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Purity Identification of Chemical Emasculation Hybrid Rape (*Brassica napus*) Qinyou 88 with InDel Markers

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Abstract InDel (insertion deletion length polymorphism) markers is designed for PCR detection based on the Insertion or deletion of DNA fragments of different sizes according to the sequence of a locus between different individuals of related species, which is more and more applied in crop genetic research due to its rich polymorphism, operational simplicity and reliability of results. In this study, Qinyou 88 and the parents were analyzed through InDel marker, and four pairs of candidate primers were obtained to identify the seed purity of Qinyou 88 with complementary band type and stable amplification results. Two pairs of primers ID 2 and ID 15 were verified through identified the high purity hybrids and parents, and the consistency reached 95.5%, 100% and 100%; 97.5%, 99.5 and 100%, respectively. By comparing field identification results with molecular marker identification results, the average identification results of ID 2 and ID 15 primers and field identification results were 96.14% and 95.98%, nevertheless, the analysis deviation between the two pairs of primers was only 0.84. The consistency rate between the molecular identification results and field identification results and the two pairs of primers was very high, reaching an extremely significant level. The results show that it is feasible and reliable to analyze the seed purity of Qinyou 88 using InDel molecular marker.

Keywords *Brassica napus*; Qinyou 88; Insertion deletion length polymorphism; Purity identification

Rapeseed (*Brassica napus*) is the third largest oil crop in the world followed by soybean and peanut. It is also one of the most important oil crops in China and an important source of edible vegetable oil. In recent years, with the improvement of people's living standards, the demand for vegetable oil has continued to grow, leading to the continuous growth of China's oil imports. At present, more than 60% of China's oil demand depends on imports (Wang and Yin, 2014). Under the background of the sharp reduction of cultivated land and the significant decline of rapeseed area in the world, cultivating rapeseed varieties with high and stable yield is a powerful guarantee to deal with the insufficient supply of oil crops and ensure the safety of national edible oil. Since the cultivation and promotion of "Qinyou 2", the first cytoplasmic male sterile three-line hybrid rapeseed in the world (Li and Tian, 2015), hybrid rapeseed has shown great heterosis. In the past decades, a large number of hybrid rapeseed have entered the market, enriched the germplasm resources of rapeseed and ensured the yield and quality of rapeseed. However, because the fertility of cytoplasmic male sterile lines is greatly affected by the environment, it is often accompanied by the production of micro pollen at the beginning of flowering, at the same time, the cultivation cycle is long, and a lot of human and material resources are consumed in cross selection, which is gradually replaced by the model of "high oil" + "chemical killing", which has emerged in recent years, a breeding method in which parents with high oil content are induced with chemical inducers to obtain sterile lines, and then other high oil content materials are used to provide pollen for cross selection, which has been widely used for its fast selection, low cost and wide range of cross matching. At present, we have used this method to breed a number of high-quality rapeseed varieties, and has been widely promoted and applied, such as Qinyou 33, Qinyou 19, Qinzayou 4, Qinzayou 19. Qinyou 88 (http://www.sxhrrc.com/news/313.html) (Zhang et al., 2016) is a hybrid

rapeseed variety of Guoshenyou 2013022 and Shannshenyou 2014005 chemical emasculation bred by Hybrid Rapeseed Research Center of Shaanxi Province. This variety is semi-winter, with an oil content of 47.02%, and an average yield of more than 250 kg per mu. The suitable planting areas are winter rapeseed areas in Jiangsu Province and Anhui Province, north of Huaihe River, Guanzhong region of Shaanxi Province, Yuncheng region of Shanxi Province, Longnan region of Gansu Province, etc. The exploitation of the application potential of excellent varieties must be guaranteed by the quality of high-purity seeds. Any change in the process of seed production will affect the purity of seeds. If the purity of varieties is too low, it will seriously affect the real output of varieties, produce wrong data, affect the trust of dealers and farmers in varieties, and thus cause adverse consequences for variety promotion. Therefore, seed purity is the most important quality parameter of rapeseed hybrids (Chen et al., 2002). It is well known that the time between harvesting and packaging of rapeseed hybrids and marketing is generally less than six months, and rapid and accurate identification of the purity of hybrids is essential to ensure high and stable yields (Mu et al., 2010). The traditional purity identification methods of hybrids are mainly field identification and esterase isozyme identification (Luo et al., 2011), but both have certain drawbacks. Field identification has long cycle, which is limited by environmental constraints, and requires specialized breeders to identify hybrids from heterozygotes at different developmental stages. While esterase isoenzymes can distinguish some hybrids, they do not accurately reflect seed purity in more closely related hybrids.

