horticulturae

MDPI

Article

Study on Fertility Identification of Monogerm Binary Male-Sterile Lines in Sugar Beet (*Beta vulgaris* L.) Using Molecular Markers

Ruxiao Song [1], Zedong Wu [1,*] and Linlin Sun [2,*]

[1] Academy of Modern Agriculture and Ecological Environment, Heilongjiang University, Harbin 150080, China; srx000626@163.com
[2] Xinjiang Shihezi Academy of Agricultural Sciences, Shihezi 832011, China
* Correspondence: 1997009@hlju.edu.cn (Z.W.); nkysun2018@163.com (L.S.)

Abstract

Sugar beet (*Beta vulgaris* L.) is one of the most important sugar crops and potential energy crops in China. The utilization of its heterosis is crucial for breaking through the bottlenecks in yield and quality, while the fertility identification of binary male-sterile lines is the core link to ensure the purity of hybrid seeds. Due to its indeterminate inflorescence, artificial emasculation of sugar beet is not feasible, which significantly increases the difficulty in hybrid seed production. To rapidly and accurately identify the fertility composition of monogerm binary male-sterile lines of sugar beet, ensure the maternal line purity in sugar beet hybrid seed production, and improve breeding efficiency, this study conducted fertility identification using molecular marker technology with 7 monogerm binary male-sterile line germplasm resources (297 plants) provided by three research institutions in different regions of China. Genomic DNA was extracted from young sugar beet leaves by the CTAB method. The cytoplasmic fertility types were identified using the TR1 primer, and the fertility gene composition at the nuclear *Rf1* locus was verified by the s17 molecular marker combined with *Hap* II and *Hind* III double digestion. The results showed that in the cytoplasmic fertility identification, the proportion of S-type cytoplasm in Lines 2 to 7 reached 100%, indicating stable sterility without maintainer line contamination; Line 1 had 93.33% S-type cytoplasm, mixed with 6.67% N-type cytoplasm. For the nuclear *Rf1* locus identification, 93.27% (277 plants) of the tested materials yielded the target 1800 bp band by PCR amplification, which were preliminarily identified as homozygous recessive type. Among them, Lines 1 to 3 all showed a single 1800 bp band pattern, indicating homozygous and consistent nuclear fertility genotypes; 20 plants (6.73%) in Lines 4 to 7 exhibited a composite 1800/1300 bp band pattern, suggesting the presence of restorer allele contamination in some lines. Genotype analysis based on 35 enzyme-digested verification samples revealed that the 4/4 genotype had the highest proportion. This study realized the rapid and accurate identification of cytoplasmic and nuclear fertility in monogerm binary male-sterile lines of sugar beet through molecular marker technology, clarified the fertility purity status of 7 germplasm resources, and verified the application value of this technology in the fertility identification of sugar beet binary male-sterile lines. These results provide a scientific basis and technical support for controlling maternal line purity and improving breeding efficiency in sugar beet hybrid seed production.

Keywords: sugar beet; cytoplasmic male sterility; molecular marker-assisted identification

Check for updates

Academic Editor: Beppe Benedetto Consentino

Received: 21 January 2026
Revised: 23 February 2026
Accepted: 27 February 2026
Published: 1 March 2026

Copyright: © 2026 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license.

1. Introduction

Sugar beet *(Beta vulgaris* L.) is a biennial herbaceous plant belonging to the Amaranthaceae family, with its origin traced to the European coast around 8500 BCE. As a sugar crop widely cultivated across the globe, it is derived from the wild species Beta maritima, native to the Mediterranean coast. Andreas Marggraf first discovered that the roots of sugar beets contain the same crystals as those present in sugarcane stems [1]. As one of the most important sugar-making raw materials globally, sugar beet is the world's second-largest sugar crop after sugarcane. Currently, sugar produced from sugar beet accounts for 30% of the world's total sugar output [2,3], playing a crucial role in meeting global sugar demand [4]. Although China is not the country of origin of sugar beet, the history of sugar beet cultivation in China can be traced back to the early 20th century. Sugar beet exhibits high drought tolerance, cold hardiness, and salt tolerance. It is mainly cultivated in the relatively cool northwest, northeast, and northern China regions, serving as the primary sugar crop in northern China [5]. Its sugar is accumulated in the taproot, whose sugar content typically ranges from 13% to 20% [6].

By-products generated during the sugar production process from sugar beet also exhibit considerable reuse value [7–9]. Beyond its primary application in sugar extraction, sugar beet is widely utilized in the food processing and brewing industries, can also be directly used as animal feed, and possesses potential as an energy material [10–13], demonstrating diverse practical values [14]. Additionally, sugar beet is rich in nutritional value [15] and plays an important role in safeguarding food security and dietary health [16,17].

As a sugar crop and potential energy crop in northern China, its industrial development directly impacts the economic efficiency of the sugar manufacturing industry and the nation's sugar supply security [18]. As China gradually expands its sugar beet cultivation scale, sugar manufacturers are placing increasingly higher demands on raw materials. Leveraging hybrid vigor to overcome the yield and quality limitations of traditional varieties has become a key focus in sugar beet breeding [19].

