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Diagnostic Set of SSR Markers for Molecular Fingerprinting of Parental Lines and Hybrid Purity Testing in Castor (*Ricinus communis* L.)

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ABSTRACT

Microsatellite markers were used for molecular fingerprinting of varieties, parental lines of hybrids released from IIOR and testing the genetic purity of hybrid seed lot of castor. The DNA markers will allow accurate, speedy variety identification and assessing genetic purity of hybrids compared to grow-out test. In this study 110 simple sequence repeats (SSR) markers were employed for fingerprinting 2 popular castor varieties, 2 hybrids and their parental lines. 15 unique alleles were identified for 6 parental lines and a total of 132 alleles was observed with 56 polymorphic markers. The analysis of plant-to plant variation within the parental lines of the hybrid DCH177 and DCH519, using informative markers indicated residual heterozygosity at two marker loci. This highlights the practical utility of SSR markers in maintaining the genetic purity of the parental lines and assessing the genetic purity of the castor hybrids.

Key words: *Castor, Fingerprinting, Hybrid purity, Parental lines, SSR markers*

INTRODUCTION

Castor is one of the most important non edible oilseed crop grown throughout the world. India ranks first in area, production and productivity among the major producing countries viz., China and Brazil. The hybrid seed production in castor is primarily based on two line system which involves pistillate line and male line. Pistillate lines are environmentally sensitive and shows interspersed stamens (IFS) which leads to selfing of the female line. As the castor is highly cross pollinated the contamination of the pollen source, parental seed impurity and selfing of the female lines will lower the genetic purity of the hybrids. Estimation and maintenance of genetic purity of the parental lines and hybrids is essential to exploit high level of heterosis and successful adoption of hybrids. Conventionally, morphological descriptors are routinely used for establishing the identity of parental lines. But these morphological descriptors suffer from many drawbacks such as influence of environment on trait expression, epistatic interactions, pleiotrophic effects etc. Molecular markers have potential in achieving this goal as it is rapid, reliable and cost effective as it is based on DNA sequence variation provide unbiased means of identifying crop varieties and parental lines of hybrids[1].

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Among the various DNA based markers of choice SSRs are ideal markers because of their abundance, co-dominance, reproducibility, cost-effective and uniform distribution throughout the genome. Molecular fingerprinting of varieties and parental lines is also essential for their protection (IP) and prevention of unauthorized commercial use. These markers are particularly useful for developing unique DNA profiles of varieties and parental lines which would unambiguously distinguish with other genotypes in order to obtain plant protection.

Genetic purity in hybrid seed production is of critical importance, as low purity seed would cause severe losses to the farmers[2]. The routinely used method for assessing genetic purity is the grow out test (GOT), involving representative sample of the seed lot. But it is a time and resource consuming exercise, and is also influenced by environmental factors[3]. The molecular markers for hybrid purity assessment is more rapid, accurate and cost effective method involving less amount of DNA. The main objective of the present study was to differentiate parental lines by employing SSR markers. Use of these markers in the assessment, maintenance and testing of the genetic purity of the parental lines and hybrid seeds for commercialization.

MATERIAL AND METHODS

Plant materials

For the purpose or molecular characterization, two varieties and four parental lines of castor hybrids released for commercial cultivation from IIOR were used (Table 1). The parental lines and F_1s of hybrids were obtained from seed production unit, Indian Institute of Oilseeds Research (ICAR-IIOR), Hyderabad. For marker analysis, seeds were germinated in paper towels under aseptic conditions in IIOR, Hyderabad. A random sample of 100 seeds per seed lot of DCH177 and DCH519 produced at IIOR seed production plot grown for grow-out test was used for testing their genetic purity.

DNA extraction and PCR amplification

Genomic DNA was isolated following the procedure of Doyle and Doyle[4] with slight modifications. For fingerprinting, DNA from the bulk of leaf samples of 10 individual plants was used. Quantification of DNA was accomplished by analysing the DNA on 0.8% Agarose gel using diluted uncut λ DNA as standard. Based on our earlier studies SSR markers with high Polymorphic Information Content (PIC) was selected for fingerprinting studies. DNA amplification was carried out in a 10 µl reaction mixture containing 1×PCR assay buffer containing 1.5 mM $Mgcl_2$, 0.2 mM dNTPs, 0.5 pm forward and reverse primers, 0.1 unit of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India) and 25 ng of genomic DNA template. The amplification was carried out in a thermal cycler with following conditions 94 °C for 5 min for initial denaturation, 35 cycles of 94 °C for 30 seconds for denaturation, 55 °C for 30 seconds for annealing and 72 °C for 30 sec for extension followed by 72 °C for 5 min for final extension. PCR products were separated on 6% Polyacrylamide gels using 1× TBE buffer and visualised after silver staining and photographed with camera. The size of the amplified fragments was determined by using 100 bp DNA ladder (Bangalore Genei Pvt. Ltd., Bangalore, India).