At the end of the last century, with the application of PCR technology, the identification of plants at the DNA level has become a reality. The molecular marker technology that arose from this has been widely used in the identification of seed authenticity. With its advantages of fast, accurate and good reproducibility, it has become an inevitable choice for seed purity analysis (Zhou et al., 2010). And with the continuous development of sequencing technology, the method applied to the purity detection of varieties has changed from the initial RAPD (Random amplified polymorphic DNA) (Wang et al., 2009), SSR (Simple sequence repeat) (Li et al., 2013) and other molecular markers have gradually changed into more convenient InDel markers (Insertion deletion length polymorphism) (Meng et al., 2012). InDel refers to the insertion or deletion of nucleotide fragments of different sizes in the sequence of the same site of the genome between closely related species or different individuals of the same species (Weber et al., 2002). It is a phenomenon of gap generated by homologous sequence alignment. In most cases, it is impossible to determine the ancestral sequence (Mills et al., 2006), and it is difficult to determine which sequence has the insertion or deletion mutation at the vacancy site, so they are generally referred to as InDel. InDel marker is based on PCR amplification technology, which is essentially a length polymorphism marker (Hyten et al., 2010). Its amplification products are stable, rich in polymorphism and easy to identify genotype. It has been rapidly applied in animal and plant population genetic analysis, molecular assisted breeding and other fields. Compared with SSR marker, InDel marker has a clear and simple band pattern, and its stability and product separation effect are significantly better than SSR marker (Feng et al., 2005). However, it has not been reported to identify the purity of rapeseed hybrids using InDel markers. In order to make the InDel marker more widely used in the identification of rapeseed germplasm resources and variety purity identification, Qinyou 88 and the parents were analyzed through InDel marker developed by rapeseed resequencing in this study, and the purity of Qinyou 88 seeds were identified, so as to provide quality assurance for Qinyou 88 seeds to enter the market.

1 Results and Analysis

1.1 Primer screening

Qinyou 88 parents were analyzed through InDel marker and showed that the 120 pairs of primer amplification products can produce stable and clear bands, and the bands of amplification products are between 1~4, of which 12 pairs of primers can distinguish the parents. Using the above primers to amplify Qinyou 88 and its parents respectively, 4 pairs of complementary band primers (ID 2, ID 14, ID 15 and ID 39) that can distinguish Qinyou 88 and its parents respectively were obtained (Figure 1). The amplification products are clear and polymorphic, and the molecular weight of the product ranges from tens to hundreds of bp. After comparing 4 pairs of primers that can clearly distinguish Qinyou 88 from its parents, the amplified bands of ID 39 primer are relatively fuzzy

and have dragging. The amplified fragment of ID 14 primer is small and not clear enough. Therefore, ID 2 and ID 15 were selected as candidate primers for seed identification of Qinyou 88 in this study.