Previous studies have confirmed that sugar beet hybrids exhibit prominent advantages compared with conventional varieties: the yield is increased by 15–20%, the root sugar content is raised by 1.2–1.8 percentage points, and the stress resistance is significantly superior, enabling better adaptation to adverse growth conditions. In the sugar beet heterosis utilization system, the monogerm binary sterile line serves as the female parent supporting hybrid seed production. It is essentially a stable fertility system formed through specific pollination combinations. Its core function is to provide a stable sterile female parent for hybrid production, ensure efficient pollination by male parent pollen during hybridization, and avoid the reduction in hybrid purity caused by female selfing, which would restrict the breeding process [20].

The development of monogerm binary male-sterile lines of sugar beet is achieved by pollinating alloplasmic monogerm male-sterile lines of sugar beet with monogerm maintainer lines [21]. Therefore, the number of female parents available for hybrid seed production depends on the quantities of monogerm CMS (cytoplasmic male-sterile) lines and maintainer lines. Traditionally, the identification of male-sterile lines or maintainer lines has been performed using the hybridization method: to identify a male-sterile line, the candidate line must be pollinated with a known maintainer line. After harvesting the seeds, they are subjected to southern multiplication to cultivate mother roots, which are then vernalized to induce flowering. If no pollen is produced after flowering, the line is confirmed as a CMS line. For the identification of maintainer lines, the candidate line is used to pollinate a known male-sterile line. If the progeny exhibit male sterility, the candidate line is confirmed as a homozygous maintainer line. This traditional identification method not only takes approximately three years but also requires a substantial investment

of material and financial resources. In contrast, marker-assisted breeding technology can accurately identify the fertility type of individual plants in sugar beet lines or varieties, as well as the composition of fertility-related genes in their nuclei within a short period. Furthermore, the fertility identification of sugar beet binary male-sterile lines is a core link in ensuring the purity of their use as female parents for hybrids and improving seed production efficiency, which requires simultaneous verification of CMS traits and nuclear recessive maintainer genes. Among these methods, based on the polymorphism of the mitochondrial TR1 locus, PCR amplification is performed on sugar beet monogerm binary male-sterile lines using TR1 primers, and the cytoplasmic type is determined according to the size of the amplified bands: if the band size is less than 500 bp, the cytoplasmic type is indicated to be the sterile S-type; when the amplified band size exceeds 500 bp, the cytoplasmic type is indicated to be the fertile N-type [22,23]. The identification of nuclear-related fertility genes is mainly achieved using s17 primers, combined with restriction enzyme digestion analysis using two restriction enzymes, *Hap* II and *Hind* III [24].

The identification of nuclear-related fertility genes is mainly achieved using the s17 primer and requires restriction enzyme digestion analysis with two enzymes, *Hap* II and *Hind* III. The amplification products of the s17 primer typically fall into three types: 1800 bp, 1300 bp, and the 1800 bp/1300 bp combined type. Among these, only the 1800 bp amplification band can proceed to subsequent restriction enzyme digestion identification, while the other types can be directly excluded. For the 1800 bp amplification product, further genotypic identification is needed via double restriction enzyme digestion with *Hap* II and *Hind* III. According to Taguchi et al. [22], three band patterns may appear in the restriction enzyme digestion products, each corresponding to a different allele combination: a single 1800 bp band (corresponding to the 5/5 allele combination), three bands of 1800 bp + 1000 bp + 700 bp (corresponding to the 4/5 genotype), and two bands of 1000 bp + 700 bp (corresponding to the 4/4 genotype). It should be noted that this study only confirmed that the 4/4 type is identical to the known homozygous recessive nuclear sterile genotype (*rf1*/*rf1*), i.e., linked to the *bvORF20L* non-restorer allele; however, no clear conclusion has yet been drawn regarding whether the 5/5 and 4/5 types are associated with the recessive nuclear sterile gene. Although the formation of different band patterns arises from variations in individual bases, whether these variations affect the fertility phenotype remains to be verified [23]. Therefore, in this study, only the 4/4 type, which exhibits two bands of 1000 bp + 700 bp, was identified as the homozygous recessive nuclear sterile genotype (*rf1*/*rf1*); the 5/5 and 4/5 types were initially regarded as potential recessive nuclear sterile genotypes, requiring further verification through subsequent field experiments. However, whether other restriction digestion patterns also harbor homozygous recessive genes associated with nuclear sterility remains to be investigated. Currently, molecular marker-assisted fertility testing has been studied in rice [25], soybean [26] and wheat [27].

In this study, a marker-assisted identification method was used to identify the fertility genotypes of 7 monogerm binary male-sterile lines of sugar beet developed by three different scientific research groups. This study systematically analyzes the fertility status of seven sugar beet germplasm resources using molecular marker technology, verifies the application value of this approach in identifying monogerm binary male-sterile lines, and provides a scientific basis and technical reference for sugar beet breeding practice.

