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ARTICLE

Evaluation, Validation, and Application of Sex-Specific Molecular Marker in Kiwifruit (*Actinidia* spp.)

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ABSTRACT: The genus *Actinidia* is primarily functionally dioecious, and early sex identification plays a crucial role in improving breeding efficiency and reducing production costs. In this study, the accuracy of three sex-linked molecular markers (*SyGI* [*Shy Girl*], *FrBy* [*Friendly Boy*], and *SmY1*) in sex identification was evaluated in various *Actinidia* species. The selected marker products were subsequently cloned and sequenced in six wild *Actinidia* species. Ninety-six wild *A. chinensis chinensis* accessions and 74 *A. chinensis deliciosa* accessions, most of which were wild, with only one cultivated, were used for comprehensive primer validation. Thirty-three juvenile *A. chinensis chinensis* hybrid seedlings were used for practical application tests. The results showed that the marker *SyGI* accurately identified the sex of 20 samples from six *Actinidia* species and 96 *A. chinensis chinensis* accessions with 100% reliability. For *Actinidia chinensis deliciosa*, the identification accuracy reached 98.65%. Sequence analysis revealed that *SyGI* shared the highest similarity with the male-specific genomic region. Furthermore, *SyGI* achieved 100% accuracy in identifying the sex of 33 juvenile *A. chinensis chinensis* individuals. The findings confirm that the *SyGI* marker possesses high accuracy, strong specificity, and broad applicability, making it a valuable tool for kiwifruit breeding programs. The cloned sequences from wild *Actinidia* species also provide important references for future research on the mechanisms of sexual evolution and determination.

KEYWORDS: *Actinidia*; dioecious; sex identification; *SyGI*; accuracy; specificity; applicability

1 Introduction

Kiwifruit (*Actinidia* Lindl.) is a perennial, dioecious, and economically important fruit crop native to China [1]. Often referred to as the "world's precious fruit" and the "king of fruits", kiwifruit is highly valued for its exceptional nutritional content, health benefits, and medicinal properties [2,3]. As a major global fruit crop, its market demand continues to rise steadily alongside the expansion of the kiwifruit industry.

Species within the *Actinidia* genus are primarily functionally dioecious, bearing Type I unisexual flowers. In these flowers, males possess vestigial pistils, while females have abortive stamens, resulting in the presence of only one functional reproductive organ per flower [4]. Based on the development of unisexual flowers, Mayer and Charlesworth (1991) classified dioecious plants with Type I unisexual flowers as showing cryptic dioecy [5].

Kiwifruit is a perennial, cryptically dioecious woody plant, and distinguishing between male and female plants at the seedling stage is not possible through morphology. Moreover, the juvenile phase lasts more than four years, with male individuals often comprising half or more of the progeny [6]. In breeding and improvement programs, growing seedlings to maturity involves considerable time and financial investment, especially when male plants must later be removed. Therefore, developing and applying accurate methods for early sex identification is essential for improving breeding efficiency and minimizing production costs in kiwifruit cultivation.

Extensive research has been dedicated to developing sex identification techniques in kiwifruit, employing approaches such as morphological characterization, physiological and biochemical analyses, and molecular biology methods [7–9]. Among these, DNA-based molecular markers have become the predominant tool due to their speed and reliable accuracy. Molecular markers, such as SSR, ISSR, RAPD, and sex-linked gene loci, have frequently been used for early sex identification in dioecious plants. For example, in persimmons, the male-linked gene *OGI*, located on the Y chromosome, plays a crucial role in sex determination and has been used for early sex identification of persimmon plants [10,11]. In kiwifruit, a variety of molecular markers have been developed and applied for sex identification, including RAPD markers (SmY, SmX), SCAR markers (SmY1, SmX), SSR markers (A001, A002, A003), etc. [12–14]. However, most of these identified molecular markers are polymorphic, and their linkage to sex remains inconclusive. In recent years, the practical performance of some molecular markers has proven inconsistent. For example, the male-linked RAPD marker S1032-850 has shown poor reliability during subsequent validation [15]. Among these markers, the SCAR marker SmY1 has demonstrated high accuracy for sex identification in *A. chinensis chinensis* [15,16]; however, its effectiveness in other *Actinidia* species remains uncertain. SmY1 is a marker of male sex in kiwifruit. It was originally developed as a RAPD marker by Harvey et al., and was later converted into a more stable SCAR marker by Gill et al. [12,13]. The present study investigated the use of SmY1 for identification in different *Actinidia* species to explore its universal applicability.

Understanding the mechanisms of sex determination is essential for guiding the development of reliable molecular markers in dioecious plants. Key areas of research include the study of sex chromosomes, sex-determining genes, sex-determination regions, and their associated regulatory elements. Research into plant sex determination is relatively recent and marked by considerable variability and evolutionary instability. In most plants, sex chromosomes remain at early evolutionary stages, retaining high levels of sequence homology [17]. The prevailing hypothesis posits that sex chromosomes originate from ancestral autosomes [18]. In the early stages of this transition, sex chromosomes are morphologically indistinguishable from autosomes. Over time, they undergo structural and functional differentiation, eventually forming heteromorphic sex chromosomes. This evolutionary process involves divergence between X (or Z) and Y (or W) chromosomes, as well as between sex chromosomes and autosomes [19].

In kiwifruit, studies have shown that the X and Y chromosomes in *A. chinensis chinensis* are similar in size and structure, aligning with Stage 2 of the six-phase model of sex chromosome evolution proposed by Ming et al. [20]. The subtelomeric region of the Y chromosome contains sex-determining loci that do not undergo recombination with the corresponding X chromosome—an established feature of sex-determining loci [21]. Additionally, a small male-specific region has been identified on the proto-Y chromosome, accompanied by the appearance of exclusively male or female plants. This pattern of sexual differentiation is comparable to that observed in asparagus (*Asparagus officinalis*), suggesting that *Actinidia* sex chromosomes are still in an early evolutionary phase.

Regarding genetic models of sex determination, Charlesworth B and Charlesworth D [22] proposed the two-gene mutation hypothesis, which suggests that at least two mutations are needed to develop a

functional Y chromosome: a dominant mutation that suppresses female development (SuF), and a recessive mutation causing male sterility [23,24]. Kiwifruit employs an XY sex-determination system consistent with this two-gene sex-determination model [25].