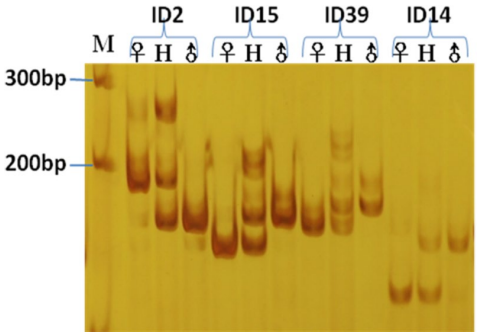

Figure 1 The amplification of candidate primer for Qinyou 88 and parents

Note: M: DL1500 marker; H: Qinyou 88; ♀: Female; ♂: Male

1.2 Determination of the consistency and stability of candidate primers in Qinyou 88 and its parent standard samples

200 artificially emasculated Qinyou 88 and parent seed DNA were selected respectively, and the consistency and stability were determined with the help of candidate primers ID 2 and ID 15. The results are as follows (Figure 2A; Figure 2B; Figure 2C; Figure 3A; Figure 3B; Figure 3C; Table 1).

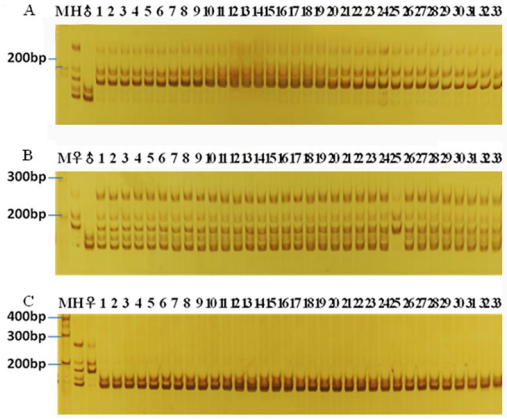

Figure 2 The amplification of female (A), Qinyou 88 (B) and male (C) by ID2 primer pair

Note: M: DL1500 DNA Maker; H: Qinyou 88; ♀: Female; ♂: Male; All the plant were female in A; 25 was female, the other were hybrid; All the plant were male in C

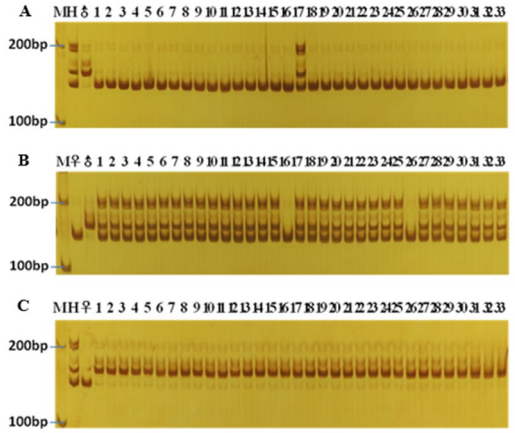

Figure 3 The amplification of female (A), Qinyou 88 (B) and male (C) by ID 15 primer pair

Note: M: DL1500 marker DNA Maker; H: Qinyou 88; ♀: Female; ♂: Male; 17 was hybrid and the other were female in A; 16 and 26 were female, the other were hybrid in B; All the plant were male in C

Table 1 The comparison of the consistency of primer ID 2 and ID 15 primers among with high purity Qin you 88 and its parents

Sample name	Number	Consistent result of ID 2				Consistent result of ID 15				Analytical bias
		F	H	M	P	F	H	M	P	
F	198	198	0	0	100	197	1	0	99.5	0.5
H	200	6	191	3	95.5	3	194	3	97.5	2
M	197	0	0	197	100	0	0	197	100	0

ID2 and ID15 were used to amplify the 200 Qinyou 88 and their parents, and the clear and stable product bands were statistically analyzed. Of which, the consistency ratios of ID2 hybrids and parents were 95.5% for hybrids, 100% for female parents, and 100% for male parents, respectively. The consistency ratios of ID 15 hybrids and parents were 97.5% for hybrids, 99.5% for female parents and 100% for male parents, respectively. The analytical bias of two pairs of primers for hybrid, female parent and male parent were 2, 0.5 and 0, respectively.

