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Development and Purity Identification of InDel Marker Based on Re-resequencing of "modilong" Wax Gourd

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Abstract In order to establish a simple, economic, accurate and reliable method to identify the purity of wax gourd hybrid seeds, an InDel marker characterized by significant differences was developed via whole genome re-sequencing of parent plants of 'modilong' wax gourd; Then the developed InDel molecular marker was used to identify the purity of hybrid seeds of 'modilong' taking the DNA of its hybrid seeds and parent plants as test DNA, and the obtained results were compared with field identification results. A total of 466 pairs of InDel markers were screened by genome re-resequencing of parent plants of 'modilong', and 26 of which with distinct differences were selected to be amplified and both got the strip. Among them, 7 pairs of primers can clearly distinguish the purity of wax gourd samples. The purity of InDel molecular marker identification was more than 99% consistent with the results of field plot planting identification, indicating that the purity results obtained by different methods were highly consistent. The InDel molecular marker screened in this study could be used as the purity identification of wax gourd hybrids.

Keywords Wax gourd; Seed purity; Indel molecular marker

Wax gourd (*Benincasa hispida* (Thunb.) Cogn.) is an important vegetable crop of the Cucurbitaceae family, which is rich in nutrients, with medicinal and health benefits, and is a healthy food with high potassium and low sodium (Jiang et al., 2014). Wax gourd has good storage and transportation, long shelf life and high yield, which has become one of the main vegetables to regulate the annual balanced supply of the market (Jiang et al., 2013). China leads the world in the cultivation of wax gourd, with an annual sowing area of more than 320 000 hm^2. The purity of hybrid wax gourd varieties is the basis to ensure the heterosis, and also the main index to measure the seed quality of hybrid wax gourd. In the process of hybrid seed production of wax gourd, artificial emasculation is not complete or omitted, and false hybrids often appear, which leads to the decline of heterosis of hybrid varieties and huge economic losses to product. Therefore, to effectively improve the production quality of good varieties, the establishment of a set of rapid and accurate methods for identifying the purity of seeds of wax gourd hybrids is one of the urgent problems that need to be solved during the production and operation of wax gourd (Liu et al., 2019). Traditional field identification of seed purity methods is affected by various factors and have limitations in terms of timeliness and accuracy, while molecular markers can facilitate standardization, scaling and rapid identification of the purity of commodity species. In particular, the publication of wax gourd genome can provide feasibility for rapid screening of molecular markers (Xie et al., 2019). The currently reported molecular markers for indoor purity identification of wax gourd are RAPD (Lu et al., 2010) and SSR (He et al., 2017; Chen et al., 2020).

In recent years, with the rapid development of genome sequencing technology, the third generation of new molecular markers, including InDel and SNP, have emerged, which have the characteristics of economy, practicality, high specificity and good stability. InDel is widely distributed, dense and numerous in the genome. In terms of distribution density, InDel is second only to SNP, but much higher than SSR. However, SNP is limited by genotyping technology, requiring special equipment in small and medium-sized detection, with high cost and complex operation. Therefore, the application prospect of InDel marker is broad (Lu et al., 2019). InDel refers to the difference between two parents due to nucleotide insertion or deletion, which can be used to distinguish two

parents (Wang et al., 2019). InDel marker has good stability, high polymorphism and simple banding pattern, which is easier to be applied to animal and plant population genetic analysis, molecular assisted breeding and other fields (Ji et al., 2019). At present, it has been used to detect the seed purity of corn (Yao et al., 2020, Jiangsu Agricultural Sciences, 48(1): 79-84), tomato (Zhang et al., 2019), Chinese cabbage (Liu et al., 2019) and other crops, and the results are accurate and reliable.

1 Results and Analysis

1.1 Development of InDel marker and screening verification of 'Modilong' based on resequencing

Through the whole genome resequencing of the parents of 'Modilong', after comparison of the reference genome, the InDel markers were developed by using GATK, Primer 6.0 and other software. A total of 466 pairs of InDel markers were screened, of which 26 pairs were selected for primer synthesis and amplification (Table 1). The results showed that clear bands could be amplified, including chr640818838, chr41295559, chr1252341989, chr1223923504, chr717248263, chr413443261 and chr245098788, can amplify bands with significant differences among 12 male parent (L2), female parent (D6) and F1, which can be used to distinguish the purity of commercial varieties of 'Modilong' (Table 2; Figure 1).

