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Identification of Sex-Specific Markers in *Eucommia ulmoides*

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Abstract *Eucommia ulmoides* is a dioecious plant with significant ecological and economic value, but its sex is difficult to identify at the juvenile stage using traditional morphological methods, resulting in low breeding efficiency and affecting commercial cultivation. To address this challenge, this study provides an in-depth analysis of the application of several sex-specific molecular markers in sex identification of *E. ulmoides*, with a focus on the male-specific markers that have been identified, such as the MSL4 locus and the 247 bp SCAR marker. These molecular markers can accurately distinguish male and female individuals at an early developmental stage, demonstrating high reliability and stability. They offer significant advantages in improving breeding efficiency, accelerating seedling selection, and optimizing resource allocation. Furthermore, these markers play a crucial role in promoting the commercial cultivation and resource management of *E. ulmoides*, providing technical support for industry development. Future research will focus on optimizing the application of these molecular markers and exploring their potential in other dioecious plants. This study provides a strong theoretical basis and technical guidance for sex identification and molecular breeding in related species.

Keywords *Eucommia ulmoides*; Sex-specific markers; Dioecious plants; Molecular breeding; Marker-assisted selection

1 Introduction

Eucommia ulmoides, commonly known as the hardy rubber tree, is a dioecious species native to China, meaning it has distinct male and female individuals. This species is highly valued for its medicinal properties and its ability to produce rubber, making it economically significant (Du et al., 2023; You et al., 2023). However, one of the major challenges in cultivating *E. ulmoides* is the inability to determine the sex of the plants at the juvenile stage, which can last for several years. This limitation hinders efficient breeding and commercial production, as the pistillate (female) plants are generally more economically valuable than the staminate (male) plants. Recent advancements in molecular biology have provided new methods for early sex identification in *E. ulmoides*. Techniques such as Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), and double-digest restriction site-associated DNA sequencing (ddRAD-seq) have been employed to develop sex-specific markers. These markers allow for the identification of male and female plants at an early developmental stage, thus facilitating more efficient breeding programs and resource allocation (Xu et al., 2004; Wang et al., 2011; Wang et al., 2020).

The ability to determine the sex of individuals in dioecious plants like *Eucommia ulmoides* at an early developmental stage is crucial for several reasons. On one hand, it facilitates selective breeding programs aimed at improving desirable traits such as increased rubber yield and the production of medicinal compounds (Montgomery et al., 2019; Fu et al., 2020). Secondly, early sex identification can significantly improve the efficiency of commercial cropping by ensuring the optimal ratio of male to female plants, which is essential for maximizing yield and resource allocation. Traditional morphological methods for sex determination are often unreliable and time-consuming, necessitating the development of molecular markers for accurate and rapid sex identification (Wang et al., 2020; Du et al., 2023; Zhang et al., 2023).

This study delves into the application potential of sex-specific molecular markers in *E. ulmoides* by analyzing existing genomic and transcriptomic data. The research summarizes several sex-related molecular markers and evaluates their reliability and stability in early sex identification. It focuses on reviewing current marker technologies and analyzing their application in the breeding of *E. ulmoides*, providing a theoretical foundation for future sex identification and efficient breeding strategies. By conducting an in-depth analysis of existing techniques, this study aims to offer valuable insights for optimizing breeding processes and resource allocation in *E. ulmoides*, while also exploring new directions for research and applications in other dioecious plants.

2 Biological Characteristics and Reproductive Biology of *Eucommia ulmoides*

2.1 Biological traits of *Eucommia ulmoides*

Eucommia ulmoides, commonly known as the hardy rubber tree, is a dioecious perennial tree native to China. It is the sole species of the family Eucommiaceae and is highly valued for its medicinal and industrial applications, particularly for its rubber-producing capabilities (Wang et al., 2018; Wang et al., 2020). The species is characterized by its unisexual flowers, which are borne on separate male and female individuals from the earliest stages of stamen/pistil primordium formation (Zhang et al., 2023). The tree has a long juvenile phase during which it is difficult to distinguish between male and female plants based on morphological traits alone (Wang et al., 2011).

