ulmoides* and can also provide many useful molecular markers. This study expounds the general structure, gene content and arrangement pattern of the chloroplast genome of *Eucommia ulmoides*, and analyzes several types of molecular markers extracted from the chloroplast genome. The development and application of these markers can be used to study genetic diversity, population structure and evolutionary relationships, and help protect genetic resources. This study also introduced the commonly used methods for chloroplast labeling analysis, discussed some technical difficulties, summarized the current research progress and new applications, and proposed some future research directions. This study aims to provide a scientific basis for a more comprehensive understanding of the chloroplast genome and molecular markers of *Eucommia ulmoides*.

Keywords *Eucommia ulmoides*; Chloroplast genome; Molecular markers; Genetic diversity; Conservation genetics

1 Introduction

Eucommia ulmoides, also known as hardy rubber tree, is of great value in both industrial and medicinal fields (Wang et al., 2019). It is the sole member of the Eucommulaceae family and holds an important position in traditional Chinese medicine, especially being very common in the treatment of orthopedic diseases (Zhu et al., 2020). *Eucommia ulmoides* can also produce rubber, and this unique ability has attracted the attention of many scientists and enterprises (Li et al., 2020). However, *Eucommia ulmoides* is dioecious, that is to say, each tree is either male or female. This characteristic has brought a lot of troubles to breeding and cultivation (Wang et al., 2019).

The structure of the chloroplast genome is relatively stable, which is very helpful for studying the genetic relationship and classification among plants (Yu et al., 2015). The research conducted by Zhang et al. in 2023 demonstrated that the chloroplast genome of *Eucommia ulmoides* has been fully sequenced, revealing a typical tetrad structure with 135 genes, containing a wealth of useful genetic information. These data can be used to develop molecular markers, which are of great value for the breeding and conservation of *Eucommia ulmoides*. Meng et al. in the same year, that is, in 2023, believed that the chloroplast genome is involved in photosynthesis and other important metabolic processes and is a crucial research object in plant physiology and biochemistry studies.

This study analyzed the molecular markers obtained from the chloroplast genome of *Eucommia ulmoides*. By summarizing existing research, it understood the role of these genetic markers in breeding, species conservation, and in-depth study of the specific traits of *Eucommia ulmoides*. It emphasized their importance in the entire field of plant genomics and their application potential in enhancing the industrial and medicinal value of *Eucommia ulmoides*. This study aims to provide a foundation for the subsequent scientific research and development of *Eucommia ulmoides*.

2 Chloroplast Genome Structure in *Eucommia ulmoides*

2.1 General features

The chloroplast genome of *Eucommia ulmoides* has a typical four-segment structure, which is divided into a large single-copy (LSC) region, a small single-copy region (SSC), and two inverted repeat (IR) regions. The total length of its genome is approximately 163 586 base pairs (Wang et al., 2018). Liu et al. (2022) found that the GC content of the entire genome was 38.4%. From the perspective of base composition, adenine (A) and thymine (T) each account for 30.8%, while cytosine (C) and guanine (G) each account for 19.2%. This structure is similar to that of most other angiosperms, indicating that the chloroplast genome of *Eucommia ulmoides* has strong conservation.

2.2 Gene content and organization

The chloroplast genome of *Eucommia ulmoides* contains 135 genes, among which 89 are protein-coding genes, 38 are transfer RNA (tRNA) genes, and 8 are ribosomal RNA (rRNA) genes. The quantity and arrangement of these genes are relatively stable, and most of them are related to photosynthesis, transcription and translation. Some SSRs and SNPs were also found in the chloroplast genome of *Eucommia ulmoides*. These variant regions are very helpful for studying genetic diversity and developing molecular markers (Zhang et al., 2016). Zhong et al. demonstrated in their 2022 study that their presence also indicates that there are some mutation hotspots and potential genetic variations in *Eucommia ulmoides*.