Table 1 26 InDel markers of 'modilong' developed based on whole gene resequencing

Gene ID	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temperature (℃)	Length of the female parent (bp)	Length of the male parent (bp)
chr126151816	'GCCTCCTTCGTCTCTTCCC	'GCAGCATTCTGGGACAAGG	59	273	276
chr163310183	'TGCCAAACCACTTGTAGCCA	'ACACGGATGGAATCGGGT	60	267	269
chr257526795	'GCATGCGCTGAAGCTAGG	'TTCGATGGACGCATGCGA	59	100	98
chr245098788	'GCGTCCGACATTTGCTGC	'GGGAGCTAAGCGATGCGT	60	267	330
chr345311405	'GAATTCCGCTGCGCCATC	'TCGGGATTCACCAGTTTGTGT	60	277	269
chr41262028	'AGATCATGCGCTCCAACGA	'TTGAACAATCGGGGCCCC	59.5	270	272
chr412955559	'ACGAGGGGTGGATTCTTCA	'GCGTTCAGCTGGGTTCAC	59	272	303
chr413443261	'CCCATTCTCGTCTCTTCCCC	'TCTCCTGAATGCGGACGC	60	248	266
chr523661005	'TGGCTGTGAAAGGTGTGCA	'GGGAGTGTGGCCGATCAC	60	170	172
chr551026677	'ACCCGCCTTAGACAAAAGGT	'GCTTCGCGTTGTGGATGG	59.5	276	279
chr618300055	'CGATCGTACACCCGCACA	'GGTCTTCTCGGCGCACTA	59	91	95
chr640818838	'TTTTCACTTAGCCACCCCAA	'GGTCGGCAATCTAACGAGGA	59.5	114	135
chr611116630	'TCACAGCCAGCCTTGACC	'CCGAGGTCGAACACAGGG	59.5	215	212
Chr717248263	'AGCGATCAAGACAATCCTCGA	'CGTTCTTTCGTTGCTCATGGA	59	243	297
chr742564327	'GCGCTAAATTTGCGGGCT	'GACCAGGTGGAGTGGCAC	59	207	209
chr817904254	'CAGGGACGACACCACCAC	'ATCGCACATTGTCGCCCA	60	276	279
chr855536135	'CCCCTGGGTTACCTACATCC	'TGGTCAGTTTGTCGGCTCA	59	243	245
chr934860344	'ACGTCAAATCGCGTGTCAG	'CCGCCCCATGGACATCTC	59	245	256
chr992107509	'TCATATCGCGTCCAATCCAGT	'CCCCCTCAAGTTGGAGCA	58.5	248	250
chr1045610178	'ACTTTGTGGAAGCAGTCGC	'TGTGGGTGTCCAGCTCAC	59	259	262
chr1021383373	'TCGCTCCGCTTAGCCCTA	'GCTCCAACTCCGTATCAAGGT	60	229	231
chr1152371587	'GGCCCTTCTTTTTGCGCC	'CAGTAGCCCAACACCGCA	60	274	119
chr1221699690	'CCAATGCCTCACCCTCGG	'AGGGATGCGTTGGTGGTG	60	198	204
chr1223923504	'TGTTGTGCACGTGCTTGC	'CGAGTGGCGGCTGGATAG	60	214	261
chr1252341989	'GCACCGTGGGGCTGATAG	'AGTGGGCGTTAGTGATGGA	60	205	257
chr1252532776	'CACCCAAGCCTCCAGTGG	'CGGAGGTTTGGGTGGCTT	60	183	189

1.2 Field and Laboratory Identification of Purity of 'Modilong'

The seeds of 'Modilong' used in the test were from the Seed Production Base of Hunan Xingshu Seed Co., Ltd. Seven pairs of InDel primers, such as chr640818838, chr1252341989, chr1223923504, were used to amplify the seeds of 'Modilong'. The electrophoretogram showed "Parental Complementary Type", and the bands were clear, which could clearly distinguish hybrids from non-hybrid (Figure 2). Among them, the purity of DNA template amplified by primer chr640818838 was 100%. Primer chr1252341989 had two non-hybrids. Primer chr1223923504 had three non-hybrids, and the purity of the amplified samples with other primers was 100%.