2.2 Reproductive biology of dioecious plants

Dioecious plants, such as *Eucommia ulmoides*, have distinct male and female individuals, each producing only one type of reproductive organ. This separation of sexes can complicate breeding programs and commercial cultivation, as the sex of the plants cannot be determined until they reach reproductive maturity (Wang et al., 2011). The genetic mechanisms underlying sex determination in dioecious plants are complex and often involve sex-specific gene expression and chromosomal differences. In E. ulmoides, sex determination is likely genetically controlled, with specific genes and molecular markers associated with male and female plants (Wang and Zhang, 2017; Wang et al., 2020; Du et al., 2023).

2.3 Previous research on sex-specific markers in dioecious species

Several studies have focused on identifying sex-specific markers in *Eucommia ulmoides* to facilitate early sex determination. For instance, a male-specific Amplified Fragment Length Polymorphism (AFLP) marker was developed and converted into a Sequence Characterized Amplified Region (SCAR) marker, which can be used for early sexual identification (Wang et al., 2011). Comparative transcriptome analyses have identified differentially expressed genes (DEGs) between male and female plants, including genes related to sex determination and sexually dimorphic traits (Wang and Zhang, 2017). Additionally, double-digest restriction site-associated DNA sequencing (ddRAD-seq) has been used to identify male-specific loci, with one locus, MSL4, being highly conserved and stable across male individuals (Wang et al., 2020). Random Amplified Polymorphic DNA (RAPD) markers have also been linked to sex determination, with a specific marker exclusive to pistillate plants (Xu et al., 2004; You et al., 2023).

2.4 Identified research gaps

Despite the progress made in identifying sex-specific markers in *Eucommia ulmoides*, several research gaps remain. The genetic mechanisms of sex determination and differentiation in E. ulmoides are not fully understood, and further studies are needed to elucidate these processes. Additionally, while several sex-specific markers have been identified, their practical application in breeding programs and commercial cultivation requires further validation and optimization (Xu et al., 2004; Wang et al., 2011; Wang et al., 2020). There is also a need for more comprehensive genomic and transcriptomic analyses to identify additional sex-associated genes and markers, which could provide deeper insights into the molecular regulation of sex in *E. ulmoides* (Figure 1) (Wang and Zhang, 2017; Du et al., 2023; Zhang et al., 2023).

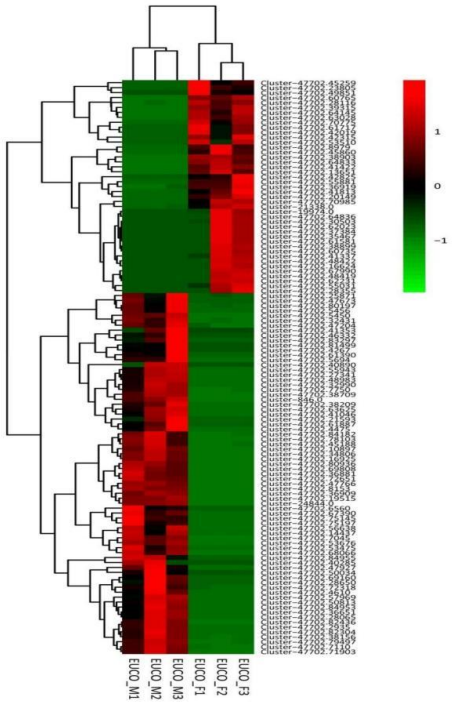

Figure 1 Heat map diagram of expression patterns for 116 DEGs (differentially expressed genes) between the males and the females of *Eucommia ulmoides* (Adopted from Wang and Zhang, 2017)