2.3 Comparison with other plant species

The phylogenetic study by Jin et al. (2022) indicates that *Eucommia ulmoides* is closely related to *Aucuba japonica*, both belonging to the Oleocales order. This is also supported by the similarity of their chloroplast genomes. Li et al. (2014a) found that the genome of *Eucommia ulmoides* also exhibited some interesting difference patterns. Most SNPS occurred in gene regions, while insertions and deletions (Indels) were mainly concentrated in the blank areas between genes. This pattern also shows a similar phenomenon in other angiosperms, indicating that the chloroplast genomes of different plants have been relatively conserved during the evolutionary process. The chloroplast genome of *Eucommia ulmoides* has a typical quad structure, rich genes and obvious genetic variations.

3 Molecular Markers from Chloroplast Genomes

3.1 Types of molecular markers

3.1.1 Simple sequence repeats (SSRs)

Simple sequence repeats (also known as microsatellites) are short and repetitive DNA sequences that usually have a high degree of diversity. Researchers have identified and developed some SSR markers in the chloroplast genome of *Eucommia ulmoides* that can be used as molecular markers. Jin et al. (2020) developed eight polymorphic chloroplast SSR (cpSSR) loci, which can be used to study the population genetics of *Eucommia ulmoides*.

3.1.2 Single nucleotide polymorphisms (SNPs)

Single nucleotide polymorphism (SNPs) refers to the change of a certain base in a DNA sequence. Many such SNPs have been discovered in the chloroplast genome of *Eucommia ulmoides*. Meng et al. identified 75 SNPs in their 2023 study, among which 59 were located in genetic regions. Among the 40 SNPs presumed to belong to the coding region, all are synonymous mutations, indicating that they do not change the structure of the protein.

3.1.3 Insertions and deletions (Indels)

Sometimes, DNA sequences may undergo some changes due to the addition or deletion of certain bases, which are known as insertions and deletions (Indels). Zhong et al. (2022) identified 80 such Indels in the chloroplast genome of *Eucommia ulmoides*, with the majority occurring in the regions between genes. These Indels can be used as “molecular markers”, which are helpful for phylogenetic research and analyzing the relationships among different populations.

3.2 Development and identification of markers

Researchers conducted a comprehensive genomic analysis to develop and identify molecular markers in the chloroplast genome of *Eucommia ulmoides*. They obtained the complete chloroplast genome by screening methods and then compared different samples to identify the regions with differences. In their 2020 study, Li et al. discovered 71 mutated chloroplast DNA fragments and selected 20 loci from them for population genetic analysis. High-throughput sequencing technologies such as PacBio and Hi-C make genome assembly more accurate and make it easier to find reliable markers (Figure 1).

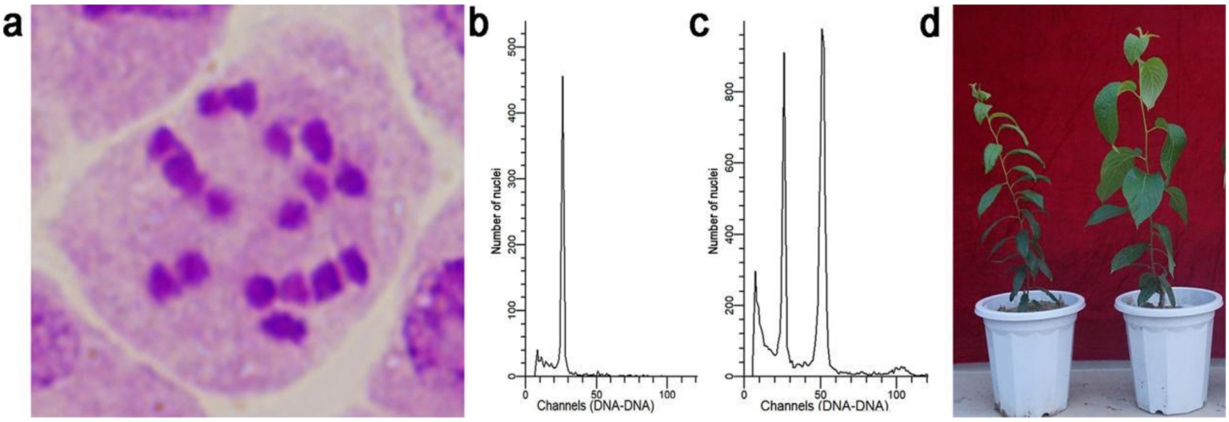

Figure 1 Karyotypic analysis of haploid plants generated through parthenogenesis under high-temperature treatments (48 °C for 6 h and 54 °C for 4 h) in *E. ulmoides* (Adopted from Li et al., 2020)

