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Germination Genetics of SSR Polymorphism Marker and Purity Identification of Hybrid Seeds in Wax Gourd (*Benincasa hispida*)

Jieying Chen, Zhengguo Liu ✉, Chunling Wang, Qingming Chen, Zhikui Cheng, Lianlian Ma, Jiquan Gou
Agricultural College, Guangxi University, Nanning, 530001, P.R. China
✉ Corresponding author email: liu-zhengguo@126.com
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Abstract Seed purity identification is an indispensable process before seeds are used in production and is the key to ensuring seed quality. Developing an accurate, effective, inexpensive and convenient method for Wax gourd seeds purity identification can provide technical support for seed quality. The co-dominant primers of XD-18 and XD-99 were chosen to test the seed purity of 'Jinyuan 2', 'Jinyuan 3' and 'Lvxianzi 2' 3 Wax Gourd hybrids in this research. The F_2 generations of Wax Gourd hybrids was used to analyze whether the 2 SSR markers were in accordance with Mendelian separation law. If it is in accordance with Mendelian separation, then 2 pairs of primers were used to carry out SSR molecular marker purity identification of hybrid seeds of Wax gourd, and a comparison experiment was performed between the SSR molecular marker purity identification and the field planting purity identification. The results showed that the 2 SSR markers were in accordance with the Mendelian separation law of 1: 2: 1 in the F_2 generation population and SSR molecular marker purity identification of Wax gourd hybrid seeds was highly consistent with the field planting purity identification. Therefore, this study provides a more accurate technical system for the purity identification of Wax gourd seeds and other crop hybrid seeds.
Keywords Wax gourd; SSR marker; Mendelian separation; Hybrid purity

The wax gourd (*Benincasa hispida* Cogn.) is an annual climbing plant of Cucurbitaceae. Chieh-qua (*Benincasa hispida* Cogn. var. chieh-qua How.) is generally considered as a variety of the wax gourd, a common vegetable in southern China and cultivated in India, Vietnam, Thailand and other Asian countries. Wax gourd hybrid has been widely used in production, but the purity of hybrid seeds will be reduced due to lax isolation of artificial seed production, delayed emasculation, improper cross pollination and other factors. The methods of hybrid purity identification mainly include cytology, morphology, biochemical marker and molecular marker, among which molecular marker is a detectable polymorphic site of DNA sequence, which can directly reflect the differences of DNA.

SSR (Simple sequence repeat) molecular markers have the advantages of co-dominance, good repetitiveness, high polymorphism, and are not affected by conditions such as external environment, tissue and development. It has been repeatedly used in rice (Moorthy et al., 2011), broccoli (Yu et al., 2013), cauliflower (Zhao et al., 2012), melon (Ai et al., 2005; http://en.cnki.com.cn/Article_en/CJFDTotal-FZZW2006S2005.htm), watermelon (Li et al., 2015, Chinese Agricultural Science Bulletin, 31(33): 177-185; Lu et al., 2018), maize (Li et al., 2018), cotton (Selvakumar et al., 2010; Fu et al., 2017), sunflower (Pallavi et al., 2011) and other crops hybrid seed purity identification. SSR molecular markers of wax gourd were mainly applied in genetic analysis of pericarp color (Zhu et al., 2016) and genetic diversity analysis of germplasm resources. At present, most of the studies using molecular markers to identify the purity of hybrid seeds have not carried out genetic analysis of co-dominant markers, which may lead to two results: first, the co-dominant markers used for purity identification may not conform to Mendelian separation. Second, if the parental inbred lines are selected only according to the plant morphology, the co-dominant markers of their parents may not be completely homozygous, and if such molecular markers are used for purity identification, the purity error will be too large. The genetic analysis of codominant markers and the use of SSR codominant

markers in accordance with Mendelian separation for seed purity identification can improve the accuracy of identification and reduce the purity error.

Therefore, in this study, 'Jinyuan 2', 'Jinyuan 3' wax gourd and 'Lvxianzi 2' Chieh-qua and their parents and F_2 generation were used as experimental materials. Firstly, the F_2 generations was used to analyze whether the co-dominant marker was in accordance with Mendelian separation, and then the marker was applied to the purity identification of the F_1 generation. And it was compared with grow out test (GOT) to provide a new and accurate technical system for the identification of hybrid seed purity.