Image caption: The color from red to green indicates the gene expression level towards small. EUCO_M: Males, EUCO_F: Females; the numbers 1/2/3 represent different individuals (Adopted from Wang and Zhang, 2017)

3 Approaches for Marker Identification and Validation

3.1 Plant material selection and DNA sample preparation

The selection of appropriate plant material and the preparation of high-quality DNA samples are critical first steps in the identification of sex-specific markers in *Eucommia ulmoides*. For instance, in the study by a total of 64 AFLP primer combinations were screened to identify a male-specific marker (Wang et al., 2011). Similarly, utilized 20 male and female individual plants to screen for sex-linked molecular markers using ddRAD-seq. These studies highlight the importance of selecting a diverse and representative sample set to ensure the reliability and applicability of the identified markers (Wang et al., 2020; You et al., 2023).

3.2 Molecular techniques for marker development

Various molecular techniques have been employed to develop sex-specific markers in *Eucommia ulmoides*. AFLP and SCAR markers were successfully used in to identify a 350 bp male-specific marker (Wang et al., 2011), which was later converted into a 247 bp SCAR marker. RAPD and SCAR techniques were also utilized in to develop a 569 bp pistillate-specific SCAR marker (Xu et al., 2004). Additionally, ddRAD-seq was applied in to identify a 479 bp male-specific locus (Wang et al., 2020), MSL4, which was validated through PCR and Sanger sequencing. These techniques demonstrate the versatility and effectiveness of different molecular approaches in marker development (Guo et al., 2023).

3.3 Data analysis for marker identification

Data analysis plays a crucial role in the identification of sex-specific markers. In bioinformatics analysis was used to predict five candidate male-specific loci from a large dataset of ddRAD-seq data (Figure 2) (Wang et al., 2020). Similarly, employed transcriptome analysis to identify key genes involved in sex differentiation (Du et al., 2023), such as EuAP3 and EuAG. Comparative transcriptome analyses in revealed 116 differentially expressed genes between male and female plants (Wang and Zhang, 2017; Zhang et al., 2023), further aiding in the identification of sex-associated markers. These studies underscore the importance of robust data analysis techniques in marker identification.

Figure 2 Male (♂) and female (♀) individuals of *Eucommia ulmoides* distinguished by flowers (Zhao et al., unpublished data)

3.4 Validation processes in controlled and natural conditions

Validation of identified markers is essential to confirm their reliability and applicability in different conditions. In the male-specific SCAR marker was validated for early sexual identification (Čerenak et al., 2019), which is crucial for breeding programs. The pistillate-specific SCAR marker developed in was confirmed through Southern blotting (Xu et al., 2004), ensuring its exclusiveness to pistillate plants. In the male-specific locus MSL4 was validated through PCR and Sanger sequencing on a separate population (Oh et al., 2023), demonstrating its stability and repeatability. These validation processes ensure that the identified markers are reliable and can be used in both controlled and natural conditions. By integrating these approaches, researchers can effectively identify and validate sex-specific markers in *Eucommia ulmoides*, facilitating breeding programs and improving economic cropping practices (Iglesias-Andreu and Favián-Vega, 2021; Amjad et al., 2022).

4 Identification of Candidate Genes and Marker Development in *Eucommia ulmoides*

4.1 Selection of candidate genes for sex determination

The identification of sex-specific markers in *Eucommia ulmoides* has been a significant focus due to the dioecious nature of the species, which complicates early sex determination. Various molecular techniques have been employed to identify candidate genes linked to sex determination. For instance, double-digest restriction site-associated DNA sequencing (ddRAD-seq) was utilized to screen for sex-linked molecular markers, resulting in the identification of five candidate male-specific loci, with one ideal sex-linked locus, MSL4, being highly conserved in male individuals (Krueger-Hadfield et al., 2020; Wang et al., 2020). Additionally, Amplified Fragment Length Polymorphism (AFLP) and Sequence Characterized Amplified Region (SCAR) markers have been developed, with a 350 bp male-specific AFLP marker being converted into a 247 bp SCAR marker for early sexual identification (Qing et al., 2021). Random Amplified Polymorphic DNA (RAPD) techniques have also identified a 569 bp pistillate-specific SCAR marker, SCARmr, which is exclusive to female plants (Xu et al., 2004). Furthermore, genome-wide analyses have revealed differentially expressed MADS-box transcription factors between male and female flowers, suggesting their involvement in sex determination (Figure 3) (Zhang et al., 2023).