Image caption: a Somatic chromosome number of the haploids (2n=x=17). b Ploidy levels obtained from 3-week-old first leaf samples from haploid plants by flow cytometric analysis. c Ploidy levels obtained from 3-week-old first leaf samples from a mixture of haploid and diploid plants by flow cytometric analysis. d A haploid plant (left) and diploid plant (right) of *E. ulmoides* (Adopted from Li et al., 2020)

Li et al. (2020) treated *Eucommia ulmoides* at two different high temperatures, namely heating at 48°C for 6 hours and 54°C for 4 hours, to induce parthenogenesis. The results showed that this method could indeed enable *Eucommia ulmoides* to form haploids. Through karyotype analysis, they found that the somatic chromosome number of these haploids was 2n = x = 17. They analyzed the samples with three-week-old leaves using a flow cytometer and concluded that haploid plants have clear ploidy characteristics. Plants with haploid and diploid mixtures have different levels of ploidy. High-temperature treatment is an effective method that can be used to obtain Eucommia haploid plants, which is helpful for breeding and genetic research.

3.3 Applications of chloroplast markers

Chloroplast markers is helpful for understanding the genetic diversity, population distribution and evolutionary process of *Eucommia ulmoides*. The cpSSR loci and SNP markers identified in the relevant research can be used in conservation genomics to formulate plans for protecting this endangered plant. These markers are also very useful in phylogenetic research. Scientists have also discovered through the analysis of chloroplast genomes that *Eucommia ulmoides* and *Aucuba japonica* in Japan are “sister species”. Liu et al. (2022) established a high-density genetic map using SNP markers, which is helpful for identifying QTLS related to growth traits.

4 Key Applications of Chloroplast Molecular Markers

4.1 Genetic diversity and population structure

4.1.1 Assessing genetic variation

Dong et al. (2012) identified 71 polymorphic chloroplast DNA fragments in their early studies, among which 20 sites were selected as molecular markers that might be used in subsequent studies. Scientists have also developed more polymorphic chloroplast SSR markers (cpSSR), making the assessment of genetic differences more accurate. Firetti et al. (2017) hold that these markers are beneficial for researchers to conduct a more detailed analysis of the genetic diversity of *Eucommia ulmoides* and to understand its evolutionary process and environmental adaptability.

4.1.2 Population differentiation

In their 2015 study, Yu et al. used ISSR and SRAP markers and found that there were significant genetic differences among *Eucommia ulmoides* populations in different regions (Figure 2). The results of molecular variation analysis (AMOVA) show that most genetic variations occur within the same population, indicating that local genetic diversity is also very important. Li et al. (2019) demonstrated in their study that these research results indicated that chloroplast markers were helpful for identifying different genetic populations and understanding the genetic structure of *Eucommia ulmoides* populations.