Data analysis

The marker characteristics viz., number of alleles (NA), major allele frequency and polymorphism information content (PIC) were obtained using the software program Power Marker version 3.25[5].

RESULTS AND DISCUSSION

Fingerprinting of parental lines

For fingerprinting of the six parental lines, 102 SSR markers developed in ICAR-IIOR were used and 56 polymorphic markers (54.9%) were identified. A total of 132 alleles were amplified and number of alleles per locus ranged from 2 to 5 with an average of 3 and 16 loci amplified highest number of alleles (≥3) (Table 2, Figure 1). The number of alleles detected in the present study corresponded well with earlier reports. However, Allan *et al*., [6] and Bajay *et al*., [7] obtained higher number of alleles (8) as compared to present

study, because of inclusion of more genotypes in their study. The number of alleles detected by a single SSR locus varies depending upon the fingerprinting techniques and materials used in the studies. Fifteen unique alleles (12.7 %) were observed for the five parental lines 48-1, DCS-107, DCS-9, DPC-9 and M-574 parental lines and for DCS-78, no unique allele was observed (Table 3). An allele that was observed only in one of the 6 castor parental lines was considered to be a unique allele. Unique alleles are important because they may be diagnostic of a particular genotype and useful for breeding purpose and also protecting the germplasm. The major allele frequency ranged from0.33 to 0.83 with an average of 0.60. The average PIC value of each locus was 0.43, with the highest of 0.67 for 7 primers (mRcDOR11, mRcDOR49, mRcDOR103, mRcDOR130, RCM12532, RCM12601 and RCM 13335) and the lowest of 0.24 for 12 primers (mRcDOR8, mRcDOR24, mRcDOR29, mRcDOR54, mRcDOR90, mRcDOR151, mRcDOR159, mRcDOR203, RCM13315, RCM13360, RCM13405 and RCM13531). All the markers amplifying unique alleles showed low level polymorphism information content (PIC) values. Nine markers were observed polymorphic for the parental lines of the hybrid DCH 177 and 10 markers for DCH519 (Table 4). Figure 5 shows the amplification of unique alleles in 48-1, DCS-107, DCS-9, DPC-9 and M-574 by SSR primers. Similarly, others also detected unique alleles in different crops[8]. For any given marker, multiple alleles were negligible indicating presence of homogeneity in the germplasm. The utility of SSR fingerprints for plant variety protection is known in many crops [9, 10, 11, 12, 13]. As per the UPOV guidelines for registration of new plant varieties, DUS testing based on some morphological characters and in some cases isozyme markers are used. The DUS testing is effected by limited number of phenotype traits and genotype and environment interactions. Therefore, the molecular markers which are available in large number for most of the species which are repeatable across the labs and environments is being considered.

Assessment of genetic heterogeneity within parental lines

The genetic heterogeneity, if may within the parental line could lead to lack of uniformity in the commercial F_1 seeds resulting in to poor acceptance of the hybrid produced. Since SSR markers are co-dominant, they can be used for unambiguous identification of both the homozygotes and the heterozygotes at a particular marker locus. Thus, these markers have the potential to test genetic heterogeneity with the parental lines. Out of 30, i.e., 15 plants each of the parental line DCS9 X DPC9 (DCH177), DCS78 X M574 (DCH519) analysed using 10 polymorphic SSR markers, a 2 plants at the marker locus mRcDOR151 were found to be heterozygous in case of DPC9. However, no plant-to-plant variation was observed with DCS9, M574 and DCS 78. The heterogeneity in the parental lines will result in the breakdown of hybrids in the longer run. However, the extent of heterogeneity and the marker loci under question may vary depending upon pedigree and maintenance of the parental line.