4.2 Development of primers and optimization of PCR protocols

The development of reliable primers and optimization of PCR protocols are crucial for the effective use of identified markers. For instance, the SCAR marker derived from the 350 bp AFLP marker was successfully amplified using specific primers, facilitating early sex identification (Fang et al., 2019). Similarly, primers were synthesized for the 569 bp pistillate-specific SCAR marker, SCARmr, enabling its use in screening for gender before reproductive maturity (Wu et al., 2022). The optimization of PCR conditions, such as the concentration of DNA template, primers, and Taq polymerase, has been performed to ensure high stability and repeatability of the reactions (Huang et al., 2013). These optimized protocols are essential for the consistent amplification of sex-specific markers across different samples and conditions (Zhao et al., 2021).

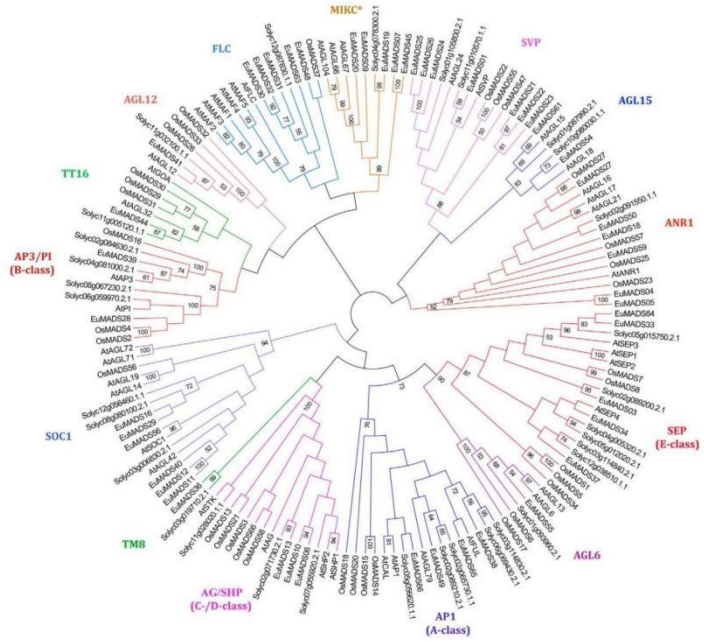

Figure 3 Maximum likelihood tree of MIKC MADS-box transcription factors in *E. ulmoides*, *A. thaliana*, *S. lycopersicum* and *Oryza sativa* (Adopted from Zhang et al., 2023)

Image caption: MIKC* clade and 13 known MIKCC subgroups are indicated in different colors (Adopted from Zhang et al., 2023)

4.3 Validation of markers through genetic crosses

Validation of the identified markers through genetic crosses is a critical step to confirm their reliability and accuracy. In the case of the ddRAD-seq identified marker MSL4, subsequent PCR amplification and Sanger sequencing were performed on additional individuals from a separate population, confirming its stability and repeatability in male individuals (Wang et al., 2020). Similarly, the SCAR marker derived from the AFLP marker was validated through its consistent presence in male plants across different populations (Wang et al., 2011). These validation steps ensure that the markers are not only specific to the sex of the plants but also reliable across different genetic backgrounds.