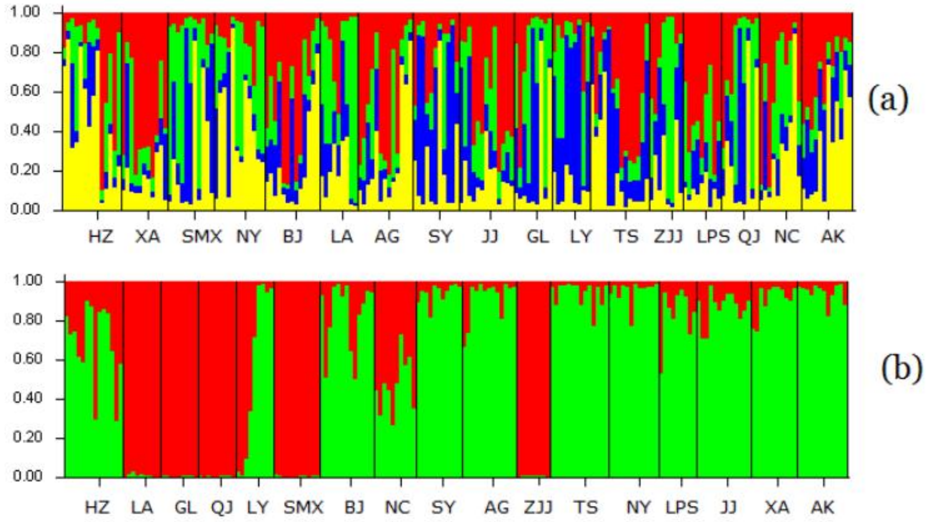

Figure 2 Population structure of *Eucommia ulmoides* prepared using the STRUCTURE program (Adopted from Yu et al., 2015)
Image caption: as revealed using inter-simple sequence repeat markers (a); sequence-related amplified polymorphism markers (b) (Adopted from Yu et al., 2015)

Yu et al. (2015) analyzed the population structure of *Eucommia ulmoides* using the STRUCTURE program and two molecular markers, ISSR and SRAP, and found that there were obvious genetic groups within the population of *Eucommia ulmoides*, indicating that it has a high genetic diversity. The ISSR marking shows a complex population structure with multiple small subpopulations. The SRAP markers more clearly show the differentiation among the main genetic populations, indicating that combining different types of molecular markers can provide a more comprehensive understanding of the genetic situation of *Eucommia ulmoides*. This study emphasizes that the use of multiple molecular markers can more accurately analyze the population structure and genetic diversity of plants.

4.1.3 Phylogeographic studies

Chloroplast DNA is single-parent inheritance with a slow rate of change and is very useful in systematic geography research. Hu et al. (2019) hold that this type of marking is useful for understanding the historical changes and evolutionary process of a species in space. In the study of *Eucommia ulmoides*, the analysis of chloroplast genomes confirmed that it is a “sister species” to *Aucuba japonica* in Japan, providing new clues for understanding the systematic geographical history of *Eucommia ulmoides*. Moghaddam et al. (2021) demonstrated that such research could help clarify how *Eucommia ulmoides* was previously distributed and possibly migrated.

4.2 Phylogenetic and evolutionary studies

In 2023, Du et al. discovered the genetic relationship between *Eucommia ulmoides* and other plants through complete chloroplast genome analysis, and it is clustered in the same branch as *Aucuba japonica* in Japan. Some chloroplast regions with particularly large variations have also been identified, which is beneficial for studying the relationships between species with relatively close classifications. Walker et al. (2014) demonstrated in their early research that these markers laid the foundation for studying the evolutionary process and genetic ties of *Eucommia ulmoides* in a larger plant phylogenetic context.

4.3 Conservation genetics

Yu et al. (2015) obtained data on the genetic diversity and population structure of *Eucommia ulmoides* through chloroplast molecular markers. This information is useful for identifying different genetic populations and regions with high genetic diversity, and can also provide a basis for conservation efforts. These data can also be used to establish a “core germplasm bank” to preserve the most crucial genetic resources. Chloroplast labeling can also be used to guide breeding work and improve the genetic stability of *Eucommia ulmoides* populations. Zhou et al. (2021) demonstrated that chloroplast molecular markers provide significant data support and research tools for studying the genetic differences, phylogeny, and geographical history of *Eucommia ulmoides*.

5 Methodologies for Analyzing Chloroplast Markers

5.1 DNA extraction and amplification

To analyze chloroplast markers, high-quality DNA should be extracted first. The modified CTAB method is very effective for *Eucommia ulmoides* and can extract genomic DNA with high purity and good integrity, facilitating subsequent experiments (Dong et al., 2012). After extracting the DNA, amplification is the next step. Researchers usually use polymerase chain reaction (PCR) to amplify certain regions of the chloroplast genome. In some experiments, especially when the initial amount of DNA is not large, a technique called “multi-primer loop amplification” is also used to enrich chloroplast DNA (Atherton et al., 2010). This method can not only increase the quantity of chloroplast DNA, but also improve its quality, which is beneficial to the subsequent high-throughput sequencing.