In research on sex-determining regions (SDRs) and sex-determining genes, the sex-specific loci have been identified in several dioecious species, including white campion (*Silene latifolia*) [26], papaya (*Carica papaya*) [27], garden asparagus (*Asparagus officinalis*) [28], poplar (*Populus* spp.) [29,30], and hemp (*Cannabis sativa*) [31]. Advances in kiwifruit genomics have recently led to the identification of two key sex-determining genes: *SyGI* (*Shy Girl*), which suppresses female development, and *FrBy* (*Friendly Boy*), a masculinizing factor. These genes act together to regulate sexual dimorphism in *Actinidia* [25,32]. Recently, several reports have described the use of the *SyGI* and *FrBy* genes for sex identification in kiwifruit [33–35]. The design of the *SyGI* primers employed in these studies was based on the coding DNA sequence (CDS) of the *SyGI* gene. However, *SyGI* has a homolog, *Achn384741*, located on an autosome, with a sequence similarity of 99% [36], resulting in strong resemblance between the CDS regions of *SyGI* and *Achn384741*. Fang et al. [35] observed poor amplification efficiencies for primers designed for the exonic region of *SyGI*, suggesting the presence of additional homologs of the *SyGI* sequence within the kiwifruit genome. The present study selected a pair of specific primers from a recent study [36] that could avoid interference from homologous sequences during amplification and exclusively amplified the non-coding region sequences upstream of the CDS region of the *SyGI* gene.

Kiwifruit have significant genetic diversity, complex ploidy levels, and high heterozygosity [37]. Wild kiwifruit show marked genetic diversity and variability, thereby representing a crucial breeding resource [38–40]. While De Mori et al. have evaluated sex-specific molecular markers, including *SyGI* and *FrBy*, for several *Actinidia* species, there is limited information on the validation of the *SyGI* and *FrBy* markers for large-scale sex identification in wild kiwifruit [33]. Therefore, it is essential to investigate the applicability of these two sex-determining genes in various wild kiwifruit populations.

This study focused on the sex-linked SmY1 marker, as well as the recently reported *SyGI* and *FrBy* molecular markers. While SmY1 has been shown to be highly accurate in several studies, its universal applicability is unknown. These markers were investigated in different wild kiwifruit species to evaluate their suitability. Male and female plants from six *Actinidia* species were used to screen for highly conserved and accurate male-specific molecular marker. The identified marker products were cloned and analyzed through sequence alignment. Their reliability and broad applicability were further evaluated through large-scale validation in two important wild species, *A. chinensis chinensis* and *A. chinensis deliciosa*. The primary objective was to identify and validate robust sex-specific molecular marker with high universality and accuracy across *Actinidia* species. The selected marker aims to enable rapid and reliable sex identification in kiwifruit, thereby improving breeding efficiency and reducing associated time and costs. Additionally, comparative cloning in various wild *Actinidia* species will provide a molecular basis for further investigation into mechanisms of sex determination within the genus.

2 Materials and Methods

2.1 Plant Materials

Female and male individuals from six *Actinidia* species (*A. chinensis chinensis*, *A. chinensis deliciosa*, *A. eriantha*, *A. arguta*, *A. hemsleyana* and *A. callosa*) were selected for initial primer screening (Table 1). For the molecular cloning of sex-specific marker products, leaf samples were collected from male individuals of six wild *Actinidia* species (*A. chinensis chinensis*, *A. chinensis deliciosa*, *A. eriantha*, *A. arguta*, *A. hemsleyana*, and *A. callosa*) (Table 2). For further validation and application of the molecular marker, leaf samples

were collected from two groups: (1) 96 mature wild *A. chinensis chinensis* and 74 mature *A. chinensis deliciosa* plants, including both sexes, with geographical distributions shown in Table S1; (2) 33 two-year-old juvenile hybrid *A. chinensis chinensis* seedlings of unknown sex (Table S2). Young leaves from all specimens were immediately flash-frozen in liquid nitrogen and stored at −80°C for subsequent molecular analysis. Thirty-three of the seedlings shown in Table S2 were used for field verification when they were three years old. All plant materials were sourced from the kiwifruit breeding base of the Sichuan Provincial Academy of Natural Resource Sciences, located in Sichuan Province, China.

Table 1: Plant materials used for sex-specific molecular marker screening in *Actinidia*.

Number	Sample Name	Species	Sex	Source of Materials
1	JXQS-EA-04238	*A. eriantha*	female	Wild material; Jiangxi Province, China
2	JXQS-EA-04243	*A. eriantha*	male	Wild material; Jiangxi Province, China
3	JXfujun-04212-OP01	*A. arguta*	female	Wild material; Jiangxi Province, China
4	HNNX-AE-03098-OP02	*A. arguta*	female	Wild material; Henan Province, China
5	HNNX-AE-03098-OP07	*A. arguta*	male	Wild material; Henan Province, China
6	JXfujun-04212-OP04	*A. hemsleyana*	male	Wild material; Jiangxi Province, China
7	JXfujun-04212-OP10	*A. hemsleyana*	female	Wild material; Jiangxi Province, China
8	JXFX-CF-04146	*A. callosa*	female	Wild material; Jiangxi Province, China
9	TJ-CF-10	*A. callosa*	male	Wild material; Tongjiang County, Sichuan Province, China
10	FD-DA-2	*A. chinensis deliciosa*	female	Wild material; Fengdu County, Chongqing City, China
11	WS-DA-20	*A. chinensis deliciosa*	male	Wild material; Wushan mountains, Chongqing City, China
12	TJ-DD-19	*A. chinensis deliciosa*	female	Wild material; Tongjiang County, Sichuan Province, China
13	Emei03103	*A. chinensis deliciosa*	female	Wild material; Mount Emei, Sichuan Province, China
14	ChengMi	*A. chinensis deliciosa*	female	Cultivar
15	WS-DA-58	*A. chinensis deliciosa*	female	Wild material; Wushan mountains, Chongqing City, China
16	WS-DA-17	*A. chinensis deliciosa*	male	Wild material; Wushan mountains, Chongqing City, China
17	PZ-DA-7	*A. chinensis deliciosa*	male	Wild material; Pengzhou City, Sichuan Province, China
18	TJ-DA-15♂	*A. chinensis deliciosa*	female	Wild material; Tongjiang County, Sichuan Province, China
19	HNNX-CK-03095	*A. chinensis chinensis*	male	Wild material; Henan Province, China
20	HNNX-CK-03086	*A. chinensis chinensis*	female	Wild material; Henan Province, China

Table 2: Plant materials used for molecular cloning of sex-specific marker products.

Sample Name	Species	Sex	Source of Materials
04266	*A. chinensis chinensis*	male	Wild material; Jiangxi Province, China
WS-DA-54	*A. chinensis deliciosa*	male	Wild material; Wushan mountains, Chongqing City, China
JXQS-EA-04243	*A. eriantha*	male	Wild material; Jiangxi Province, China
HNNX-AE-03098-OP07	*A. arguta*	male	Wild material; Henan Province, China
JXfujun-04212-OP04	*A. hemsleyana*	male	Wild material; Jiangxi Province, China
TJ-CF-10	*A. callosa*	male	Wild material; Tongjiang County, Sichuan Province, China

2.2 Methods

2.2.1 DNA Extraction

Genomic DNA was extracted from kiwifruit leaf tissue using the Plant Genomic DNA Extraction Kit (Product No. DN32; BIOFIT BIOTECHNOLOGIES Co., Ltd., China), following the manufacturer's protocol. DNA purity and concentration were measured using a microvolume spectrophotometer. Extracted DNA samples were stored at −80°C for later use.