2. Materials and Methods

2.1. Plant Materials

The experimental materials consisted of 7 monogerm binary male-sterile line germplasm resources (297 individual plants) provided by the Jilin Academy of Agricultural Sciences, the Shihezi Sugar Beet Research Institute, and the Sugar Beet Improvement Team

of Heilongjiang University. The experimental mother root was planted in April 2025 at the Heilongjiang University Experimental Base in Hulan District, Harbin City, Heilongjiang Province. Seven monogerm binary male-sterile line germplasm resources were planted in ridges by strain, with a plant spacing of 0.4 m and a row spacing of 0.68 m. Detailed information on the strains used in the field planting experiment is shown in (Table 1).

Table 1. Specific information of test strains.

Product Name	Origin of the Product Line	Product Code	Number of Plants
X4141	Jilin Academy of Agricultural Sciences	1	30
Single embryo sterile line of Shi2	Shihezi Sugar Beet Research Institute	2	13
Single embryo sterile line of Shi1	Shihezi Sugar Beet Research Institute	3	14
Ring 91	Heilongjiang University Beet Improvement Team	4	60
Ring 92	Heilongjiang University Beet Improvement Team	5	60
Ring 96	Heilongjiang University Beet Improvement Team	6	60
Ring 97	Heilongjiang University Beet Improvement Team	7	60

2.2. Test Reagents and Instruments

Test reagents: 2 × CTAB buffer (Solarbio Science & Technology Co., Ltd., Beijing, China); DNA extraction reagent (Biotopped Technology Co., Ltd., Beijing, China); isopropanol (Tianjin Fuyu Biochemical Co., Ltd., Tianjin, China); TE buffer (Solarbio Science & Technology Co., Ltd., Beijing, China); 2 × Rapid Taq Master Mix (Biotopped Technology Co., Ltd., Beijing, China); Agarose (Solarbio Science & Technology Co., Ltd., Beijing, China); *Hap* II enzyme (Harbin Baoshide Biotech Co., Ltd., Harbin, China); *Hind* III enzyme (Harbin Baoshide Biotech Co., Ltd., Harbin, China); 10 × M Buffer (Harbin Baoshide Biotech Co., Ltd., Harbin, China); 50 × TAE solution (Solarbio Science & Technology Co., Ltd., Beijing, China); Gold View nucleic acid dye (Solarbio Science & Technology Co., Ltd., Beijing, China); 2000 bp marker (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China); absolute ethanol (Tianjin Fuyu Biochemical Co., Ltd., Tianjin, China).

Experimental Instruments: High-speed cryogenic tissue grinder (Wuhan Servicebio Technology Co., Ltd., Wuhan, China); HB120-S LED Digital Display Heating Metal Bath (Dragon Laboratory Instruments (Beijing) Co., Ltd., Beijing, China); MINIP-2500 Microplate Centrifuge (Hangzhou Mio Instrument Co., Ltd., Hangzhou, China); high-speed centrifuge (Changsha Intai Instrument Co., Ltd., Changsha, China); electronic balance (Sartorius Scientific Instruments (Beijing) Co., Ltd., Beijing, China); MV-100 vortex mixer (Wuhan Servicebio Technology Co., Ltd., Wuhan, China); Bio-Rad Power Pac 3000 electrophoresis system (Bio-Rad Laboratories, Inc., Hercules, CA, USA); Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA); Bio-Rad gel imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA); NanoDrop 2000/2000c UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA); SIM-F124 Ice Maker (Sanyo Electric Co., Ltd., Osaka, Japan).

2.3. DNA Template Preparation

This experiment uses molecular marker technology to identify the fertility genotype of sweet menu embryo binary sterile lines. The cytoplasmic fertility type was identified

using TR1 primers, while the nuclear fertility type was determined via the s17 molecular marker. Following PCR amplification of the DNA templates, 1% agarose gel electrophoresis was performed. All primers were synthesized by Shanghai Sengong Biotech Co., Ltd. The specific primer sequences are listed in (Table 2).

Table 2. DNA molecular markers used in the experiment.

Primer Name	Tag Type	The Nucleotide Sequence of PCR Primers	References
TR1	VNTR	5′-AGAACTTCGATAGGCGAGAGG-3′ 5′-GCAATTTTCAGGGCATGAACC-3′	[23]
s17	CAPS	5′-CAATCTGTGGTGCTGACCAAA-3′ 5′-GATTAAAGAGGGCTGCTGAAGCCGAGA-3′	[22]

Genomic DNA was extracted from young sugar beet leaves sampled from the field using the CTAB method [28]. The concentration and purity of the extracted DNA were determined with a micro-volume UV–visible spectrophotometer. The DNA was diluted to 200 ng/μL and stored at 4 °C for subsequent use, while the remaining genomic DNA stock solution was cryopreserved at −20 °C.

2.4. PCR Reaction System and Program

PCR reaction system (10 μL): 2 × Rapid Taq Master Mix 5 μL, forward and reverse primers 0.2 μL each, DNA diluent 1 μ L, ddH2O 3.6 μL.

PCR reaction program: pre-denaturation at 95 °C for 3 min; denaturation at 94 °C for 25 s, annealing at 60 °C for 25 s, extension at 72 °C for 2 min, a total of 25 cycles; extension at 72 °C for 5 min.