1.3 Comparative analysis of InDel marker identification results and morphological identification results

In the winter of 2016, three Qinyou 88 hybrids mixed with different numbers of parents were listed one by one, identified by ID primers, and morphological identification was carried out in the field with breeders from budding stage to early flowering stage in 2017. Among them, ID 2 (Figure 4A) primer lane 30 is the female parent, lane 12 is the male parent, and the others are hybrids. ID 15 (Figure 4B) lanes 16 and 30 are female parents, lane 12 is male parent, and the others are hybrids. Among 198 female parent samples, 198 were identified as female parent with ID 2, 197 were identified as female parent with ID 15, and 1 was identified as hybrid with ID 15 (Table 2). Among the 200 hybrid samples, 191 were identified as hybrids, 6 female parents and 3 male parents with ID 2, and 194 were identified as hybrids, 3 female parents and 3 male parents with ID 15. Among the 197 male parent samples, 197 were identified as male parent with ID 2, the others were 0, 197 were identified as male parent with ID 15, and the others were 0.

The correspondence ratio between the detection results of the two pairs of primers and the field morphological identification results is more than 97%, and the identification results of the two pairs of primers are consistent in some individual plants, but only inconsistent with the field identification results. The analysis showed that the analytical bias between the two pairs of primers is only 1.01, which is considered that the two pairs of primers can be used to detect the purity of Qinyou 88 seeds.

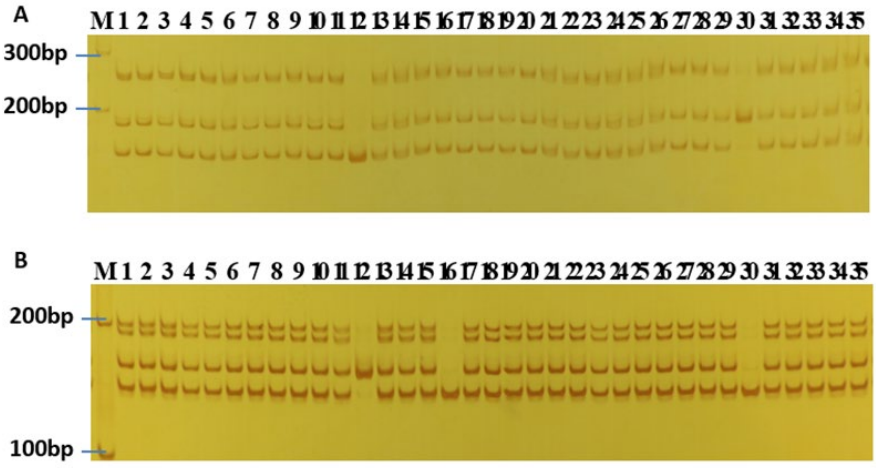

Figure 4 The identification results of ID 2 and ID 15 for Qinyou 88 in field grown plants

Note: M: DL1 500 DNA Maker; 30 was female, 12 was male and the other were hybrid in A; 16, 30 were female, 12 was male and the other were hybrid in B

Table 2 The analysis of consistency between ID primer and the field identification and analysis result of primers

Sample	Number	ID2		ID15		Analytical bias of two primers
		Corresponding plants	Correspondence ratio (%)	Corresponding plants	Correspondence ratio (%)	
Qinyou88-1	198	193	97.47	195	98.48	1.01
Qinyou88-2	200	190	95	189	94.5	0.5
Qinyou88-3	198	190	95.96	188	94.95	1.01
Average	-	-	96.14	-	95.98	0.84