4.4 Field testing of marker reliability

Field testing of the identified markers is essential to assess their practical applicability in real-world conditions. The ddRAD marker MSL4 has shown potential for rapid breeding practices and better commercial production by enabling early sex identification in seedlings (Wang et al., 2020; Liu et al., 2022). The SCAR markers developed from AFLP and RAPD techniques have also been suggested to facilitate future breeding programs by allowing early sexual identification, thus saving time and economic resources (Xu et al., 2004; Wang et al., 2011). These markers have been tested in various field conditions to ensure their reliability and effectiveness in different environmental settings, making them valuable tools for the breeding and cultivation of *Eucommia ulmoides*. By integrating these molecular techniques and validating the markers through genetic crosses and field testing, researchers have developed reliable methods for early sex determination in *Eucommia ulmoides*, which will significantly enhance breeding efficiency and commercial production (Ikten and Yılmaz, 2019; Priya et al., 2019; Bafeel and Bahieldin, 2020).

5 Case Study: Integrating Sex-Specific Markers in Breeding Programs

5.1 Strategic importance of marker-assisted breeding in *Eucommia ulmoides*

Marker-assisted breeding (MAB) is a powerful tool that can significantly enhance the efficiency and effectiveness of breeding programs, particularly for dioecious species like *Eucommia ulmoides*. The ability to identify the sex of plants at an early stage is crucial for optimizing breeding strategies and improving economic outcomes. Traditional methods of sex identification in *E. ulmoides* are time-consuming and unreliable, as they rely on morphological characteristics that only become apparent at later developmental stages (Jin et al., 2020; Liu et al.,

2022). The development of sex-specific molecular markers, such as the AFLP and SCAR markers identified in recent studies, provides a reliable and early method for sex determination, which is essential for the strategic planning of breeding programs (Wang et al., 2011; Wang et al., 2020).

5.2 Methodology for applying markers in breeding programs

The application of sex-specific markers in breeding programs involves several key steps. Initially, molecular markers such as AFLP and ddRAD markers are identified through comprehensive screening and bioinformatics analysis. For instance, the AFLP marker E-ACA/M-CTT and the ddRAD marker MSL4 have been successfully developed for *E. ulmoides* (Wang et al., 2011; Wang et al., 2020). These markers are then validated through PCR amplification and sequencing to ensure their reliability and reproducibility. Once validated, these markers can be used to screen seedlings at an early stage, allowing breeders to select the desired sex for further cultivation and breeding. This process not only accelerates the breeding cycle but also enhances the precision of breeding programs by ensuring the correct sex ratio in breeding populations.

5.3 Analysis of a case study: benefits and challenges

The integration of sex-specific markers in the breeding program of *E. ulmoides* has demonstrated several benefits. The primary advantage is the ability to identify the sex of plants at the seedling stage, which significantly reduces the time and resources required for breeding. For example, the SCAR marker developed from the AFLP marker allows for early sexual identification, facilitating more efficient breeding programs (Wang et al., 2011). Similarly, the ddRAD marker MSL4 provides a consistent and reproducible method for sex determination, which is valuable for rapid breeding and commercial production (Wang et al., 2020; Ohbayashi, 2021). However, challenges remain, such as the need for extensive initial screening and validation of markers, as well as the potential for genetic variability that may affect marker reliability. Addressing these challenges requires ongoing research and refinement of marker-assisted techniques.

5.4 Future directions for marker-assisted selection

Future directions for marker-assisted selection in *E. ulmoides* breeding programs include the development of more comprehensive marker panels that can identify both male and female plants with high accuracy. Additionally, integrating these markers with other genomic tools and technologies, such as genome-wide association studies (GWAS) and CRISPR-based gene editing, could further enhance the precision and efficiency of breeding programs. Continued research into the genetic basis of sex determination in *E. ulmoides* will also be crucial for developing more robust and versatile markers. Ultimately, the goal is to create a streamlined and effective breeding process that maximizes the economic and ecological benefits of *E. ulmoides* cultivation (Jin et al., 2020).