2.5. Electrophoresis

After PCR amplification, 1% agarose gel electrophoresis containing Gold View is used, and a 2000 bp marker is used as the DNA molecular weight standard for comparison. The sample size is 5 μL, and electrophoresis is carried out for 30 min under a constant voltage of 120 V. Finally, the strips are observed and photographed in the gel imager.

2.6. Enzyme Digestion Validation Methods Related to the Rf1 Site

If the molecular marker band size corresponding to the *Rf1* locus associated with sugar beet nuclear fertility is 1800 bp, the PCR product requires further restriction enzyme digestion (using *Hap* II and *Hind* III) to identify its fertility genotype.

The total reaction volume for the *Rf1* locus genotyping double-digestion reaction is 10 μL. The reaction mixture consists of 5 μL of s17 amplification product, 0.25 μL each of *Hap* II and *Hind* III enzymes, 2 μL of 10× M Buffer, and 2.5 μL of ddH_2O.

The restriction enzyme digestion protocol was as follows: 37 °C for 3 h. The PCR amplification products were detected by 1% agarose gel electrophoresis, followed by band observation and photography using a gel documentation system.

3. Results

3.1. Analysis of Cytoplasmic Fertility Type Identification Results

In this experiment, using the polymorphism of the mitochondrial TR1 locus, TR1 primers were selected to identify the cytoplasmic fertility genotypes of 7 monogerm binary sterile lines (297 plants) of sugar beet. The results of genotype amplification showed that there were two types of amplification bands: those below 500 bp and those above 500 bp, corresponding to sterile cytoplasm (S-type) and fertile cytoplasm (N-type), respectively. Among them, 93.33% of the amplification bands of Line 1 were S-type cytoplasm, and 6.67%

of the amplification bands were above 500 bp, representing fertile N-type cytoplasm. The amplification band types of Lines 2 to 7 were all S-type cytoplasm.

Among all lines, the proportion of S-type cytoplasm was the highest: 93.33% in Line 1 and 100% in Lines 2 to 7. In contrast, the proportion of N-type cytoplasm was the lowest, accounting for only 6.67% in Line 1. Partial electrophoresis detection results are shown in Figure 1. These results indicate that the CMS traits of Lines 2 to 7 were generally stable. However, the presence of partial N-type cytoplasm in Line 1 suggests that this line was contaminated with maintainer lines.

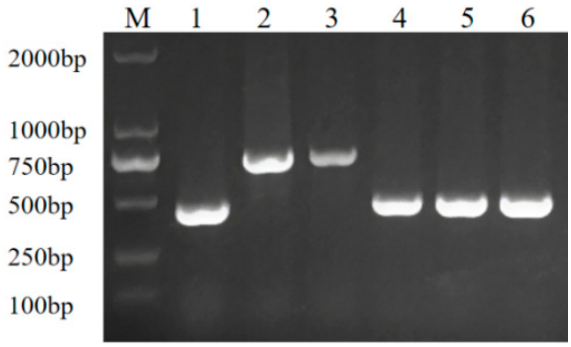


Figure 1. Gel electrophoretic map of some plants in strain 1 amplified by TR1 primer.

3.2. Identification and Analysis of Nuclear Genome Rf1 Locus Genotype

Using CAPS molecular markers (s17 primers) associated with the *Rf1* locus, PCR amplification was performed on 297 DNA templates of the tested materials, yielding two types of bands: a 1800 bp single band and a 1300 bp/1800 bp composite band type. A total of 277 tested materials showed a single 1800 bp amplified band. Lines 1 to 3 exclusively exhibited the 1800 bp band, indicating stable fertility genes. In contrast, a small number of materials (20 plants in total) from Lines 4 to 7 displayed the 1300 bp/1800 bp composite band type, suggesting genetic admixture in the fertility-related genes. Detailed amplification results of each line are presented in Table 3, and partial electrophoresis detection results are shown in Figure 2.

Table 3. Statistical Analysis of s17 Primer Amplification Results for 297 Beet Germplasm Resources.

Primer	Product Code	1800 bp (Number of Plants)	1800/1300 bp (Number of Plants)
s17	1	30	0
	2	13	0
	3	14	0
	4	48	12
	5	57	3
	6	56	4
	7	59	1
Total		277	20

Note: Seven lines were tested, with product codes 1–7.

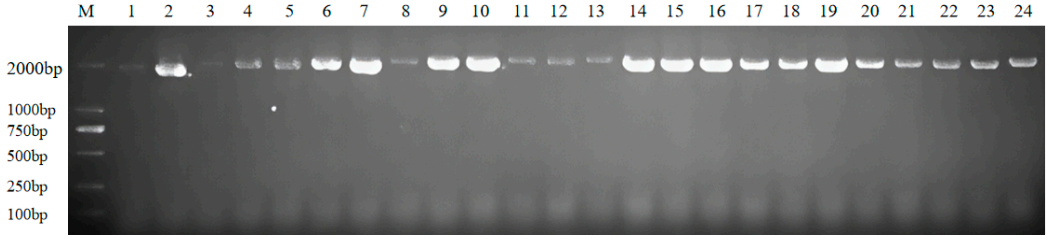


Figure 2. Gel electrophoresis of partial plants of strain 4 and 5 amplified by s17 primer.