2 Discussion

Seed purity is an important basis for seed grade evaluation. So far, field morphological identification is still the most authoritative method for seed purity identification. However, with the maturing of microspore culture and molecular marker assisted selection technology, as well as the introduction of the regulation on variety registration of rapeseed as a non-major crop, the breeding process of rapeseed is faster and faster, and the number of new varieties registered is increasing sharply. At the same time, to cultivate good new rapeseed varieties in a short time, breeders often focus on using a small number of backbone parents for selection, which makes the genetic diversity of varieties gradually narrow. If they are not professionals engaged in breeding, it is often difficult to distinguish these subtle differences by using the traditional field planting form identification method. In recent years, molecular marker technology has developed rapidly, with a variety of marker types, rich polymorphism, good stability, simple operation, and can accurately and effectively identify a large number of heterotopic spots. Therefore, it is widely used in seed purity identification of a variety of crops (Tang et al., 2007; Li et al., 2008; Tian et al., 2010; Song et al., 2010; Miao et al., 2011). While the InDel marker is a molecular marker developed on the basis of sequencing the species. It not only has the advantages of other molecular markers, but also shows rich polymorphic sites. And because it is developed in the genome information of the species to be tested, it can more accurately identify varieties. It has been applied in Chinese cabbage and other crops (Lan et al., 2011; Shen et al., 2011; Zhang et al., 2012; Xue et al., 2014; Hu et al., 2016; Yang et al., 2018), but so far, it has not been reported that the InDel marker is used in the purity identification of rapeseed hybrids.

Because InDel markers are developed based on parental genome information, compared with RAPD, SSR and other molecular markers, it has a high detection efficiency. It can detect target strains only through dozens or hundreds of primers, which greatly improves the efficiency of primer screening. In this study, only 10 pairs of parental differential primers screened from 120 pairs of InDel markers were used. After further screening, 4 pairs of parental complementary primers were obtained, and the detection efficiency was as high as 3%. While RAPD (Liu et al., 2000) and SSR (Mu et al., 2010) need to screen hundreds of primers to obtain 1 to 2 pairs of candidate primers. Compared with them, the efficiency of InDel is greatly improved, and the band type is simple and easy to identify. It can be expected that InDel will be more and more used in germplasm identification in future research.

In this study, two pairs of parental complementary InDel markers with high stability and large band differences were selected to verify the consistency between Qinyou 88 hybrids and parents. The consistency of the two pairs of InDel markers for hybrids, female parents and male parents was higher than 95%. The analysis deviation between the two pairs of primers was only 2, 0.5 and 0, respectively, and the consistency of the two pairs of InDel markers reached a very significant level. The analysis of the deviation between the two primers showed that the primers were highly consistent with the male parent detection, and the consistency reached 100%. However, there was a certain deviation in the detection of hybrid and female parent. It was considered that there may be partial heterozygosity in the internal gene loci of female parent in the parent (Tang et al., 2008), which affected the detection efficiency. While in hybrids, the deviation was as high as 2. However, through analysis, it was found that the inconsistent individual test results were all female parent bands, which may be caused by pollen drift during emasculation. At the same time, by detecting the consistency between hybrids and parents, we could identify the existing parents, eliminate the parents that are inconsistent with the target band type, and purify the parents

quickly and accurately. By comparing the field identification and InDel molecular marker identification of some hybrid parent seeds in Qinyou 88 hybrid, the correspondence ratio between the two pairs of primers and the identification results of three samples in the field is 94.95%~98.48%, which seems that the correspondence ratio between the two pairs of primers and the identification results in the field is not particularly high. Through further analysis of the identification results of the two pairs of primers, it was found that except for the differences between the identification results of two pairs of primers for a single plant, the identification results of the other single plants were the same with the two pairs of primers, and the deviation between the identification results of the two pairs of primers was only 0.5~1.01. The analysis results showed that the reason why the correspondence ratio with the field identification results was not high may be that the field identification depended on visual observation to distinguish hybrids and alien plants, but only hybrids have different phenotypes in different growth periods, showing super parental phenotype in a certain period, maternal phenotype or paternal phenotype in a certain period, which brings great interference to the surveyors and leads to objective results. Through the co-planting of Qinyou 88 and its parents, it was found that Qinyou 88 tended to maternal phenotype in most periods of the growth cycle, so it may be that some individual plants were mistaken for hybrids in the identification process, resulting in the correspondence ratio between the molecular identification results and the field identification could not reach 100%. Through the mutual verification between two pairs of introducers, it was considered that the InDel molecular marker primer was more reliable and stable for the purity identification of Qinyou 88 seeds, and the identification cycle was shorter than that of the field, with high reliability.