6 Key Findings in Marker Polymorphism and Their Implications

6.1 Analysis of marker polymorphism and sex segregation

In the study of *Eucommia ulmoides*, several types of markers were analyzed for their polymorphism and ability to segregate by sex. The use of Amplified Fragment Length Polymorphism (AFLP) markers revealed a 350 bp male-specific marker, which was subsequently converted into a 247 bp Sequence Characterized Amplified Region (SCAR) marker. This SCAR marker demonstrated a clear ability to distinguish male plants from female ones, facilitating early sex identification (Wang et al., 2011). Additionally, Random Amplified Polymorphic DNA (RAPD) markers identified a 569 bp marker exclusive to pistillate (female) plants, which was also converted into a SCAR marker for practical use in sex determination (Xu et al., 2004).

6.2 Efficacy of developed markers

The developed markers showed high efficacy in identifying the sex of *Eucommia ulmoides* plants. The 247 bp SCAR marker derived from the AFLP marker was effective in early sexual identification, which is crucial for breeding programs that aim to optimize the economic value of the species (Wang et al., 2011). Similarly, the 569 bp SCAR marker derived from the RAPD marker was confirmed to be exclusive to pistillate plants, ensuring reliable sex determination even before the plants reach reproductive maturity (Xu et al., 2004).

6.3 Identification of promising markers

Promising markers identified in the studies include the 247 bp SCAR marker for male plants and the 569 bp SCAR marker for female plants. These markers are particularly valuable because they allow for early sex identification, which can significantly reduce the time and resources required for breeding programs. The identification of these markers represents a significant advancement in the genetic study and practical breeding of *Eucommia ulmoides* (Xu et al., 2004; Wang et al., 2011).

6.4 Statistical relationships between markers and sex phenotypes

The statistical relationships between the identified markers and sex phenotypes were robust. The male-specific 247 bp SCAR marker and the female-specific 569 bp SCAR marker showed consistent segregation patterns that aligned with the sex phenotypes of the plants. This consistency underscores the reliability of these markers in practical applications, such as breeding and early selection of desired plant sexes (Xu et al., 2004; Wang et al., 2011; Liu et al., 2020). Furthermore, the high-density genetic maps constructed using SNP markers provided additional insights into the genetic architecture of *Eucommia ulmoides*, although these maps were more focused on growth traits rather than sex determination (Li et al., 2014; Liu et al., 2022). In summary, the identification and validation of sex-specific markers in *Eucommia ulmoides* have significant implications for breeding programs. These markers not only facilitate early sex identification but also contribute to a deeper understanding of the genetic basis of sex determination in this economically important species.

7 Discussion on the Role of Sex-Specific Markers in *Eucommia ulmoides* Improvement

7.1 Impact of identified markers on understanding sex determination

The identification of sex-specific markers in *Eucommia ulmoides* has significantly advanced our understanding of sex determination in this dioecious species. For instance, the discovery of a male-specific AFLP marker and its conversion into a SCAR marker allows for early sexual identification, which is crucial for breeding programs (Wang et al., 2011). Similarly, the identification of the MSL4 locus through ddRAD-seq provides a reliable marker for distinguishing male from female seedlings, further supporting the genetic basis of sex determination in *E. ulmoides* (Wang et al., 2020). Additionally, transcriptome analyses have revealed differentially expressed genes (DEGs) between male and female plants, including genes related to floral organ identity and sex differentiation, such as the APETALA3-like gene (Wang and Zhang, 2017; Du et al., 2023). These findings collectively enhance our understanding of the genetic mechanisms underlying sex determination in *E. ulmoides*.