Seven commercial varieties of 'Modilong' were selected. The purity identification results of InDel markers were different from those of field identification, but the difference was not obvious. At present, the purity of national standard wax gourd for field use is 96% (Zhao et al., 1996, National Standards of the People's Republic of China: Melon Seeds, GB16715-1996). The mean value of indoor identification results and field identification showed that the purity of sample 1 is not up to standard, while the purity of sample 2-7 meets the national standard, which can be applied to the promotion and planting of commercial varieties.

Table 2 Molecular Markers and Field Identification of Purity of 'Modilong' Hybrids

Primer	Sample						
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7
chr640818838	92.10 %	97.70 %	98.76 %	98.70 %	100 %	96.40 %	100 %
chr412955559	93.6 %	96.5 %	98 %	98.3 %	100 %	96.0 %	100 %
chr1252341989	94 %	97 %	99 %	96 %	100 %	96.5 %	100 %
chr1223923504	93.0 %	98.7 %	98.2 %	99 %	100 %	96 %	100 %
chr717248263	92.10 %	97.70 %	98.76 %	98.70 %	100 %	96.40 %	100 %
chr413443261	92 %	96.9 %	98 %	98 %	100 %	95.8 %	100 %
chr245098788	92 %	97.3 %	97.6 %	98.7 %	100 %	97 %	100 %
Field identification results	93 %	97 %	98 %	98.5 %	100 %	96 %	100 %
Coincidence degree	99.3 %	99.8 %	99.3 %	99.5 %	100 %	99.3 %	100 %

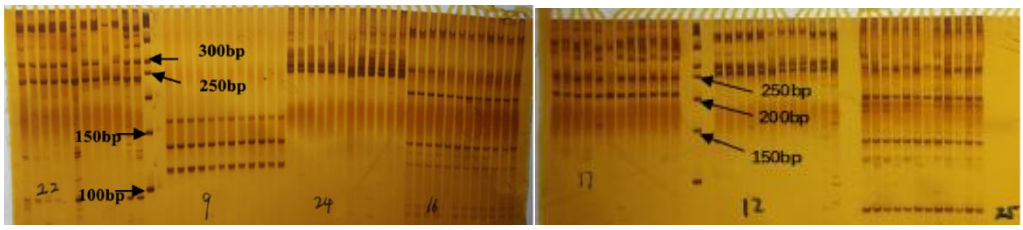

Figure 1 Electrophoretic patterns of 7 InDel-labeled PCR products of 'Modilong'

Note: Primer 9 corresponds to chr640818838; Primer 12 corresponds to chr412955559; Primer 16 corresponds to chr1252341989; Primer 17 corresponds to chr1223923504; Primer 22 corresponds to chr717248263; 24 The primer corresponds to chr413443261; the primer No. 25 corresponds to chr245098788, and the Marker is MF228 of Polymei

2 Discussion

At present, the Heterosis of wax gourd and chieh-qua has been widely used in production. The rapid and accurate identification of the purity of wax gourd is not only the technical guarantee to ensure the seed quality, but also the premise to commercialize the purity detection of varieties. It can buy time for enterprises to sell seeds, thus ensuring the interests of farmers (Chen et al., 2020). In China, the field identification method is basically used to identify the hybrid purity of wax gourd commercial varieties, that is, to judge whether the seeds are hybrid according to the morphological performance of crops. There is long detection cycle, time-consuming and labor-consuming, large land occupation area, and a limited number of morphological features that can be used to identify the purity of hybrid varieties, and most of the morphological features are vulnerable to environmental impact, so there is a certain possibility of misjudgment. It has the advantages of accuracy and rapidity to find the differences between parents through re-sequencing technology, but the markers developed by re-sequencing are not 100% different in practical application, which may be the illusion of sequence differences between the two samples, such as sequencing depth and data filtering (Zhang et al., 2016). In this study, the 26 pairs of primers selected have great differences, but there are 7 pairs of electrophoretic pairs that can be clearly distinguished during electrophoresis. Of course, it is also possible to prolong the electrophoresis time or increase the concentration of polyacrylamide gel to increase the difference in purity.