Testing genetic purity of hybrid seeds

Assessing the genetic purity of hybrid seed is a critical requisite for its commercial use. As castor is a highly cross pollinated crop, outcrossing, selfing of the female parent and physical admixture during the handling of the harvested material during hybrid seed production, leading to decline in genetic purity. Genetic purity up to 85% is essential for certified seed (hybrid) as per the certification standards in India. Generally, genetic purity of castor hybrid is assessed by grow out test i.e., raising the representative samples up to 60 days. After seed production, produce has to be kept for 60 days for quality assessment which is laborious, expensive and time consuming. Considering the disadvantages of grows out test, rapid and reliable methods using molecular markers are getting attention for genetic purity testing. Now several molecular markers i.e., RAPD, ISSR, AFLP, SSR etc. are being used. SSR markers are extensively used because of their co-dominant nature. In the present study, 110

SSR markers were screened for polymorphism in parental lines of DCH177 (DPC-9 and DCS-9) and DCH519 (M-574 and DCS-78). Out of 110 SSR markers tested, 24 markers showed polymorphism between DCS-9 and DPC-9 parental lines of hybrid DCH-177, and 13 between DCS-78 and M-574 parental lines of hybrid DCH-519 (Table 4). Single polymorphic marker should be sufficient to ascertain the genetic purity of the hybrids. But in cross pollinated crops like castor, more polymorphic markers should be used. To test the genetic purity of DCH177 two SSR markers mRcDOR103 and mRcDOR130 which is polymorphic between parental lines DCS9 and DPC9 were used. Similarly, for DCH 519 two markers mRcDOR181 and mRcDOR206 were used. Some of the markers are polymorphic for both the hybrid parental lines like mRcDOR49 and thus cannot be used for distinguishing the two hybrids in case they contaminate each other. Gouri Shankar *et al.,*[14] also reported hybrid purity assessment of castor hybrids PCH111 and PCH 222 using EST-SSR markers. Pranavi *et al.,*[15] also reported hybrid purity assessment of castor hybrids. Hybrid purity testing using SSR markers are similarly done in other crops like Safflower[9], Rice[10], Maize[11], Flax[12], Sorghum[13]. Admixtures of the parental lines can be identified using single polymorphic marker, however contamination of female parent with unknown male parent can be detected reliably by using more number of polymorphic markers. So a combination of two or three markers are used for assessing hybrid purity. These polymorphic SSRs were then assessed using bulk DNA of both hybrids and parents. For all the markers, parents showed single allele whereas hybrid showed both the parental alleles indicating the heterozygosity of the hybrid. The markers were further tested with DNA of 100 individual plants to assess their utility in hybrid purity assessment. All the individual hybrid plants showed both the alleles specific to the parents in DCH519, while in DCH177 four samples showed parental band (female) indicating selfed plant. Figure 2 depicted the individual plant assay of each hybrid DCH-177 and DCH-519 with mRcDOR-130 and mRcDOR-206 respectively. The results confirmed the utility of these markers in genetic purity assessment of the hybrids. The usage of molecular markers will greatly supplement the complete procedure of testing genetic purity of hybrid seeds by way of saving one full season and thus reducing the cost of hybrid seed.

Table 1: List of castor varieties and parents of hybrids used for DNA fingerprinting

S.No	Genotype	Pedigree	Morphological characters
1	48-1	HO x MD	Red, double bloom, non-spiny, normal plant type
2	DCS-107	DCH-177 x JI-133	Green, spiny, double bloom, normal plant type
3	DCS-9	240x Bhagya	Red, spiny, double bloom, normal plant type
4	DPC-9	VP-1 x 128-1 (Bhagya x CO-1)	Green, spiny, zero bloom, pistillate line, normal plant type
5	DCH-177 (H)	DPC-9 x DCS-9	Hybrid
6	DCS78	Male version of DPC-11	Green, spiny, double bloom, normal plant type
7	M-574	Mutant from VP-1	Green, spiny, triple bloom, pistillate line, dwarf plant type
8	DCH-519(H)	M-574x DCS-78	Hybrid