3.3. Identification and Analysis of Enzyme Digestion Validation Results

Using the s17 primer, the genotypes of the *Rf1* locus in the nucleus of sugar beet monogerm binary male-sterile lines were identified. Following double digestion of the PCR amplicons with restriction enzymes *Hap* II and *Hind* III, three band patterns were generated: 1800 bp (genotype 5/5), 1800 bp + 1000 bp + 700 bp (genotype 4/5), and 1000 bp + 700 bp (genotype 4/4). Detailed results are presented in Table 4.

Table 4. Statistics of Double Restriction Enzyme Digestion Results of s17 Amplicons from Partial Sugar Beet Germplasm Resources.

Digestion	Product Code	5/5 (%)	4/5 (%)	4/4 (%)
Hap II and *Hind* III double digestion	1	100	0	0
	2	0	100	0
	3	100	0	0
	4	0	0	100
	5	0	0	100
	6	0	0	100
	7	0	20.34	79.66

In this experiment, 5 plants were randomly selected from each of the 7 sugar beet monogerm binary male-sterile lines (35 plants in total) to verify the 1800 bp amplicons from the s17 PCR amplification via double digestion with restriction enzymes *Hap* II and *Hind* III. A total of three distinct genotypes were obtained: 6 plants of genotype 5/5, accounting for 17.1%; 3 plants of genotype 4/5, accounting for 8.6%; and 26 plants of genotype 4/4, accounting for 74.3%. It should be noted that this genotype frequency is only representative of the verified samples and cannot be extrapolated to all 277 plants. In the present study, only genotype 4/4 was identified as a candidate material carrying homozygous recessive nuclear sterility-related genes; both genotypes 5/5 and 4/5 were derived from 1800 bp amplicons and were initially determined to be potentially different mutation types of the recessive nuclear sterility gene, which require further verification and exploration in subsequent research. The results of partial restriction enzyme digestion and amplification are presented in Figure 3.

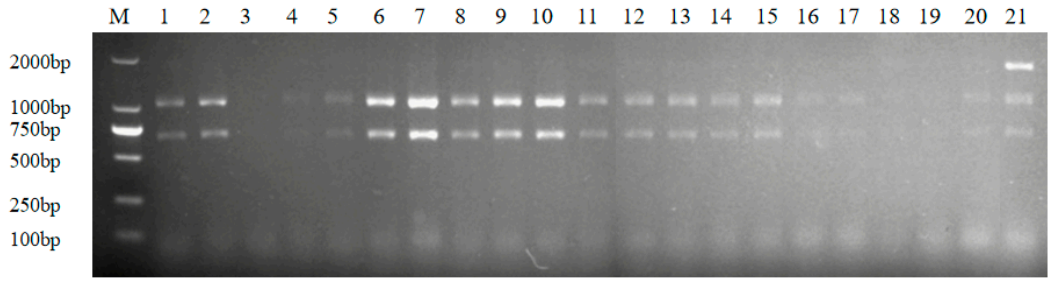


Figure 3. Gel electrophoresis of s17 double restriction amplification results from strain 4 to strain 7.

4. Discussion

The cytoplasmic types and fertility genotypes at the nuclear *Rf1* locus of 297 plants from seven monogerm diploid CMS lines of sugar beet, provided by three Chinese research institutions, were systematically identified using TR1 and s17 molecular markers. Cytoplasmic identification results showed that Lines 2 to 7 had a 100% proportion of S-type cytoplasm with stable sterility, indicating that no maintainer lines were mixed into the CMS lines during their propagation. In Line 1, 6.67% of N-type cytoplasm was detected, suggesting that maintainer lines were inadvertently mixed into the diploid CMS line during its development; this was inferred to be associated with the admixture of maintainer lines into

the maternal CMS line. This contamination was mainly caused by mechanical admixture during seed production or harvesting. When CMS lines and maintainer lines were planted in close proximity, harvest contamination was likely to occur. A single maintainer seed mixed into the CMS line could develop into a maintainer mother root, which would further produce a large number of maintainer seeds, eventually leading to a certain proportion of maintainer plants in the diploid CMS population. It should be emphasized that the admixture of maintainer lines into CMS lines is one of the key risks affecting the purity of sugar beet hybrids, whereas the reverse admixture (i.e., CMS lines into maintainer lines) has no adverse impact on the stability and purity of diploid CMS lines.

Similarly, in the identification of the *Rf1* locus in the nuclear genome, a target band of 1800 bp was amplified in 93.27% (277 plants) of the materials, indicating that most materials possessed the expected nuclear fertility genotype. However, a composite band pattern of 1800/1300 bp was detected in 6.73% (20 plants) of Lines 4 to 7, suggesting that restorer alleles had introgressed. This contamination phenomenon may be attributed to two aspects: first, during the development of monogerm diploid CMS lines, since sugar beet is an outcrossing crop with a pollen dispersal distance of up to 1000–2000 m, insufficient isolation distance between CMS lines and restorer lines during planting may lead to pollination by foreign restorer pollen, resulting in nuclear gene contamination; second, in the nuclear fertility gene background of maintainer lines themselves, individual plants may retain restorer genes, although this probability is relatively low. Regardless of the pathway, it will cause some individuals in the diploid CMS lines (used as the female parent of hybrids) to carry restorer genes and produce pollen, thereby affecting the overall purity of sugar beet hybrids.