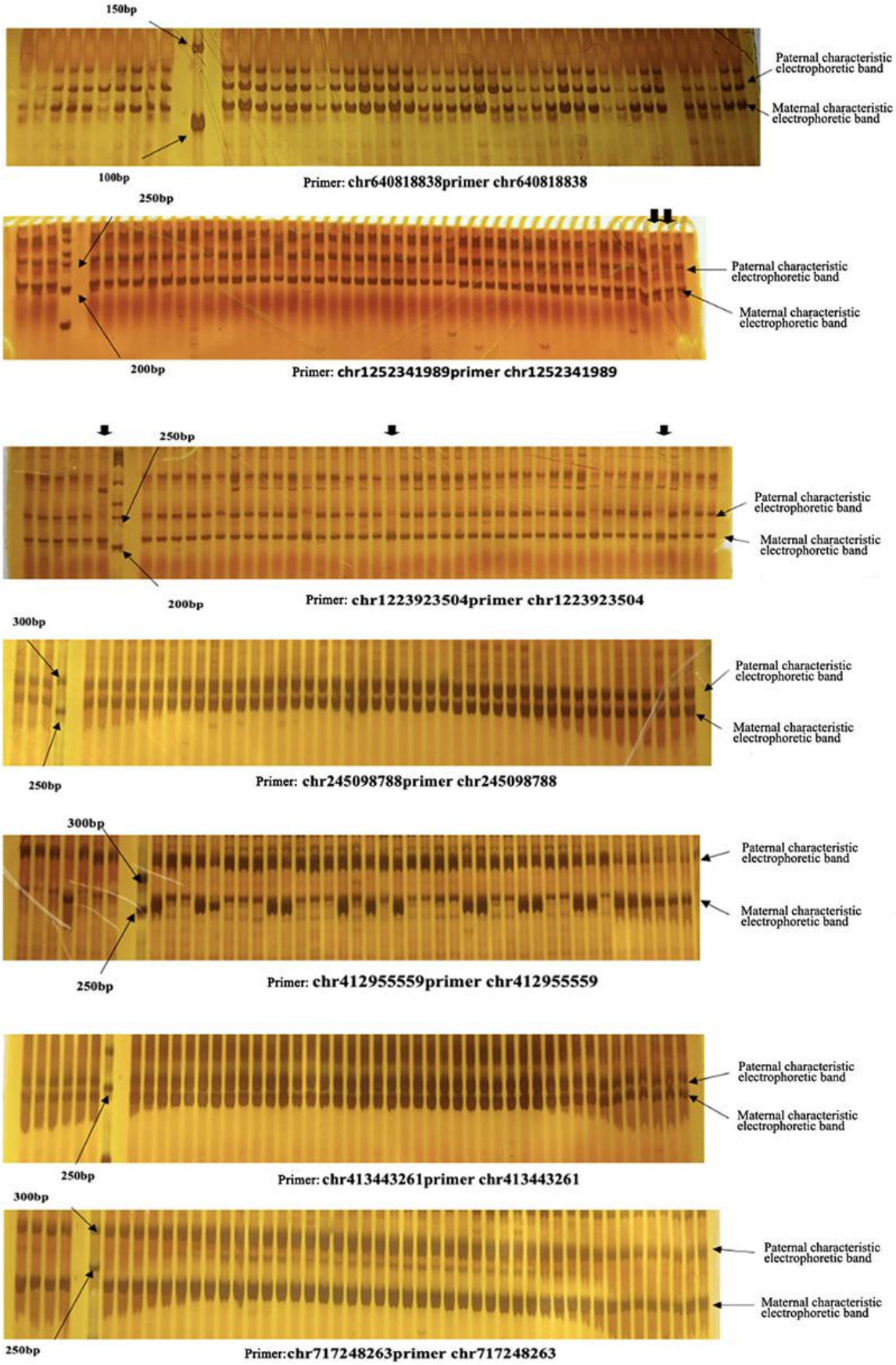

Figure 2 The electrophoresis pattern of the purity identification of the hybrids of 'Modilong' using each InDel marker
Note: The lanes marked with black arrows are non-hybrid