2.2.2 Selection of Sex-Linked Molecular Markers

Sex-linked molecular markers previously reported in *Actinidia* spp. were selected based on published studies. These markers are listed in Table 3.

Table 3: Primers for sex-linked molecular markers used in kiwifruit.

Primers	Forward Primer Sequences	Reverse Primer Sequences	Product Size (bp)	Source References
SyGI	CAAATACAACATCTGAATTTGTTTCTTA	TCTAAATGCCATGATCAATTCTCACTAATTATCAC	308	Akagi et al., 2018 [36]
SmY1	TCGCAATTCGTTAGGGATGATGCG	CATAATCAACCATCCATAAAAACCAT	770	Gill et al., 1998 [13]
FRBY	ATGGCAAAGTGGTTCTCTCTCCAT	TTAACAAACCCAAACCCTAAAATAAAC	723	Akagi et al., 2019 [25]

2.2.3 PCR Amplification

Polymerase chain reaction (PCR) was conducted in a 50 μL reaction volume to amplify target sequences (SmY1, *SyGI*, and *FRBY*). Each reaction contained 1 μL of template DNA, 25 μL of 2× EasyTaq® PCR SuperMix, 1 μL of each primer (10 μM), and 22 μL nuclease-free water. The thermal cycling protocol was as follows: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at the primer-specific temperature for 30 s (*SyGI*: 58.5°C for 30 s; SmY1 and *FRBY*: 58°C for 30 s), and extension at 72°C for 20–60 s (*SyGI*: 72°C for 30 s; SmY1 and *FRBY*: 72°C for 45 s); followed by a final extension at 72°C for 10 min.

2.2.4 Molecular Cloning and Sequence Analysis of Sex-Specific Marker

PCR amplification was conducted using genomic DNA from male individuals of six *Actinidia* species (*A. chinensis chinensis*, *A. chinensis deliciosa*, *A. eriantha*, *A. arguta*, *A. hemsleyana*, and *A. callosa*) with primers specific to the *SyGI* marker. The reaction conditions and PCR system were identical to those described in Section 2.2.3. Amplification products were separated via electrophoresis on 1% agarose gels, and bands corresponding to the expected size were excised and purified.

Purified PCR fragments were ligated into the pClone007 Versatile Simple Vector using a commercial cloning kit and subsequently transformed into competent Escherichia coli cells. Positive colonies were selected from each transformation and verified by colony PCR. A total of 12 confirmed positive clones were submitted for sequencing to Tsingke Biotechnology Co., Ltd. (China).

The resulting sequences were aligned using DNAMAN software (DNAMAN Application 6.0.3.99) and analyzed using BLAST (Basic Local Alignment Search Tool) against the NCBI nucleotide database to assess sequence specificity and homology.

By aligning the cloned sequences with their corresponding reference sequences which have the highest similarity (GenBank: LC260493.1, LC260495.1, LC260498.1) [36], the cloned sequences were identified as the 308-bp (*A. chinensis chinensis*, *A. chinensis deliciosa*, *A. arguta*, and *A. hemsleyana*) or 307-bp (*A. eriantha and A. callosa*) upstream sequence of the start codon. As an extension of the core findings of this study, we conducted a comparative analysis of cis-acting elements within the candidate proximal regulatory sequence of the *SyGI* gene cloned from six wild *Actinidia* species. Additionally, we analyzed the impact of sequence variations on these cis-acting elements. This extension study aimed to preliminarily explore: (1) whether the sequence variations across different species lead to the generation or disruption of cis-acting elements; (2) how these variations influence the distribution and composition of conserved cis-acting elements. Through the above comparative analysis, specific candidate sites and hypotheses can be provided to understand the natural variation in *SyGI* expression regulation. The cis-acting elements in this region were identified and analyzed using the PlantCARE website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) [41].

3 Results and Analysis

3.1 Primer Evaluation and Selection

PCR amplification results using *SyGI*, SmY1, and *FRBY* primers across six *Actinidia* species (*A. chinensis chinensis*, *A. chinensis deliciosa*, *A. eriantha*, *A. arguta*, *A. hemsleyana*, and *A. callosa*) are shown in Fig. 1. Notably, the *SyGI* primers consistently amplified a single, clear band in all male samples, with no amplification observed in female plants. This marker accurately distinguished male and female individuals across all six species, achieving 100% identification accuracy.

In contrast, the SmY1 primers yielded target bands in only three male *A. chinensis deliciosa* and one male *A. chinensis chinensis* accessions, indicating limited applicability and lower accuracy. The *FRBY* primers produced multiple nonspecific bands in the female samples, undermining their specificity and reliability for sex identification. Based on these comparative results, *SyGI* was identified as the most specific and accurate molecular marker and was selected for use in subsequent analyses.

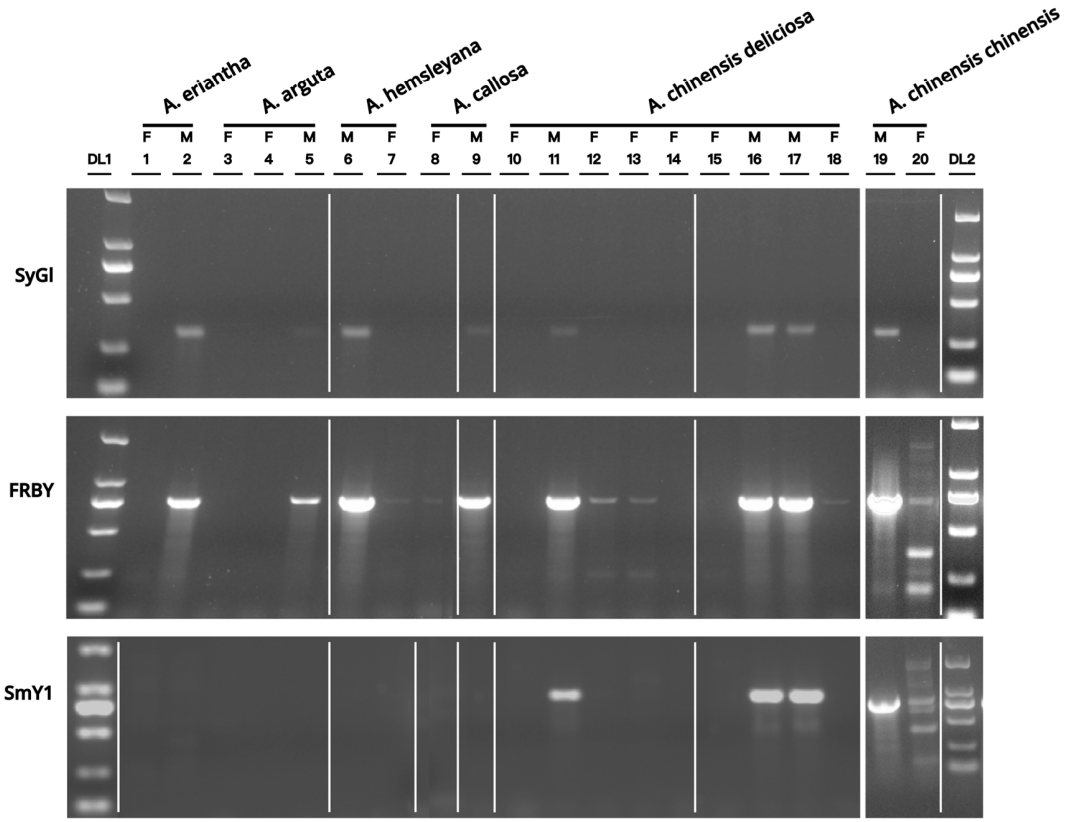

Figure 1: Electrophoresis of PCR products from sex-linked molecular markers. F: female sample. M: male sample. DL1-2: DL2000 DNA marker. 1–20: Plant materials used for the screening of sex-specific moleculars in *Actinidia* (details are shown in Table 1). The lanes were rearranged from the same gel for ease of comparison. White lines indicate the borders where lanes were rearranged.