At the allelic level, an amplification band of 1800 bp obtained using the s17 marker in this study was basically identified as carrying homozygous recessive nuclear sterility-related genes. Further restriction enzyme digestion showed that only the 4/4 genotype was confirmed as a candidate material carrying homozygous recessive nuclear sterility-related genes; currently, whether the 5/5 and 4/5 genotypes represent homozygous recessive sterility genes remains unclear and requires further verification. This genotyping result was consistent with the findings of Moritani et al. [24], who studied the *Rf1* locus in sugar beet using CAPS markers—they also found that the dd(L) genotype (corresponding to the 4/4 genotype in this study) was highly associated with the maintainer line genotype, while no maintainer lines were detected in other band patterns, verifying the applicability of the s17 marker in monogerm sugar beet CMS lines. Meanwhile, the phenomenon of restorer gene admixture observed in this study was similar to that reported by Taguchi et al. [22] in polygerm sugar beet CMS lines, where restorer gene introgression was also identified as one of the main factors affecting the purity of CMS lines.

Notably, compared with traditional hybrid identification methods, the molecular marker system adopted in this study can complete fertility identification at the seedling stage, shortening the breeding cycle by 3 years and significantly improving breeding efficiency. However, this study also has certain limitations: due to the limited sample size for restriction enzyme digestion verification, only 5 plants were randomly selected from each line (35 plants in total) for *Hap* II + *Hind* III double enzyme digestion verification. The obtained genotype frequencies (17.1% for 5/5 genotype, 8.6% for 4/5 genotype, and 74.3% for 4/4 genotype) only reflect the distribution of the verified samples and have not been extrapolated to all 277 plants. Therefore, this study does not provide conclusions, such as the restorer gene frequency of each line, that require large-sample support. Future studies should expand the sample size and combine field phenotype surveys to further verify the universality of this marker system.

From a cross-crop perspective, male sterility is not only a core bottleneck in sugar beet breeding but also a common constraint on the genetic improvement of other high-value crops such as soybean. As a typical self-pollinating crop, the utilization of heterosis in soybean must rely on a complete CMS "three-line" system, which has not yet been maturely promoted. The main reasons are as follows: the scarcity of CMS line germplasm resources with uneven combining ability and a prolonged breeding cycle of 6–9 years; the rarity and poor stability of restorer lines—for some restorer lines, the fertility of the resulting hybrid F_1 is significantly affected by short light conditions, and the pollen fertility rate of weak restorer lines can drop sharply from 30–40% to 0.2–11.9%, resulting in almost no pod setting; and the complex interaction between CMS lines and restorer lines increases the difficulty in screening strong heterotic combinations [29]. In contrast, as a crop utilizing vegetative organs, sugar beet does not require the participation of restorer lines, leading to a relatively simplified breeding pathway. The existence of this common male sterility challenge suggests that the TR1 + s17 molecular marker identification system established in this study is not only applicable to the purity control of sugar beet CMS lines but also can provide technical reference for the breeding of CMS lines and the purification of parents in related crops such as soybean.

In summary, the TR1 + s17 molecular marker system established in this study can effectively identify the fertility genotypes of monogerm diploid CMS lines of sugar beet, thereby providing reliable technical support for the purity control of female parents in hybrid seed production. In the subsequent seed propagation process, isolation measures should be further strengthened, and parent purification should be carried out in combination with molecular markers to reduce the risk of admixture of maintainer lines and restorer genes from the source.

5. Conclusions

In this study, TR1 and s17 molecular markers were used to systematically identify the cytoplasmic and nuclear fertility genotypes of 297 plants from seven monogerm diploid CMS lines of sugar beet, provided by three Chinese research institutions. The results of cytoplasmic identification showed that the proportion of S-type cytoplasm in Lines 2 to 7 was 100% with stable sterility, and 6.67% of N-type cytoplasm was detected in Line 1, suggesting that maintainer lines had been mixed into this line during its development, mainly due to mechanical admixture during seed production or harvesting. In the identification of the nuclear *Rf1* locus, a target band of 1800 bp was amplified in 93.27% (277 plants) of the materials; among these, Lines 1 to 3 all showed a single 1800 bp band pattern with homozygous and consistent nuclear fertility genotypes. A composite band pattern of 1800/1300 bp was detected in 6.73% (20 plants) of Lines 4 to 7, indicating the introgression of restorer alleles, which may be related to foreign restorer pollen contamination or residual restorer genes in the maintainer parent lines. Double enzyme digestion verification based on 35 samples showed that only the 4/4 genotype (double bands of 1000 bp + 700 bp) could be confirmed as a candidate material carrying homozygous recessive nuclear sterility-related genes (*rf1*/*rf1*), while the corresponding relationship between the 5/5 and 4/5 genotypes and recessive nuclear sterility genes remains unclear and requires further verification through subsequent field experiments.