5.2 Sequencing techniques

High-throughput sequencing platforms such as Illumina Genome Analyzer II (GAII) have been successfully applied in the sequencing of chloroplast DNA (Wang et al., 2010). This method can conduct comprehensive sequencing of chloroplast genomes. SNPs and Indels were identified from it (Figure 3) (Wang et al., 2018). There is also a technique called “genomic screening” (shallow sequencing of the entire genome), which can also obtain the complete chloroplast genome sequence. This method is highly efficient, not costly, and can also provide sufficient data for subsequent comparative genomic analysis.

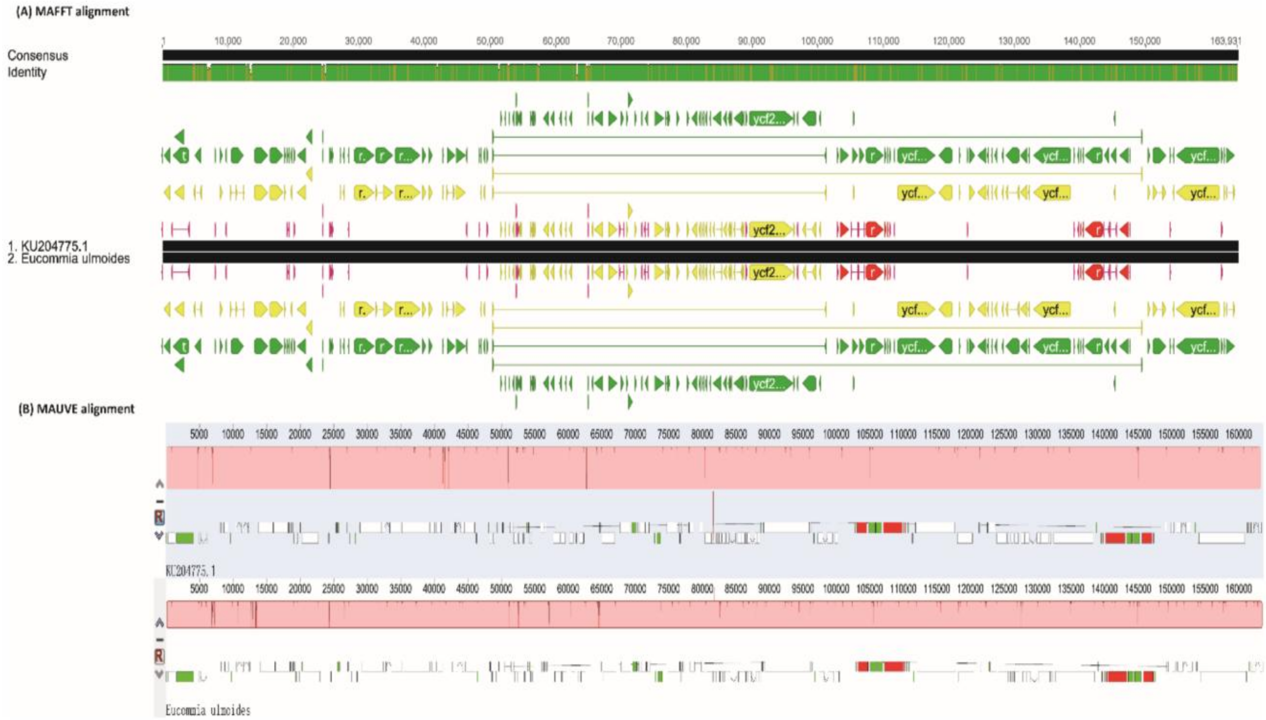

Figure 3 Conserved chloroplast genome structure in *Eucommia ulmoides* (Adopted from Wang et al., 2018)

Image caption: (A) Pairwise chloroplast genome alignments derived from Multiple Alignment using Fast Fourier Transform (MAFFT) program. The sequence identity is indicated on the top. Label KU204775.1 represents the *E. ulmoides* chloroplast genome retrieved from GenBank, while label *E. ulmoides* indicates the newly sequenced genome in this study. (B) Pairwise chloroplast genome alignments derived from MAUVE software (Adopted from Wang et al., 2018)

Wang et al. (2018) discovered that the chloroplast genomic structure of *Eucommia ulmoides* was very stable. They compared the chloroplast genome sequence (labeled as KU204775.1) obtained from the GenBank database with the newly sequenced data using the MAFFT program and found that the sequences of the two were very similar, indicating that the internal variations of the chloroplast genome of *Eucommia ulmoides* are few and the structural changes are not significant. They also conducted further analysis using MAUVE software and found that the overall structure of the genome and the genetic content remained almost unchanged, proving its conservation.