1 Results and Analysis

1.1 Parental codominant markers were obtained by screening

The polymorphic primers XD-18 (F: 5'-TatttCCTCGGGGGGCTTG-3'; R:5'-GTCATTGGATCTTGGCCACT-3') and XD-99 (F: 5'-AGTGAAAAAGTGGCGGTTTG-3'; R: 5'-TGGAGAGGTGAAAGGTGAAAGATTCG-3') were chosen to identify the purity of 'Jinyuan 2', 'Jinyuan 3' and 'Lvxianzi 2' 3 wax gourd hybrids. The results showed that XD-18 primers could simultaneously amplify different characteristic bands of the parents in 'Jinyuan 2' wax gourd (F_1 generation), and XD-99 primers could simultaneously amplify different characteristic bands of the parents in 'Jinyuan 3' wax gourd (F_1 generation) and 'Lvxianzi 2' Chieh-qua (F_1 generation).

XD-18 primers amplified 200 bp and 170 bp characteristic bands for YMY-24-1 (female parent), 180 bp and 150 bp characteristic bands for YO-16-1 (male parent), and co-dominant complementary bands of female parent and male parent were simultaneously amplified for 'Jinyuan 2' wax gourd (F_1 generation) (Figure 1A).

XD-99 primers amplified 140 bp characteristic bands for YMY-24-1 (female parent) and 200 bp characteristic bands for YS-4-1 (male parent). The parent also presented co-dominant complementary characteristic bands, and 'Jinyuan 3' (F_1 generation) also had the parent bands (Figure 1B).

XD-99 primers amplified 180 bp characteristic bands for GK-3-4-3-2-1-3 (female parent) and 220 bp characteristic bands for 7-2-1-3-2-2-1 (male parent). Both parents showed co-dominant complementary characteristic bands. Its 'Lvxianzi 2' Chieh-qua (F_1 generation) has both parental bands (Figure 1C).

The results showed that the SSR primers XD-18 and XD-19 showed co-dominant characteristics, and the F_1 generation showed complementary bands of parents, which could effectively distinguish the hybrids from their parents, indicating that the two SSR markers could be used to identify the hybrid seed purity of wax gourd.

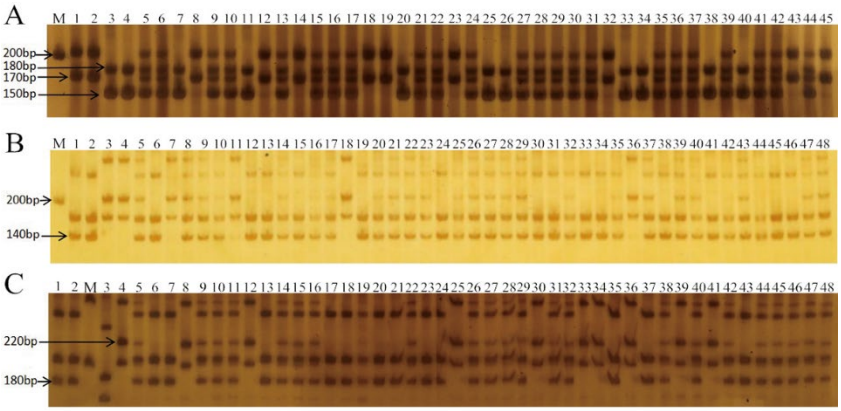

Figure 1 Partial electrophoresis amplification of parent, F_1 and F_2 different single plants of 3 varieties of wax gourd by XD-18 and XD-99 primers

Note: M: DL1000 DNA marker; A: Electrophoresis result of 'Jinyuan 2' by XD-18; 1~2: YMY-24-1 female parent; 3~4: YO-16-1 male parent; 5: F_1; 6~45; B: F_2 individuals; Electrophoresis result of 'Jinyuan 3' by XD-99; 1~2: YMY-24-1 female parent, 3~4: YS-4-1 male parent; 5: F_1, 6~48: F_2 individuals; C: Electrophoresis result of "Lvxianzi 2" by XD-99; 1~2: GK-3-4-3-2-1-3 female parent; 3: Other variety, 4: 7-2-1-3-2-2-1 male parent; 5: F_1; 6~48: F_2 individuals