3 Materials and Methods

3.1 Experimental materials

The experimental material in this study is chemical emasculation hybrid rapeseed (*Brassica napus*) Qinyou 88 (Hybrid, referred to as H) and its female parent double low chemical induced male sterile line YD2013 (Female, referred to as F) and its male parent CY1168 (Male, referred to as M) (the same below). Among them, the seeds of Qinyou 88 were from artificially emasculated hybrid seeds and sampled commercial seeds. The above seed materials were provided by Hybrid Rapeseed Research Center of Shaanxi Province, and the field morphological identification and molecular identification were also carried out here.

The primers used in this study came from resequencing the parents of the QTL mapping population of our research group, detecting the InDel site information, selecting the sequence, using primer design software primer5, independently developing InDel markers, designing pairs of primers, randomly selecting 120 pairs, which were synthesized by AuGCT.

3.2 Candidate primer screening

Qinyou 88 and its parent seeds were planted in the field. Young leaves were taken from each plant at the seedling stage and mixed. The total DNA of hybrid and parent was extracted by CTAB method (Wang et al., 2009), dried at room temperature, dissolved with appropriate ddH_2O, and stored at 4℃. The PCR amplification system was 10 μL, in which DNA template 1 μL, 10×Buffer 1 μL, Mg^{2+} 0.8 μL, dNTP 0.25 μL, 0.5 μL for upstream and downstream primers, respectively, *Taq* enzyme 0.1 μL, add ddH_2O to 10 μL. Reaction procedure was as follows: 94℃ for 5 min; 94℃ for 30 s, 55℃ for 30 s, 72℃ for 45 s, 35 cycles; Extended at 72℃ for 7 minutes and stored at 10℃ (Zhao et al., 2015). Added 1/2 volume of Loading buffer to the InDel amplification product, denatured it at 95℃ for 5 min, quickly put it into ice water for cooling, and electrophoresed it on 6% denatured polyacrylamide gel (page) at 200V constant pressure for 1.5 h. Silver staining and color development were carried out by using the simplified silver staining method, and the amplification band type was recorded.

3.3 InDel marker stability analysis

Put a double-layer filter paper into the Petri dish, randomly select 200 Qinyou 88 hybrids and 200 high-purity parents obtained by artificial hybridization, extract the DNA of a single plant after germination for 5 d, and refer to the method of Li et al. (2013). Use the specific primers obtained by screening for amplification analysis, record the genotype of a single plant amplified by each primer, and mutually verify the stability and consistency of different primers in the parents.

The correspondence ratio = (Parent to be tested or hybrid-alien plant) × 100/Parent or hybrid to be tested.

3.4 Field planting identification

The field identification test was carried out in the Experimental Base of Hybrid Rapeseed Research Center of Shaanxi Province from September 2016 to May 2017. A certain number of parents were randomly added to the samples to be tested for on-demand sowing. Each seed was planted in 15 rows, with a length of 2.0 m and a row spacing of 0.4 m. At the flowering stage, the plants were labeled one by one, and the seed purity was identified. At the same time, the young flower buds were selected respectively, and the molecular identification was carried out by using candidate primers. The identification results of molecular markers were compared and analyzed with the morphological results, and the correspondence degree between the identification results of different primers and the field identification results was compared.

Correspondence ratio (%) = (Total number of plants identified - number of plants not consistent with field identification)/Total number of plants identified × 100%.

Authors' Contributions

LBJ, SLP, WZY and GKH are the executors of the experimental design and research of this study. ZLJ, ZXP and ZWG completed the collection and analysis of field data. LBJ, ZCY and WH participated in the writing and revision of the manuscript. LDR, ZCY and WH are the designer of the experiment, guiding the experimental design, data analysis, manuscript writing and revision. All authors read and approved the final manuscript.

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