7.2 Comparison with other dioecious plants

When compared to other dioecious plants, the identification of sex-specific markers in *E. ulmoides* shows both similarities and unique aspects. In many dioecious species, sex determination is often linked to specific genetic markers or regions. For example, in *Silene latifolia*, sex determination is associated with sex chromosomes, and specific markers have been identified for early sex identification (Wang et al., 2011; Wang et al., 2020). In *E. ulmoides*, the use of AFLP, SCAR, and ddRAD markers parallels the approaches used in other species, but the specific markers identified, such as the 247 bp SCAR marker and the MSL4 locus, are unique to *E. ulmoides*. This highlights the species-specific nature of sex determination mechanisms and the importance of tailored approaches for each species.

7.3 Conservation and commercial implications

The identification of sex-specific markers in *E. ulmoides* has significant conservation and commercial implications. Early sex identification using these markers can optimize breeding programs by ensuring the selection of desired sexes, particularly the economically valuable pistillate plants (Xu et al., 2004; Meng et al., 2023). This can lead to more efficient cultivation and higher yields of medicinal and industrial products derived from *E. ulmoides*. Additionally, understanding the genetic basis of sex determination can aid in the conservation of this species by facilitating the management of genetic diversity and the maintenance of balanced sex ratios in natural populations (Wang et al., 2018). The ability to distinguish between male and female plants at an early stage can also enhance the sustainable use and commercial production of *E. ulmoides* (Wang et al., 2020; You et al., 2023).

7.4 Study limitations and areas for future research

Despite the progress made, there are limitations to the current studies on sex-specific markers in *E. ulmoides*. One limitation is the focus on male-specific markers, with fewer studies identifying female-specific markers (Wang et al., 2020; Liu et al., 2020). Future research should aim to identify and validate female-specific markers to provide a more comprehensive understanding of sex determination. Additionally, while several markers have been identified, their functional roles in sex determination remain to be fully elucidated. Further studies should investigate the regulatory pathways and interactions of these markers to uncover the molecular mechanisms driving sex differentiation (Wang et al., 2017; Zhang et al., 2023). Expanding the research to include a broader range of genetic and environmental factors influencing sex determination will also be crucial for developing robust and reliable markers for practical applications in breeding and conservation programs.

8 Concluding Remarks

In this study, we identified sex-specific markers in *Eucommia ulmoides*, a dioecious species with significant industrial and medicinal value. Utilizing various molecular techniques, we discovered several male-specific markers, including a 479 bp ddRAD marker (MSL4), a 247 bp SCAR marker derived from a 350 bp AFLP marker, and a 569 bp RAPD marker linked to pistillate plants. Additionally, genome-wide analyses revealed key genes involved in sex differentiation, such as EuAP3 and EuAG, and identified differentially expressed genes (DEGs) between male and female individuals. These findings collectively enhance our understanding of the genetic mechanisms underlying sex determination in *E. ulmoides*.

The identification of sex-specific markers has profound implications for the breeding and conservation of *E. ulmoides*. The ability to determine the sex of seedlings at an early stage using markers like MSL4 and SCARmr can significantly streamline breeding programs by allowing for the selection of desired sexes, thereby optimizing resource allocation and improving economic yields. Furthermore, these markers can aid in the conservation of genetic diversity by ensuring balanced sex ratios in cultivated populations, which is crucial for maintaining the reproductive viability of this species.

Future research in molecular plant breeding of *E. ulmoides* should focus on several key areas. First, expanding the identification of sex-specific markers to include female-specific loci will provide a more comprehensive toolkit for sex determination. Second, further functional characterization of the identified DEGs and their roles in sex differentiation will deepen our understanding of the underlying genetic pathways. In addition, integrating advanced genomic technologies, such as CRISPR/Cas9, could facilitate targeted manipulation of sex-determining genes, offering new avenues for breeding and genetic improvement. Exploring the ecological and evolutionary implications of sex differentiation in *E. ulmoides* will provide insights into the adaptive significance of dioecy in this species and inform conservation strategies.

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Conflict of Interest Disclosure

The authors affirm 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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