The difference of purity identification results between different primer sequences of "Modilong" may be because field sampling and purity sampling are not the same batch of samples, and there are some factors such as sampling errors. In addition, there are differences in DNA extraction or PCR or electrophoretic loading in the laboratory identification, resulting in shallow electrophoretic bands in some electrophoretic channels, which makes it difficult to judge hybrids and non-hybrids. Therefore, this channel is removed in the corresponding data statistics, resulting in slight differences in the purity identified by different InDel primers, but there is no significant difference between different primers, which can be used for the purity identification of wax gourd "Modilong". In this experiment, aiming at the black skin wax gourd cultivar "Modilong", by rapidly extracting the seed DNA of wax gourd, screening 7 pairs of specific primers and combining the field phenotype for variety purity identification, a more accurate and effective purity identification system was established. The time required for purity identification results was shortened from the traditional 90 d to 5~6 d, and the identification efficiency was greatly improved. The traditional field identification method can identify 1 000 plants per mu, 100 plants per sample, and the cost price is about 8 000 RMB, so the average identification cost of each sample is 800 RMB. While the application method of the invention can control the identification cost of each sample within 150 RMB, only about 20% of the traditional scheme, and the economic benefit is obvious.

3 Materials and Methods

3.1 Genome re-sequencing, InDel locus analysis and primer design of wax gourd

The total genomic DNA of wax gourd was extracted with the plant genomic DNA Extraction Kit (DP305) of Tiangen Biotech Co., Ltd., and the genome re-sequencing was completed by Beijing BerryGenomics Co., Ltd. 10x deep sequencing was carried out on the parents, respectively. After the quality control of the obtained original data was qualified, the valid sequencing data were compared with the wax gourd reference genome through BWA software (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA430006). The comparison results were deleted with SAMTOOLS. GATK software was used for InDel detection and analysis. According to the location information of InDel site on the reference genome, SnpEff software was used for annotation. The site sequences with large base number and deep sequencing depth located on the wax gourd genome are screened, and Primer 6.0 was used to design primers (Ji et al., 2019).

3.2 Extraction of genomic DNA

The DNA of 12 male parent (L2), female parent (D6) and F_1 (Modilong) were extracted by modified CTAB method (Wang et al., 2019; Yu, 2020). DNA of wax gourd seeds was extracted by alkaline method. After germination of wax gourd seeds, select about 0.5 cm root tip tissue and place it in 96 well PCR plate, add 0.1 mol/L 50 mL NaOH solution and heat it in boiling water for 5 min, then add 150 mL 0.1 mol/L Tirs-HCl for neutralization, and take 2 μL as the template for PCR amplification, and the extracted DNA can be stored at -20℃.

3.3 PCR amplification system and procedure

Using the DNA extracted by the above basic method as the template, the PCR product was obtained by PCR amplification with InDel molecular marker primers. The PCR amplification system was 10 μL, PCR system was 2×PCRmix 5 μL. F primer and R primer were 0.3 μL, respectively, template was 1.5 μL. Supplement ddH_2O to 10 μL. The PCR amplification procedure was as follows: 95℃ for 5 min, 95℃ for 30 s, 59℃~60℃ for 30 s, 72℃ for 45 s, 30~35 cycles, 72℃ for 10 min, and stored at 16℃.

3.4 Genotype statistics

The target product obtained by amplification was separated by electrophoresis with 8% non-denaturing polyacrylamide gel. After silver staining (Lin et al., 2019), the bands were observed and read on the film observation lamp, and the band types of parents and hybrid seeds were compared. The seeds with one co-dominant characteristic band for the male parent, one co-dominant for the female parent, and two characteristic bands for both parents were identified as real hybrids, The absence of any one of these bands was regarded as a false hybrid. The ratio of hybrid species to total bands is the purity of seed hybridization.

3.5 Field morphological verification

When the female flower of wax gourd opened, the preliminary investigation and statistics were carried out according to the morphological characteristics of the ovary of young wax gourd. After 20 d of female flower opening, the characteristics of wax gourd length, transverse diameter and fruit shape index were investigated again to determine the purity of 'Modilong'. Field purity=(Number of tested plants-heteromorphic plants)/Number of tested plants×l00%.

Authors' contributions

XW and WYF are the executors of the experimental design and research of this study. WRY completed data analysis and the writing of the first draft of the paper. ZHQ, ZZQ and XLL participated in the experimental design and the analysis of the experimental results. MBB is the designer and principal of the project, guiding the experimental design, data analysis, paper writing and revision. All authors read and approved the final manuscript.

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