3.2 Comparative Analysis of Cloned SyGI Sequences

Cloning and sequencing of the *SyGI* marker products revealed a 307-bp fragment in *A. eriantha* and *A. callosa*, while a 308-bp sequence was obtained from *A. chinensis chinensis*, *A. chinensis deliciosa*, *A. arguta*, and *A. hemsleyana* (Fig. 2). Sequence alignment demonstrated a high level of similarity (98.43%) among the *SyGI* sequences from male individuals across all six wild species, with few base differences.

BLAST analysis against the NCBI nucleotide database showed that the *SyGI* sequences from *A. chinensis chinensis* and *A. chinensis deliciosa* exhibited 100% identity with reference sequences LC260493.1, LC218546.1, and LC260494.1 (Fig. 3A). Sequences from *A. callosa* and *A. eriantha* also displayed high similarity to these references, with identity scores of 99.19% and 99.51%, respectively, with a few base differences between them (Fig. 3B,C).

Figure 2: Sequence comparison of cloned *SyGI* fragments from six *Actinidia* species.

Figure 3: BLAST alignment of *SyGI* sequences from six *Actinidia* species. (**A**) BLAST alignment of *SyGI* sequences from *A. chinensis chinensis* and *A. chinensis deliciosa*; (**B**) BLAST alignment of *SyGI* sequences from *A. callosa*; (**C**) BLAST alignment of *SyGI* sequences from *A. eriantha*; (**D**) BLAST alignment of *SyGI* sequences from *A. arguta*; (**E**) BLAST alignment of *SyGI* sequences from *A. hemsleyana.*

Notably, the reference sequences (LC260493.1, LC218546.1, and LC260494.1) are known male-specific Y-linked sequences from *Actinidia* [36].

Additionally, the wild *A. arguta* amplicon obtained in this study showed complete sequence identity (100%) with the *A. arguta SyGI* reference sequence (LC260498.1; Fig. 3D). The *SyGI* amplicons from *A. hemsleyana* displayed 99.68% similarity to the *A. rufa SyGI* reference sequence, with one base difference between them (LC260495.1; Fig. 3E).

3.3 Validation of Sex-Specific Molecular Marker in Kiwifruit

3.3.1 Validation of Mature Actinidia Plants

PCR amplification using *SyGI*-specific primers was carried out on 96 *A. chinensis chinensis* and 74 *A. chinensis deliciosa* accessions to assess marker performance in mature plants (most of the plants were wild, with only one cultivated; details are shown in Table S1). As shown in Fig. 4, the *SyGI* marker successfully identified the sex of all 96 *A. chinensis chinensis* samples with 100% accuracy. Specific target bands were amplified in all male samples, while no product was detected in female individuals (Fig. 4).

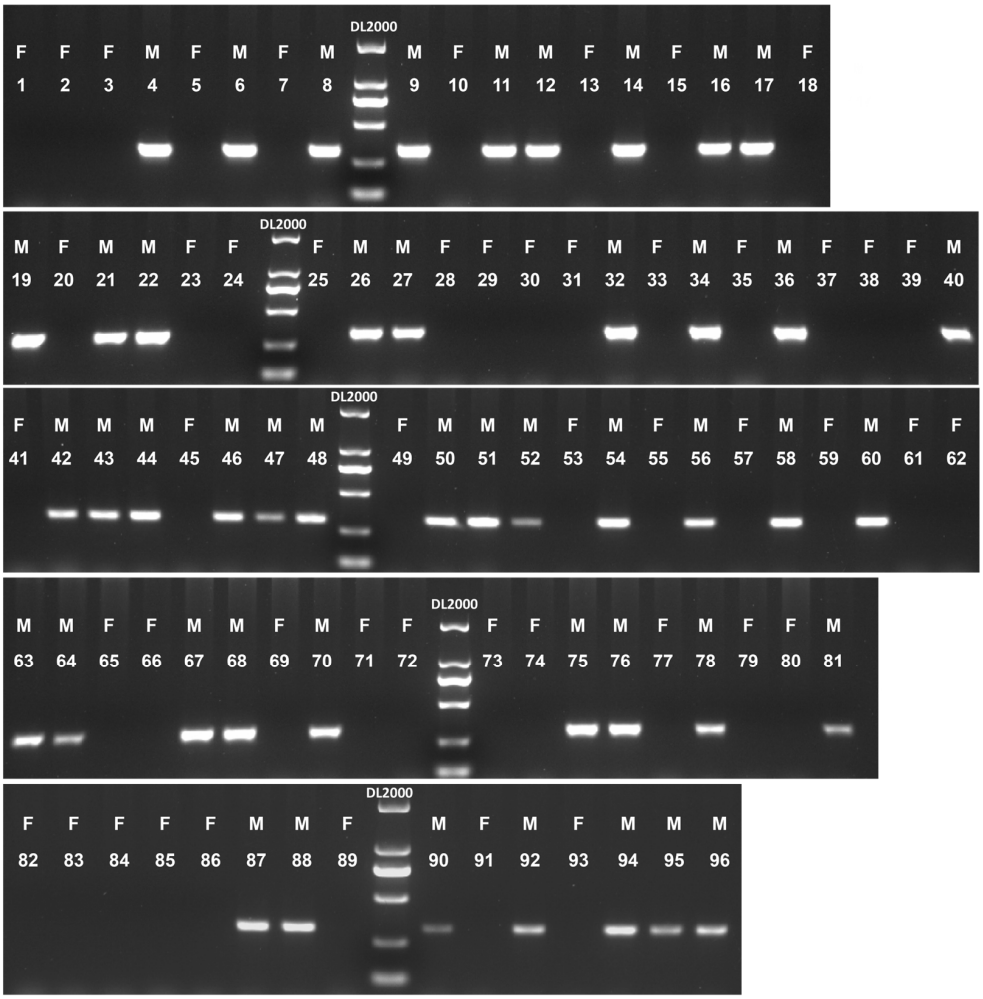

Figure 4: Sex identification of 96 mature *A. chinensis chinensis* accessions using the *SyGI* marker. F: female accession. M: male accession. DL2000: DL2000 DNA marker. 1–96: 96 *A. chinensis chinensis* samples used for validation (details are shown in Table S1).