1.2 F_2 generation genetic analysis of SSR markers

The co-dominant markers XD-18 and XD-99 were used for genetic analysis of SSR polymorphism markers in the F_2 generation of three wax gourd varieties (Figure 1). After color development, the "ABH" method was used for statistical analysis of the band types, and the chi-square test was used to detect the SSR co-dominant separation ratio. The results (Table 1) showed that the marker separation of the three F_2 generation populations did not significantly deviate from 1:2:1 Mendelian separation. These results indicated that the co-dominant markers XD-18 and XD-99 had a clear genetic mechanism and could effectively reduce the error in the identification of hybrid purity.

Table 1 The separation of co-dominant with SSR markers for 3 varieties

The co-dominant separation with SSR markers in F_2 generation	Observed sample	Observed value			Excepted separation	X^2	P value
		A-value	H-value	B-value			
Jinyuan 2 (primer XD-18)	172	36	96	40	1:2:1	2.512	0.285
Jinyuan 3 (primer XD- 99)	140	32	78	30	1:2:1	1.886	0.390
Lvxianzi 2 (primer XD-99)	180	40	96	44	1:2:1	0.978	0.613

Note: A: Female genotype; H: Heterozygous genotype; B: Male genotype; When P=0.05, X^2=5.991, df=2

1.3 Comparison of SSR purity identification and field planting purity identification

XD-18 and XD-99 primers were used for SSR purity identification of 'Jinyuan 2' (Figure 2A), 'Jinyuan 3' (Figure 2B) and 'Lvxianzi 2' (Figure 2C), and comparison experiments were performed between the SSR molecular marker purity identification and the field planting purity identification. The results (Table 2) showed that the purity identification results of SSR molecular markers were highly consistent with the purity identification results of field planting.