This study confirmed that the TR1 + s17 molecular marker system can quickly and accurately identify the fertility genotypes of monogerm diploid CMS lines of sugar beet at the seedling stage. Compared with traditional hybrid identification methods, it shortens the breeding cycle by 3 years, significantly improving breeding efficiency. To ensure the purity of three-way hybrids, it is recommended that isolation measures be strengthened during the parent propagation process and that molecular marker-assisted purification be integrated

to eliminate the risks of maintainer line admixture and restorer gene introgression from the source. Future studies should expand the sample size and combine field phenotype surveys to further verify the universality of this marker system, thereby improving the accuracy of identification.

Author Contributions: Conceptualization, Z.W. and L.S.; Methodology design, Z.W.; Supervision, L.S.; Data collection, R.S.; Data analysis, R.S.; Data interpretation, R.S.; Writing—original draft, R.S.; Writing—review and editing, all authors; Funding acquisition, Z.W. and L.S.; Final approval of the version to be published, Z.W. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by Breeding of Three-line Hybrid Sugar Beet Dominant Combination (2024DB018): National Sugar Industry Modern Agricultural Industry Technology System (CARS-17).

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

Acknowledgments: We extend our sincere thanks to our fellow team members for their collaborative efforts, as well as to all who provided guidance and support throughout this project.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Pathak, A.D.; Srivastava, S.; Misra, V.; Mall, A.K.; Srivastava, S. Evolution and history of sugar beet in the world: An overview. *Sugar Beet Cultiv. Manag. Process* **2022**, 3–10. [CrossRef]
2. Dohm, J.C.; Lange, C.; Holtgräwe, D.; Sörensen, T.R.; Borchardt, D.; Schulz, B.; Lehrach, H.; Weisshaar, B.; Himmelbauer, H. Palaeohexaploid ancestry for Caryophyllales inferred from extensive gene-based physical and genetic mapping of the sugar beet genome (*Beta vulgaris*). *Plant J.* **2012**, *70*, 528–540. [CrossRef]
3. Tomaszewska, J.; Bieliński, D.; Binczarski, M.; Berlowska, J.; Dziugan, P.; Piotrowski, J.; Stanishevsky, A.; Witońska, I.A. Products of sugar beet processing as raw materials for chemicals and biodegradable polymers. *RSC Adv.* **2018**, *8*, 3161–3177. [CrossRef] [PubMed]
4. Amirahmadi, E.; Ghorbani, M.; Krexner, T.; Hörtenhuber, S.J.; Bernas, J.; Neugschwandtner, R.W.; Konvalina, P.; Moudrý, J. Life cycle assessment of biochar and cattle manure application in sugar beet cultivation–Insights into root yields, white sugar quality, environmental aspects in field and factory phases. *J. Clean. Prod.* **2024**, *476*, 143772. [CrossRef]
5. Wang, L.; Song, B.; Ishfaq, M.; Zhao, X. Optimization of nitrogen fertilizer application enhanced sugar beet productivity and socio-ecological benefits in China: A meta-analysis. *Soil Tillage Res.* **2025**, *251*, 106547. [CrossRef]
6. Varga, I.; Lončarić, Z.; Kristek, S.; Kulundžić, A.M.; Rebekić, A.; Antunović, M. Sugar Beet Root Yield and Quality with Leaf Seasonal Dynamics in Relation to Planting Densities and Nitrogen Fertilization. *Agriculture* **2021**, *11*, 407. [CrossRef]
7. Dohm, J.C.; Minoche, A.E.; Holtgräwe, D.; Capella-Gutiérrez, S.; Zakrzewski, F.; Tafer, H.; Rupp, O.; Sörensen, T.R.; Stracke, R.; Reinhardt, R.; et al. The genome of the recently domesticated crop plant sugar beet (*Beta vulgaris*). *Nature* **2014**, *505*, 546–549. [CrossRef]
8. Romeiras, M.M.; Vieira, A.; Silva, D.N.; Moura, M.; Santos-Guerra, A.; Batista, D.; Duarte, M.C.; Paulo, O.S. Evolutionary and Biogeographic Insights on the Macaronesian *Beta-Patellifolia* Species (Amaranthaceae) from a Time-Scaled Molecular Phylogeny. *PLoS ONE* **2016**, *11*, e0152456. [CrossRef]
9. Taguchi, K.; Kuroda, Y.; Okazaki, K.; Yamasaki, M. Genetic and phenotypic assessment of sugar beet (*Beta vulgaris* L. subsp. vulgaris) elite inbred lines selected in Japan during the past 50 years. *Breed. Sci.* **2019**, *69*, 255–265. [CrossRef]
10. Dhar, B.R.; Elbeshbishy, E.; Hafez, H.; Lee, H.S. Hydrogen production from sugar beet juice using an integrated biohydrogen process of dark fermentation and microbial electrolysis cell. *Bioresour. Technol.* **2015**, *198*, 223–230. [CrossRef]
11. Misra, V.; Mall, A.K.; Pandey, H.; Srivastava, S.; Sharma, A. Advancements and prospects of CRISPR/Cas9 technologies for abiotic and biotic stresses in sugar beet. *Front. Genet.* **2023**, *14*, 1235855. [CrossRef]
12. Alavilli, H.; Yolcu, S.; Skorupa, M.; Aciksoz, S.B.; Asif, M. Salt and drought stress-mitigating approaches in sugar beet (*Beta vulgaris* L.) to improve its performance and yield. *Planta* **2023**, *258*, 30. [CrossRef]
13. Wang, L.; Zhang, Z.; Han, P.; Liang, Y.; Zhang, H.; Fu, Z.; Zhao, S.; E, Y.; Zhang, H.; Wu, X.; et al. Association analysis of agronomic traits and construction of genetic networks by resequencing of 306 sugar beet (*Beta vulgaris* L.) lines. *Sci. Rep.* **2023**, *13*, 15422. [CrossRef]