Similarly, in *A. chinensis deliciosa* (n = 74, accessions #97–170), *SyGI* accurately determined the sex in 73 out of 74 samples. One accession (#130) failed to yield a detectable amplification product. The resulting sex identification accuracy was 98.65% (Fig. 5).

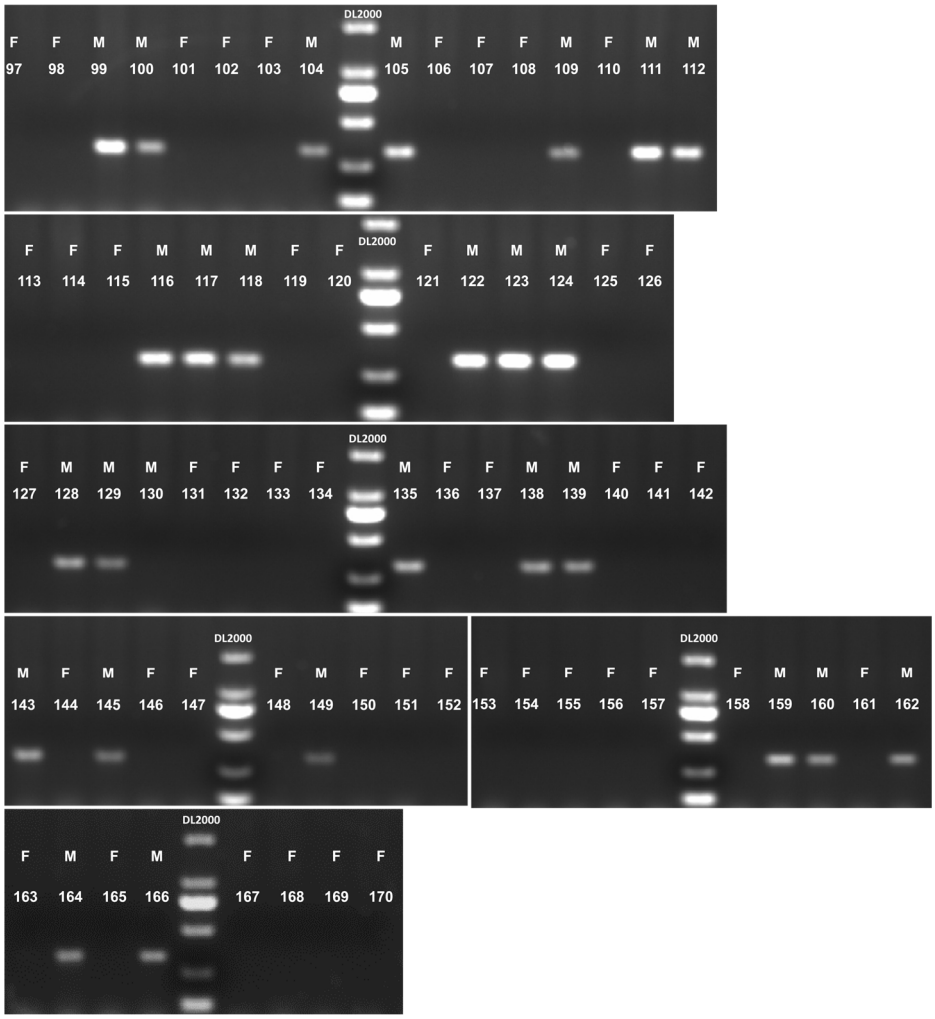

Figure 5: Sex identification of 74 mature *A. chinensis deliciosa* accessions using the *SyGI* marker. F: female accession. M: male accession. DL2000: DL2000 DNA marker. 97–170: 74 *A. chinensis deliciosa* samples used for validation (details are shown in Table S1).

3.3.2 Validation and Application of Juvenile Actinidia Plants

To assess the practical utility in early-stage breeding, the *SyGI* marker was applied to 33 juvenile *A. chinensis chinensis* seedlings (Table S2). The PCR results (Fig. 6) enabled the reliable identification of sex at the seedling stage. Subsequent field validation (Table 4) during the flowering phase confirmed complete agreement between molecular predictions and observed phenotypic sex expression, thereby validating the accuracy of the *SyGI* marker for early sex determination in *A. chinensis chinensis*.

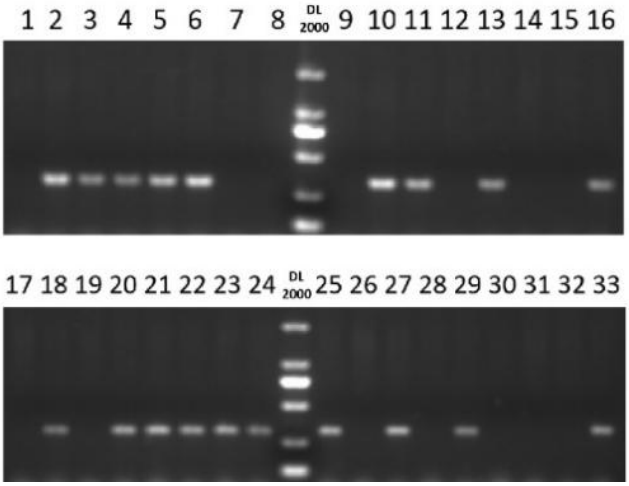

Figure 6: PCR-based sex identification of 33 juvenile *A. chinensis chinensis* seedlings using the *SyGI* marker. 1–33: 33 juvenile *A. chinensis chinensis* seedling materials (details are shown in Table S2). DL2000: DL2000 DNA marker.

Table 4: Early sex identification of juvenile *A. chinensis chinensis* seedlings.

Sample Number	Sex Type (Laboratory Identification)	Sex Type (Field Identification)
1	female	female
2	male	male
3	male	male
4	male	male
5	male	male
6	male	male
7	female	female
8	female	female
9	female	female
10	male	male
11	male	male
12	female	female
13	male	male
14	female	female
15	female	female
16	male	male
17	female	female
18	male	male
19	female	female
20	male	male
21	male	male
22	male	male
23	male	male
24	male	male
25	male	male
26	female	female
27	male	male
28	female	female
29	male	male
30	female	female
31	female	female
32	female	female
33	male	male

4 Discussion

Actinidia spp. has become an economically significant fruit crop globally. However, its dioecious reproductive system and lengthy breeding cycle present challenges for breeding programs. Early-stage sex identification is therefore critical to optimize resource allocation and reduce labor and financial inputs. Traditional sex determination methods, such as morphological and physiological-biochemical assessments, are often limited by environmental influences and developmental timing. In contrast, advances in molecular biology have enabled the development of genetic approaches that offer greater reliability for sex determination.