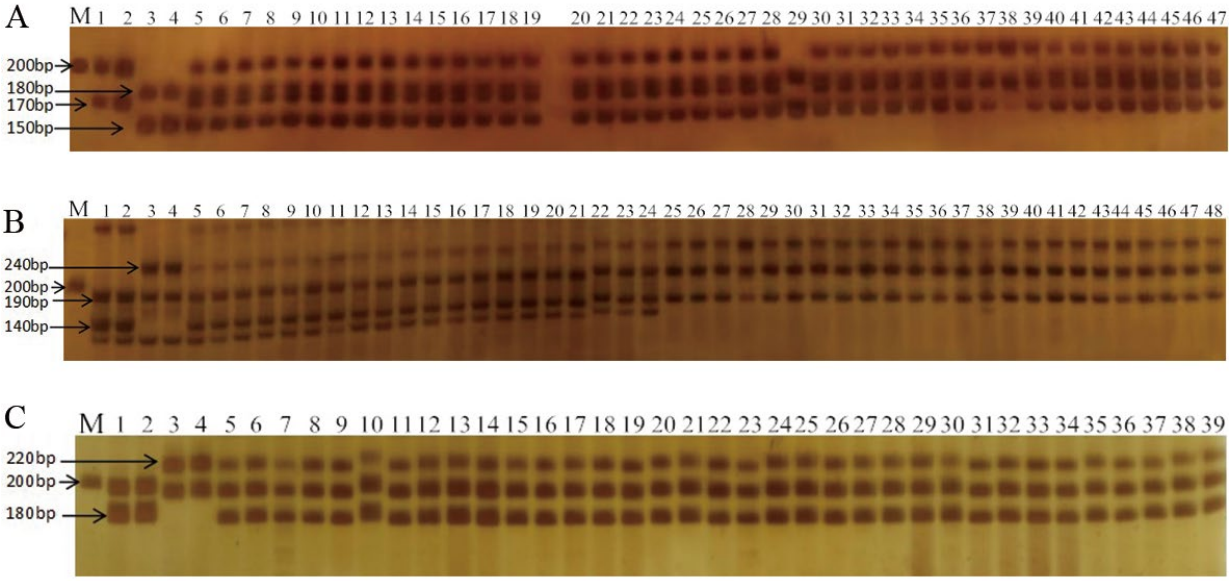

Figure 2 Purity identification of 3 wax gourd varieties by SSR primers

Note: M: DL1000 DNA marker; A: Electrophoretic results of purity identification of "Jinyuan 2"; 1~2: Female parent; 3~4: Male parent; 5~47: F_1 different individuals; B: Electrophoretic results of purity identification of "Jinyuan 3"; 1~2: Female parent; 3~4: Male parent; 5~48: F_1 different individuals; Due to the use of different 2 × *Taq* master mix (PAGE) in the front and back lanes, the first half of the lanes have one more band, which has no effect on the determination of hybrid purity; C: Electrophoretic results of purity identification of "Lvxianzi 2"; 1~2: Female parent; 3~4: Male parent; 5~39: F_1 different individuals

Table 2 Comparison of SSR purity identification and field purity identification of 3 varieties

Variety	Seed batches	SSR purity test (%)	Field purity test (%)	*P* value
Jinyuan 2	2018①	98	99	0.968
(Primer XD-18)	2018②	67	68	
Jinyuan 3	2018③	96	96	0.293
(PrimerXD-99)	2019①	95	97	
Lvxianzi 2	2018①	99	99	0.771
(PrimerXD-99)	2018②	94	96	

Note: When $p < 0.05$, the difference is significant; ①~③ represent different seed batches produced at different times and locations

2 Discussion

Seed purity identification is the key to ensure the quality of the seed, compared to cover an area of work, long cycle, high cost, affected by the environment of the field planting purity identification of hybrid approach, using SSR markers purity identification of hybrid seeds, not only time-consuming, short, low cost, and more simple, easy to operate, is the better method of hybrid seed purity identification. It is not only widely used in the purity identification of commercial crops such as rice, maize and cotton, but also in the purity identification of gourd seeds of Cucurbitaceae such as melon, watermelon and wax gourd. Ai et al. (2005) took 2 melon hybrids 'Dongfangmi 1' and '01-31' as experimental materials and screened 8 pairs of polymorphism primers from 23 pairs of SSR primers for purity identification. The experiment showed that the identification results of molecular marker purity were highly consistent with the identification results of field planting purity. Lu et al. (2018) screened three highly effective SSR markers CLSSR09643, CLSSR18153 and CLSSR01623 for the identification of watermelon hybrids purity. The purity measured by SSR marker was all over 96%, while the field planting purity was over 98%, and the two purity identification results were consistent. Chen et al. (2020) used 'Lvyou' and 'Meihua 8' wax gourd as experimental materials and used two pairs of SSR primers to verify the application feasibility of SSR molecular markers to identify the seed purity of wax gourd. However, none of the current purity identification studies used F_2 generation for genetic analysis of the screened SSR markers, which could not ensure that SSR markers in offspring were in accordance with Mendelian separation, which would affect the purity identification results of hybrids.

Due to the influence of experimental techniques and human errors, such as the determination of co-dominant homozygosity of materials, DNA extraction, the setting of DNA-PCR program, the staining of electrophoresis gel, etc., SSR markers may not show co-dominant or not accord with Mendelian separation in offspring. Liu et al. (2016) found in their study that 301 CAPS and SSR markers were co-dominant between parental parents, but there were 26 pairs of molecular markers that seriously deviated from Mendelian separation in F_2 generation. SSR markers deviated from Mendelian separation or did not conform to Mendelian separation in F_2 generation, resulting in unclear genetic mechanism, which may reduce the accuracy and feasibility of SSR markers in the identification of hybrid seed purity.

In this research, ‘Jinyuan 2’ wax gourd, ‘Jinyuan 3’ wax gourd and ‘Lvxianzi 2’ Chieh-qua were used as materials to select markers showing codominance in parents and hybrid generation. Then F_2 generation was used for genetic analysis of codominant markers, and the chi-square test was used to determine whether the codominant markers were in accordance with Mendelian segregation. If it is in accordance with Mendelian separation, then primers were used to carry out SSR molecular marker purity identification of hybrid seeds. The error of SSR purity identification was effectively reduced. After that, two markers were used to identify the purity of multiple batches of wax gourd F_1 generation. The results were compared with field morphological identification, which proved that there was no significant difference in the accuracy of SSR marker purity identification and field planting purity identification. This technology system can minimize the overall experimental error, and can be used not only for hybrid purity identification of wax gourd, but also for hybrid purity identification of other plants, laying a foundation for more rigorous and accurate hybrid seed purity identification technology in the future.