14. Ćirić, M.; Popović, V.; Prodanović, S.; Živanović, T.; Ikanović, J.; Bajić, I. Sugar beet: Perspectives for the future. *Sugar Tech* **2024**, *26*, 1208–1219. [CrossRef]
15. Khan, Z.S.; Wani, S.A.; Aijaz, T. *Beetroot: Cultivation, Processing, and Food Applications*; CRC Press: Boca Raton, FL, USA, 2026.
16. Li, H.; Li, N.; Pi, Z.; Wu, D. Analysis of Phenotypic Variation and Genetic Diversity in 107 Sugar Beet Germplasm Resources. *J. Plant Genet. Resour.* **2025**, *26*, 1956–1966.
17. Chen, S.; Zhang, C.; Liu, J.; Ni, H.; Wu, Z. Current status and prospects of the global sugar beet industry. *Sugar Tech* **2024**, *26*, 1199–1207. [CrossRef]
18. Wang, M.; Yao, J.; Wang, M.; Yang, Y.; Chen, Q.; Liu, Y.; Ren, Y.; Bai, H.; Li, X.; Xu, B.; et al. Discussion and Analysis on Development of Beet Sugar Industry in Baotou, Inner Mongolia. *Sugar Crops China* **2024**, *46*, 96–102.
19. Han, A.; Xiu, J.; Tang, G.; Chang, Y.; Shi, B.; Liang, H.; Liu, J.; Sun, F.; Li, D. Research Progress on Cytoplasmic Male Sterility in Sugar Beet. *Bot. Res.* **2025**, *14*, 10–20.
20. Zhao, R.; Pi, Z.; Kan, L.; Wu, D. Certifying Heredity Purity of Monogerm Cytoplasmic Male Sterile and Maintainer Sugar Beet. *Fujian J. Agric. Sci.* **2023**, *38*, 417–422.
21. Chen, P.; Chen, Y.; Pi, Z.; Li, N.; Wu, D. Genetic Diversity Analysis of Monogerm Cytoplasmic Male Sterile and Maintainer Lines of Sugar Beet. *Agronomy* **2024**, *14*, 2217. [CrossRef]
22. Taguchi, K.; Hiyama, H.; Yui-Kurino, R.; Muramatsu, A.; Mikami, T.; Kubo, T. Hybrid Breeding Skewed the Allelic Frequencies of Molecular Variants Derived from the Restorer of fertility 1 Locus for Cytoplasmic Male Sterility in Sugar Beet (*Beta vulgaris* L.). *Crop Sci.* **2014**, *54*, 1407–1412. [CrossRef]
23. Nishizawa, S.; Kubo, T.; Mikami, T. Variable number of tandem repeat loci in the mitochondrial genomes of beets. *Curr. Genet.* **2000**, *37*, 34–38. [CrossRef] [PubMed]
24. Moritani, M.; Taguchi, K.; Kitazaki, K.; Matsuhira, H.; Katsuyama, T.; Mikami, T.; Kubo, T. Identification of the predominant nonrestoring allele for Owen-type cytoplasmic male sterility in sugar beet (*Beta vulgaris* L.): Development of molecular markers for the maintainer genotype. *Mol. Breed.* **2013**, *32*, 91–100. [CrossRef]
25. Tang, B.; Xiao, H.; Wang, L.; Chen, Y. A Foundation Seed Production Method to Ensure Seed Purity of Rice PTGMS Lines by Fertility Identification of Plant Lines. *Hybrid. Rice* **2010**, *25*, 25–27+33.
26. Zhang, Y., III. Analyses of Traits Associated with Outcrossing and Method for Identifying Outcrossing Rate in CytoplasmicMale Sterile Soybean. Ph.D. Dissertation, Northeast Agricultural University, Harbin, China, 2018.
27. Yang, H.; Du, J.; Jia, Y.; Qin, N.; Li, Y. Prediction of fertility conversion rate of wheat BNS male sterile lines based on negative accumulated temperature in winter. *J. Gansu Agric. Univ.* **2021**, *56*, 31–35+42.
28. Yan, Y.; Zou, Y.; Wu, D.; Xing, W.; Li, X. A Method for Large-scale Rapid Extraction of DNA from Sugar Beet. *Sugar Crops China* **2018**, *40*, 44–46.
29. Bai, Y. Transformation of Cytoplasmic Male-Sterile Lines and Transcriptome Analysis of Male Sterility and F1 Male Fertility Recovery in Soybean [*Glycine max* (L.) Merr.]. Ph.D. Dissertation, Nanjing Agricultural University, Nanjing, China, 2022.

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.