In this study, the SmY1 marker showed limited accuracy and low universality in different wild species. SmY1 is a sex-linked molecular marker developed using the BSA method in diploid *A. chinensis chinensis* [12]. Therefore, it is possible that the location or sequence of sex-specific regions may vary among different kiwifruit species. The Y chromosome in kiwifruit is relatively primitive and has not fully evolved [20]. Akagi et al. investigated the evolution of dioecy in kiwifruit, showing that the sex-linked segments form a small 0.49-Mb male-specific region (MSY) on chromosome 25, flanked by zones of recombination [36]. This sex-determining region contains 61 genes. Based on the patterns of genetic diversity observed in these candidate genes, the authors concluded that dioecy likely emerged 20 million years ago in kiwifruit, possibly following an ancient polyploidization event [42]. A recent study identified the sex-determining region (SDR) of *A. chinensis chinensis* on chromosome 25 (Chr25), while the SDR of *A. eriantha* has been localized to chromosome 12 (Chr12), indicating a novel interchromosomal translocation between the sex chromosomes and autosomes in kiwifruit [43]. This study observed low chromosomal colinearity between the SDRs of *A. chinensis chinensis* and *A. eriantha.* Akagi et al. reported similar findings in *A. rufa*, *A. arguta*, and *A. polygam* [44].

Sex-determining genes are located in the sex-determining region of the sex chromosome and represent the molecular basis for the differentiation between the male and female sexes. The two-mutation model of sex determination has recently been reported in plants such as *Actinidia* spp. [25], garden asparagus (*Asparagus officinalis*) [28], and grapes (*Vitis* spp.) [45,46]. In kiwifruit, the *SyGI* and *FrBy* genes on the Y chromosome function independently as a respective suppressor of feminization (SuF) and for maintaining male (M) sex, thereby regulating sex differentiation. Previous studies have demonstrated conservation of both *SyGI* and *FrBy* in a variety of kiwifruit species [25]. However, the present study found that *FrBy* performed poorly, with some amplification of non-specific bands. The PCR amplification results revealed the presence of target bands corresponding to the size of the *FrBy* gene amplification product in female samples. De Mori et al. also observed amplification of *FrBy* in female genotypes of four different *Actinidia* species, but they ruled out the possibility of an intact and functional copy of the gene existing in these species [33]. Recently, Wen et al. also found that previously published *FrBy* primers [25] only amplified a specific band in one of the four male samples [34]. However, they subsequently designed a series of primers based on the *FrBy* sequence, leading to the successful identification of suitable primers in later studies. Collectively, these observations lead us to posit that: Sequence variations (e.g., SNPs) may exist in the primer-binding region of the *FrBy* gene in a population-specific manner, which could explain both the differences in amplification results of the same primer pair in male plants of different genetic backgrounds and the fact that redesigning primers can resolve amplification failures. On the X chromosome and/or autosomes, there may exist sequences highly homologous to the *FrBy*. The conservation of these homologous sequences likely varies across germplasms. In the wild materials tested in this study, these homologous sequences may remain well preserved, leading to the appearance of amplification bands in female samples. We will subsequently

validate this hypothesis through sequencing and genomic localization of PCR products derived from wild materials in this study.

In this study, the use of the sex-specific primers of *SyGI* was validated in a large sample of wild kiwifruit. Wild kiwifruit is known to exhibit marked genetic diversity [40,47]. A degree of gene exchange occurs among wild genotypes [38]. Wild kiwifruit is widely distributed across China. The samples of wild kiwifruit investigated in this study were sourced from Jiangxi Province, Henan Province, Sichuan Province, Zhejiang Province, and the Wuling Mountains. The majority of these materials were concentrated in Jiangxi and Henan Provinces. Jiangxi Province boasts exceptionally abundant wild kiwifruit resources, including species such as *A. chinensis chinensis* and *A. eriantha* [38]. The application of *SyGI*-specific primers to these wild kiwifruit resources revealed that the marker was highly accurate and specific in the identification of sex in these wild materials. It also can serve as a valuable reference for sex identification and genomic collinearity analyses in other dioecious crops. *SyGI* has been shown to be derived from a lineage-specific gene duplication [43]. One male *A. chinensis deliciosa* accession did not yield a positive amplification, potentially attributed to sequence deletions or structural variation in the sex-determination region, possibly due to historical recombination or hybridization events. In future follow-up studies, sequencing the *SyGI* locus in this accession may provide important insights into the genetic mechanisms underlying sex determination and evolution in *A. chinensis deliciosa*.

To assess whether *SyGI* is located within the male-specific region of the Y chromosome (MSY), cloning and sequencing of *SyGI*-amplified fragments from male individuals of six wild *Actinidia* species were performed. The comparative analysis of cloned *SyGI* sequences from six wild *Actinidia* species showed that these sequences had the highest similarity to either *Shy Girl* or the MSY sequences, confirming the male specificity of this marker. Notably, we identified SNPs in candidate proximal regulatory sequences of *SyGI* across different *Actinidia* species. The specific mechanism by which *SyGI* modulates sex determination remains unknown [48]. To explore the potential functional impact of these SNPs on the expression regulation of the *SyGI* gene, we conducted an extended analysis in this study. This extended analysis revealed lineage-specific differentiation by comparing differences in cis-acting elements within candidate proximal regulatory regions of *SyGI* gene across six wild *Actinidia* species (Fig. S1). It also directly linked key SNPs to variations in cis-acting elements (Fig. S2). These findings provided sequence evidence and hypotheses for understanding the expression regulation diversity and adaptive evolution of *SyGI*, a key gene in kiwifruit sex determination. The analysis indicated that specific SNPs directly mediated variations in key regulatory elements. For instance, in *A. callosa*, the SNPs at key sites may not only lead to the loss of a conserved MYB element but could also create a key element—AT-TATA-box, thereby potentially affecting both its developmental signal response and basal transcription efficiency [49,50]. The unique SNPs in *A. hemsleyana* and *A. arguta* may create a low-temperature response element, suggesting that their *SyGI* expression might directly sense environmental temperature signals. In *A. eriantha*, a distinct SNP may form a novel W-box near the MYB element, which could couple developmental regulation with defense response pathways [49,51]. These SNP-driven variations in the "regulatory code" indicated that natural selection may fine-tune the upstream sequence of *SyGI* to adapt to different ecological and developmental contexts. It should be noted that this analysis has limitations. Firstly, the examined "candidate proximal regulatory region" was operationally defined, and key distal regulatory elements may reside outside this region. Secondly, the conclusions were based on bioinformatic predictions, and their functional impacts require further validation through experiments such as reporter gene assays or EMSA. Nonetheless, this study translated sequence variations into concrete regulatory hypotheses, providing clear and testable targets for subsequent functional studies.

The ultimate goal of determining molecular sex markers is their application in sex identification at the early/juvenile stages in kiwifruit development. Currently, most sex identification methods for kiwifruit were developed and validated using materials of known sex [14,15], with few studies on the application of molecular sex markers in juvenile seedlings. The successful application of the *SyGI* marker for sex identification in juvenile plants used in this study confirmed the practical value of this molecular marker technology. The breeding cycle of kiwifruit could potentially be shortened by 2 to 3 years. This provides an efficient and reliable tool for the identification of kiwifruit sex.