Although 1 pair of co-dominant SSR markers is sufficient to identify hybrids from their parents, and most studies only use 1 pair of SSR markers to identify seed purity, 1 pair of SSR markers may not be able to correctly distinguish true and false hybrids in the case of mixed parents. Multiple pairs of markers are required to compare and distinguish. In this study, only one pair of co-dominant SSR markers was used for genetic analysis and identification of seed purity of a single wax gourd variety. In the future, more SSR primers should be developed and several pairs of SSR markers should be used for genetic analysis and identification of seed purity of a single wax gourd variety to further improve the accuracy of identification of hybrid purity of wax gourd.

3 Materials and Methods

3.1 Experimental materials

The wax gourd varieties and their parents and seeds of F_2 generation provided by Nanning Kenong Seedling Co., Ltd were used as materials. Varietal 1: 'Jinyuan 2' wax gourd (F_1 generation), YMY-24-1 (female parent), YO-16-1 (male parent); Variety 2: 'Jinyuan 3' wax gourd (F_1 generation), YMY-24-1 (female parent), YS-4-1 (male parent); Variety 3: ‘Lvxianzi 2’ Chieh-qua (F_1 generation), GK-3-4-3-3-4-3 (female parent), 7-2-1-7-2-1-1 (male parent). In 2018, these 3 varieties (F_1 generation) were planted in field (Nanning, Guangxi), with 30 plants for each, and the seeds of F_2 generation were obtained by self-crossing of each plant. Fifty seeds were collected from the parents of the three cultivars. There were 100 seeds in F_1 generation and the seeds were repeated for 3 times. Two hundred seeds of F_2 generation were placed in a petri dish covered with filter paper and expanded in an incubator at 30℃ to cotyledon for single DNA sample extraction.

3.2 Genomic DNA Extraction

Genomic DNA was extracted by the improved CTAB method (An et al., 2011), and purity and integrity of DNA were detected by 0.8% agarose gel electrophoresis.

3.3 PCR amplification and electrophoresis analysis

PCR (Polymerase chain reaction) reaction system 20 μL, including 10 μL of 2×*Taq* Master Mix for PAGE, 2 μL of DNA template (50 ng/μL), 2 μL positive and reverse SSR primers (50 ng/μL), 6 μL ddH_2O. The PCR amplification procedure was as follows: pre-denaturation at 95℃ for 5 min, denaturation at 95℃ for 30 s, annealing at 56℃ for 45 s, extended at 72℃ for 1 min 30 s. After 35 cycles, it was extended at 72℃ for 7 min and stored at 4℃. PCR amplified DNA was electrophoresis with 8% polyacrylamide gel, and silver staining was performed (Bassam et al., 1991) and band was recorded.

3.4 Identification of hybrid seed purity

Purity identification by SSR molecular markers: Hybrids were identified by primers with polymorphism between parent materials. In the identification results of F_1 generation, the offspring with male parent and female parent characteristic bands were true hybrids, while the offspring with only female parent or male parent characteristic bands were false hybrids or self-crosses. SSR markers were used for genetic analysis of the F_2 generation, and genotyping results (female parent genotype, heterozygous genotype, and male parent genotype) of the F_2 generation were observed and counted to calculate whether the marker separation ratio was consistent with Mendelian separation.

Field planting purity identification: 10 plants were planted by each parent as hybrid plants for comparison of phenotypic traits. 100 plants of F_1 generation were planted in each plot with 3 replicates. When the fruits were fully developed and mature, the phenotypic traits of the plants were observed and counted for purity identification.

Authors’ contributions

CJY completed the experiment design, experiment execution, data analysis, paper writing and modification of this research. LZG is the author and the person in charge of this study. He directs the experimental design, data analysis, paper writing and revision. WCL participated in data analysis and paper writing. CQM, CZK, MLL and GJQ participated in the experiment design and execution. All authors read and approved the final manuscript.

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