These results indicate that the chloroplast genome of *Eucommia ulmoides* is highly stable, which is of great significance for studying its evolutionary relationships, phylogenetic process and genetic protection. This stability also provides a good foundation for the development of molecular markers, which can be used in the research of species identification and genetic diversity.

5.3 Data analysis and interpretation

Researchers obtained a large amount of sequence data through high-throughput sequencing, de novo assembled it, reconstructed the complete chloroplast genome, and used comparative genomic analysis to identify variations like SNPs and Indels. The research of Wang et al. (2011) found that most SNPS in *Eucommia ulmoides* occurred in gene regions, and Indels were mostly concentrated in the regions between genes. Systematic genomic analysis has also been used to study evolutionary relationships, such as confirming that *Eucommia ulmoides* and *Aucuba japonica* in Japan are “sister species”. Scientists have also identified regions with particularly large variations in chloroplasts and used these regions to design primers to amplify specific target sequences, which is crucial for DNA barcoding and phylogenetic research.

6 Challenges and Limitations

6.1 Technical challenges

Liu and Zhu (2022) hold that the chloroplast genome is rather complex and of considerable scale. Only by using relatively advanced sequencing technologies and bioinformatics tools can it be accurately assembled and analyzed. New technologies such as PacBio and Hi-C have been used to complete the high-quality de novo assembly of the haploid genome of *Eucommia ulmoides*, with much better results than earlier studies. However, not all teams have the ability to acquire these advanced technologies, and the costs required prevent some research from being carried out in depth. How to identify and verify the regions with relatively large variations in the chloroplast genome is another difficulty. Some studies have identified variant sites suitable for phylogenetic analysis and DNA barcoding, but this process is time-consuming and laborious, requiring a large number of genomic alignings. In order to enable these variant sites to be smoothly amplified and sequenced in different species or populations, it becomes very necessary to develop specialized primers, which also increases a lot of technical difficulties (Yu et al., 2015).

6.2 Intraspecific variation

If the genetic differences within the same species are too large, the recognition of markers will become unstable and difficult to unify. A study analyzed the hybrid population of *Eucommia ulmoides* using ISSR and SRAP markers and found that its genetic diversity was very high, showing strong polymorphism, indicating that there was a large variation among individuals of *Eucommia ulmoides*. This difference may affect the repeatability and accuracy of molecular markers, making it difficult to draw clear conclusions when analyzing population relationships and evolutionary processes. There are also synonymous mutations and variations in repetitive regions in the chloroplast genome, which will also increase the complexity of the analysis. Jin et al. (2020) conducted a detailed comparison of the chloroplast genomes of two *Eucommia ulmoides* and found that their sequence differences were uneven. Most SNPS occurred in the gene regions, while Indels were mainly concentrated in the intergene regions.

6.3 Cross-species comparability

In their study in 2011, Nock et al. demonstrated that the evolution rate of chloroplast genes is relatively slow, making it less sensitive when studying the phylogenetic relationships of closely related species. Although some highly variable loci have been identified, the performance of these loci varies greatly among different species, which will affect their reliability in comparative studies. The chloroplast genomes of different species also have considerable differences in structure and sequence, which hinders the development of universal molecular markers. Xu et al. (2004) found in their early research between *Eucommia ulmoides* and its “sister species” - *Aucuba japonica* in Japan that there were significant differences in their chloroplast genomes, which led to the fact that the markers between the two were not necessarily interoperable. It becomes necessary to develop specific markers for a certain species or to identify areas that are relatively conservative and universal among multiple species.