Although the sex determination model of kiwifruit has been elucidated, the specific regulatory mechanisms underlying the process are not known [48]. Scientists are attempting to identify these regulatory mechanisms using approaches such as gene editing [52]. Recently, researchers have investigated the regulatory networks of sex differentiation by integrating transcriptome and metabolome analysis [53]. Plant sex is fundamentally a quantitative trait, associated with complex genetic inheritance patterns and regulatory mechanisms [54,55]. In the future, techniques such as multiplex PCR and KASP genotyping could be integrated into high-throughput kiwifruit breeding workflows. These techniques can combine multiple loci or link the loci of multiple traits with the loci of functional markers of core economic traits, thereby maximizing automation, data-driven approaches, and efficiency in breeding operations.

5 Conclusion

In summary, the *SyGI* marker selected in this study exhibits high male specificity, strong cross-species applicability, and excellent accuracy, making it a promising tool for field-based sex identification in kiwifruit. Its use can significantly enhance breeding efficiency by enabling early selection, thereby reducing time and resource investments. Furthermore, the data generated here contribute valuable insights for future development of sex-linked molecular markers and further investigations into the genetic regulation of sex determination in *Actinidia* spp.

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References

1. Wang Z, Hu G, Li Z, Zhong C, Yao X. Characterizing tetraploid populations of *Actinidia chinensis* for kiwifruit genetic improvement. Plants. 2022;11(9):1154. [CrossRef].
2. Han X, Zhang Y, Zhang Q, Ma N, Liu X, Tao W, et al. Two haplotype-resolved, gap-free genome assemblies for Actinidia latifolia and Actinidia chinensis shed light on the regulatory mechanisms of vitamin C and sucrose metabolism in kiwifruit. Mol Plant. 2023;16(2):452–70. [CrossRef].
3. Wang S, Qiu Y, Zhu F. Kiwifruit (*Actinidia* spp.): A review of chemical diversity and biological activities. Food Chem. 2021;350:128469. [CrossRef].
4. Luo L, Song W, Hua Q, Li D, Liang H, Zhang X. Research progress on plant sex-determination genes and their epigenetic regulation. Chin Bull Bot. 2024;59(02):278–90. (In Chinese). [CrossRef].
5. Mayer SS, Charlesworth D. Cryptic dioecy in flowering plants. Trends Ecol Evol. 1991;6(10):320–5. [CrossRef].
6. Khukhunaishvili RG, Dzhokhadze DI. Electrophoretic study of the proteins from *Actinidia leaves* and sex identification. Prikl Biokhim Mikrobiol. 2006;42(1):117–20. [CrossRef].
7. Zheng H, Li Y, Jin X, Zhao Y, Luo G. The variation of plant morphology and secondary metabolite of male and female *Actinidia arguta* during growth. Agric Sci J Yanbian Univ. 2018;40(04):16–21. (In Chinese). [CrossRef].
8. Biasi R, Falasca G, Speranza A, De Stradis A, Scoccianti V, Franceschetti M, et al. Biochemical and ultrastructural features related to male sterility in the dioecious species *Actinidia deliciosa*. Plant Physiol Biochem. 2001;39(5):395–406. [CrossRef].
9. Guo D, Wang R, Fang J, Zhong Y, Qi X. Development of sex-linked markers for gender identification of Actinidia arguta. Sci Rep. 2023;13(1):12780 [CrossRef].
10. Akagi T, Henry IM, Tao R, Comai L. A Y-chromosome–encoded small RNA acts as a sex determinant in persimmons. Science. 2014;346(6209):646–50. [CrossRef].
11. Zhang PX, Yang SC, Liu YF, Zhang QL, Xu LQ, Luo ZR. Validation of a male-linked gene locus (OGI) for sex identification in persimmon (*Diospyros kaki* Thunb.) and its application in F1 progeny. Plant Breed. 2016;135(6):721–7. [CrossRef].
12. Harvey CF, Gill GP, Fraser LG, McNeilage MA. Sex determination in *Actinidia*. 1. Sex-linked markers and progeny sex ratio in diploid A. chinensis. Sex Plant Reprod. 1997;10(3):149–54. [CrossRef].
13. Gill GP, Harvey CF, Gardner RC, Fraser LG. Development of sex-linked PCR markers for gender identification in *Actinidia*. Theor Appl Genet. 1998;97(3):439–45. [CrossRef].
14. Zhang Q, Liu C, Liu Y, VanBuren R, Yao X, Zhong C, et al. High-density interspecific genetic maps of kiwifruit and the identification of sex-specific markers. DNA Res. 2015;22(5):367–75. [CrossRef].
15. Zhang K, Jiang X, Wang Y, Tang W, Liu Y, Liang D, et al. Collection, evaluation and application of molecular markers for kiwifruit sex identification. J Sichuan Agric Univ. 2021;39(04):541–8. (In Chinese). [CrossRef].
16. Murakami S, Katai H, Yamada S, Taneishi M. The validity of marker-assisted selection using sex linked SCAR markers in kiwifruit (*Actinidia chinensis* cv 'rainbow red') seedlings. J Sci High Technol Agric. 2015;27(2):68–74. [CrossRef].
17. Taravella AM, Sayres MAW. Fruitful analysis of sex chromosomes reveals X-treme genetic diversity. Genome Biol. 2016;17(1):244. [CrossRef].
18. Charlesworth D. Plant sex chromosome evolution. J Exp Bot. 2013;64(2):405–20. [CrossRef].
19. Zhao Y, Zhang T, Liu C, Huang X, Yuan Z. Progress on sex determinant mechanism in horticultural plants. Acta Hortic Sin. 2018;45(11):2228–42. (In Chinese). [CrossRef].
20. Ming R, Bendahmane A, Renner SS. Sex chromosomes in land plants. Annu Rev Plant Biol. 2011;62:485–514. [CrossRef].
21. He Z, Huang H, Zhong Y. Cytogenetic study of diploid *Actinidia chinensis*—karyotype, morphology of sex chromosomes at primary differentiation stage and evolutionary significance. Acta Hortic. 2003;610:379–85. [CrossRef].
22. Charlesworth B, Charlesworth D. A model for the evolution of dioecy and gynodioecy. Am Nat. 1978;112(988):975–97. [CrossRef].
23. Charlesworth D. Plant contributions to our understanding of sex chromosome evolution. New Phytol. 2015;208(1):52–65. [CrossRef].