7 Advances and Future Directions

7.1 Technological innovations

Zhang et al. (2016) have obtained complete chloroplast genomic data through genomic screening and next-generation sequencing (NGS), revealing its sequence differences and mutation hotspots. High-throughput sequencing platforms like Roche 454 FLX+ and PacBio are helpful for better isolation and analysis of SSRS and SNPS. This information is very important for studying the genetic diversity of *Eucommia ulmoides* and conducting marker-assisted breeding. Liu et al. (2022) hold that the high-density genetic maps developed using genotyping sequencing (GBS) provide an important basis for quantitative trait locus (QTL) analysis and are beneficial for identifying genetic markers related to growth traits.

7.2 Emerging applications

Meng et al. demonstrated in their 2023 study that the molecular markers found in the chloroplast genome of *Eucommia ulmoides* are significant in conservation genomics, as they can be used to assess genetic diversity and population structure. Wang et al. (2011) also developed gender-related markers such as AFLP and SCAR in the early stage. These markers enable researchers to determine the gender of plants when they are very young and select individuals with ideal traits in advance during breeding. Qun (2004) also combined metabolomics with molecular markers to identify different chemical types and their corresponding genetic markers, which is conducive to enhancing the medicinal value and nutritional components of *Eucommia ulmoides* leaves.

7.3 Research opportunities

There are still many aspects worthy of further in-depth research on the chloroplast genome of *Eucommia ulmoides* (Taberlet et al., 1991). Yu et al. (2015) believe that future research can pay more attention to expanding the genetic linkage map and refining the QTL analysis, which is helpful to identify more growth-related traits and the genetic mechanisms behind them. The molecular markers that have been discovered still need further research to see their specific roles in metabolic processes and plants' responses to external stress. With the help of new bioinformatics tools and machine learning methods, it is also possible to analyze large amounts of genomic data more effectively, helping to make more accurate predictions and discoveries. To truly exert the role of these molecular markers, collaboration among geneticists, conservation biologists and breeding experts is indispensable (Li et al., 2014b).

8 Concluding Remarks

The study of the chloroplast genome of *Eucommia ulmoides* has provided a lot of understanding of genetic structure and brought new opportunities for the development of molecular markers. The complete chloroplast genome of *Eucommia ulmoides* has been sequenced. It has a typical tetrad structure, with a total length of 163 586 bp and a GC content of 38.4%. Researchers identified 71 polymorphic chloroplast DNA fragments in comparative genomic analysis, among which 20 sites were selected to be used as potential markers in population genetics studies. Eight polymorphic chloroplast SSR markers (cpSSR) were also developed, further enriching the tools for genetic analysis. The kinship between *Eucommia ulmoides* and *Aucuba japonica* in Japan has also confirmed , which makes the understanding of its evolutionary background clearer.

Molecular markers developed from the chloroplast genome of *Eucommia ulmoides* have helped researchers understand the genetic diversity and population structure of the species. These markers can also be applied to breeding work to screen out plants with ideal traits, thereby enhancing the yield and quality of *Eucommia ulmoides* in both medicinal and industrial applications. The high-density genetic maps established through these markers and the growth-related QTLS identified indicate that they play an important role in understanding the genetic mechanisms of key phenotypic traits.

Future research should expand the genetic resources and genomic data of *Eucommia ulmoides* more. Researchers can comprehensively understand the genetic variation of *Eucommia ulmoides* by sequencing the chloroplast genomes from different regions, or combine the chloroplast data with the nuclear genome and mitochondrial genome to gain a more complete understanding of the genetic composition of *Eucommia ulmoides*. The molecular markers and candidate genes that have been identified should also continue to undergo functional research to clarify their roles in plant growth, development and response to environmental stress. Moreover, these molecular markers need to be truly applied in breeding to enhance the economic value and ecological adaptability of *Eucommia ulmoides*. By deeply leveraging the information provided by the chloroplast genome, scientists are expected to achieve greater breakthroughs in the protection, breeding and utilization of *Eucommia ulmoides*.

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Conflict of Interest Disclosure

The author affirms that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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