24. Peng D, Wu Z. Progress on sex determination of dioecious plants. Biodivers Sci. 2022;30(3):1–12. [CrossRef].
25. Akagi T, Pilkington SM, Varkonyi-Gasic E, Henry IM, Sugano SS, Sonoda M, et al. Two Y-chromosome-encoded genes determine sex in kiwifruit. Nat Plants. 2019;5(8):801–9. [CrossRef].
26. Kazama Y, Ishii K, Aonuma W, Ikeda T, Kawamoto H, Koizumi A, et al. A new physical mapping approach refines the sex-determining gene positions on the *Silene latifolia* Y-chromosome. Sci Rep. 2016;6:18917. [CrossRef].
27. Aryal R, Ming R. Sex determination in flowering plants: *Papaya* as a model system. Plant Sci. 2014;217–218:56–62. [CrossRef].
28. Harkess A, Huang K, van der Hulst R, Tissen B, Caplan JL, Koppula A, et al. Sex determination by two Y-linked genes in garden *Asparagus*. Plant Cell. 2020;32(6):1790–6. [CrossRef].
29. Müller NA, Kersten B, Leite Montalvão AP, Mähler N, Bernhardsson C, Bräutigam K, et al. A single gene underlies the dynamic evolution of poplar sex determination. Nat Plants. 2020;6(6):630–7. [CrossRef].
30. Yang W, Wang D, Li Y, Zhang Z, Tong S, Li M, et al. A general model to explain repeated turnovers of sex determination in the Salicaceae. Mol Biol Evol. 2021;38(3):968–80. [CrossRef].
31. Prentout D, Razumova O, Rhoné B, Badouin H, Henri H, Feng C, et al. An efficient RNA-seq-based segregation analysis identifies the sex chromosomes of *Cannabis sativa*. Genome Res. 2020;30(2):164–72. [CrossRef].
32. Varkonyi-Gasic E, Wang T, Cooney J, Jeon S, Voogd C, Douglas MJ, et al. Shy Girl, a kiwifruit suppressor of feminization, restricts gynoecium development via regulation of cytokinin metabolism and signalling. New Phytol. 2021;230(4):1461–75. [CrossRef].
33. De Mori G, Testolin R, Cipriani G. A molecular protocol for early sex discrimination (ESD) in *Actinidia* spp. J Berry Res. 2022;12(2):249–66. [CrossRef].
34. Wen C, Wei J, Zhao Y, Zeng T, Li M, Zhang H, et al. Identification and development of molecular markers for sex in kiwifruit. J Sichuan Univ (Nat Sci Ed). 2025;62(02):271–8. (In Chinese). [CrossRef].
35. Fang L, Zhang H, Chen Z, Wang F, Ji X. Development of specific markers for the characterization of kiwifruit sex trait based on fluorescence capillary electrophoresi. South China Fruits. 2024;53(03):7–15. (In Chinese). [CrossRef].
36. Akagi T, Henry IM, Ohtani H, Morimoto T, Beppu K, Kataoka I, et al. A Y-encoded suppressor of feminization arose via lineage-specific duplication of a cytokinin response regulator in kiwifruit. Plant Cell. 2018;30(4):780–95. [CrossRef].
37. Qi X, Wang R, Zhang M, Lin M, Li Y, Sun L. Research progress in kiwifruit breeding. J Fruit Sci. 2024;41(11):2160–72. (In Chinese). [CrossRef].
38. He Y, Li Z, Liao G, Chen L, Zhong M, Huang C, et al. Variation in fruit quality within wild *Actinidia eriantha* germplasm. N Z J Crop Hortic Sci. 2020;48(3):153–63. [CrossRef].
39. Yao X, Wang S, Wang Z, Li D, Jiang Q, Zhang Q, et al. The genome sequencing and comparative analysis of a wild kiwifruit *Actinidia eriantha*. Mol Hortic. 2022;2(1):13. [CrossRef].
40. Ma X, Han Z, Li T, Yang Y, Su W, Li W. Genetic diversity and population structure analysis of wild kiwifruit (*Actinidia* spp.) using SRAP markers. Biotechnol Biotechnol Equip. 2024;38(1):2373845. [CrossRef].
41. Lescot M, Déhais P, Thijs G, Marchal K, Moreau Y, Van de Peer Y, et al. PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for *in silico* analysis of promoter sequences. Nucleic Acids Res. 2002;30(1):325–7. [CrossRef].
42. Caseys C. Shy girl gives kiwifruit male flowers. Plant Cell. 2018;30(4):739–40. [CrossRef].
43. Yue J, Chen Q, Zhang S, Lin Y, Ren W, Li B, et al. Origin and evolution of the kiwifruit Y chromosome. Plant Biotechnol J. 2024;22(2):287–9. [CrossRef].
44. Akagi T, Varkonyi-Gasic E, Shirasawa K, Catanach A, Henry IM, Mertten D, et al. Recurrent neo-sex chromosome evolution in kiwifruit. Nat Plants. 2023;9(3):393–402. [CrossRef].
45. Massonnet M, Cochetel N, Minio A, Vondras AM, Lin J, Muyle A, et al. The genetic basis of sex determination in grapes. Nat Commun. 2020;11:2902. [CrossRef].
46. Iocco-Corena P, Chaïb J, Torregrosa L, MacKenzie D, Thomas MR, Smith HM. VviPLATZ1 is a major factor that controls female flower morphology determination in grapevine. Nat Commun. 2021;12(1):6995. [CrossRef].

47. Guo R, Zhang YH, Zhang HJ, Landis JB, Zhang X, Wang HC, et al. Molecular phylogeography and species distribution modelling evidence of 'oceanic' adaptation for *Actinidia eriantha* with a refugium along the oceanic-continental gradient in a biodiversity hotspot. BMC Plant Biol. 2022;22(1):89. [CrossRef].
48. Ye LX, Luo MM, Wang Z, Bai FX, Luo X, Gao L, et al. Genome-wide analysis of *MADS*-box gene family in kiwifruit (*Actinidia chinensis* var. chinensis) and their potential role in floral sex differentiation. Front Genet. 2022;13:1043178. [CrossRef].
49. Dubos C, Stracke R, Grotewold E, Weisshaar B, Martin C, Lepiniec L. MYB transcription factors in *Arabidopsis*. Trends Plant Sci. 2010;15(10):573–81. [CrossRef].
50. Smale ST, Kadonaga JT. The RNA polymerase II core promoter. Annu Rev Biochem. 2003;72:449–79. [CrossRef].
51. Rushton PJ, Reinstädler A, Lipka V, Lippok B, Somssich IE. Synthetic plant promoters containing defined regulatory elements provide novel insights into pathogen- and wound-induced signaling. Plant Cell. 2002;14(4):749–62. [CrossRef].
52. De Mori G, Zaina G, Franco-Orozco B, Testolin R, De Paoli E, Cipriani G. Targeted mutagenesis of the female-suppressor SyGI gene in tetraploid kiwifruit by CRISPR/CAS9. Plants. 2020;10(1):62. [CrossRef].
53. Wang B, Tang D, Zhou J, Zhong W, Liu Q, Zhang S, et al. The regulatory network of sex differentiation in the bisexual kiwifruit 'Duie A10' unveiled by integrated transcriptome and metabolome analysis. Sci Hortic. 2025;354:114516. [CrossRef].
54. Pannell JR. Plant sex determination. Curr Biol. 2017;27(5):R191–7. [CrossRef].
55. Lu J, Shi Y, Yin X, Liu S, Liu C, Wen D, et al. The genetic mechanism of sex type, a complex quantitative trait, in *Ricinus communis* L. Ind Crops Prod. 2019;128:590–8. [CrossRef].