Table 2: SSR analysis of varieties and parents of hybrids

S.No.	Primer	Observed size (bp)	No. of Alleles	Major allele frequency	PIC
1.	mRcDOR-3	200	2	0.67	0.35
2.	mRcDOR-7	315	2	0.67	0.35
3.	mRcDOR-8	150	2	0.83	0.24
4.	mRcDOR-9	160-170	2	0.50	0.38
5.	mRcDOR-11	180 –200	4	0.33	0.67
6.	mRcDOR-13	310	2	0.50	0.54
7.	mRcDOR-24	260	2	0.83	0.24
8.	mRcDOR-25	125	2	0.50	0.38
9.	mRcDOR-26	195-210	3	0.67	0.35
10.	mRcDOR-29	270-280	3	0.83	0.24
11	mRcDOR-49	200-220	3	0.33	0.67
12.	mRcDOR-54	150	2	0.83	0.24
13.	mRcDOR-55	190	2	0.67	0.45
14.	mRcDOR-56	220-230	3	0.50	0.54
15.	mRcDOR-69	195	2	0.67	0.35
16.	mRcDOR-76	120	3	0.50	0.54
17.	mRcDOR-82	350	2	0.50	0.38
18.	mRcDOR-90	270	2	0.83	0.24
19.	mRcDOR-92	190-195	3	0.50	0.54
20.	mRcDOR-103	185-190	3	0.33	0.67
21.	mRcDOR-113	145	2	0.67	0.45
22.	mRcDOR-122	300	2	0.50	0.54
23.	mRcDOR-130	130-150	4	0.33	0.67
24.	mRcDOR-142	220	2	0.50	0.38
25.	mRcDOR-144	280	2	0.67	0.35
26.	mRcDOR-150	170-180	2	0.50	0.54
27.	mRcDOR-151	310	2	0.83	0.24
28.	mRcDOR-153	360	2	0.50	0.38
29.	mRcDOR-159	290	2	0.83	0.24
30.	mRcDOR-162	340	2	0.50	0.54
31.	mRcDOR-166	200	2	0.50	0.54
32.	mRcDOR-175	110	2	0.67	0.35
33.	mRcDOR-176	300	2	0.50	0.54
34.	mRcDOR-181	210	2	0.67	0.35
35.	mRcDOR-185	190	2	0.67	0.45
36.	mRcDOR-203	120	2	0.83	0.24
37.	mRcDOR-206	180-190	3	0.67	0.45
38.	mRcDOR-211	310	2	0.50	0.62
39.	mRcDOR-223	180	2	0.50	0.54
40.	mRcDOR-225	320-330	3	0.50	0.54
41.	mRcDOR-226	320-330	3	0.50	0.62
42.	RCM-12532	280	2	0.33	0.67
43.	RCM-12523	230-240	3	0.83	0.4
44.	RCM-12601	280-300	3	0.33	0.67
45.	RCM-12687	215	2	0.50	0.38
46.	RCM-12706	200	2	0.50	0.54
47.	RCM-12832	270	2	0.67	0.45
48.	RCM-13315	275	2	0.83	0.24
49.	RCM-13335	285-305	5	0.33	0.67
50.	RCM-13360	250	2	0.83	0.24
51.	RCM-13405	225	2	0.83	0.24
52.	RCM-13531	200	2	0.83	0.24
53.	RCM-13633	230	2	0.50	0.54
54.	RCM-13961	200	2	0.67	0.45
55.	RCM-13986	250	2	0.67	0.35
56.	RCM-13992	230-240	3	0.50	0.54
		Mean	**3**	**0.60**	**0.43**

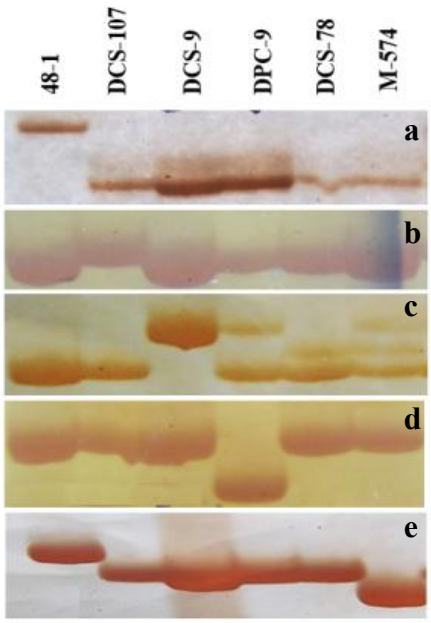
Table 3: Unique alleles identified for parental lines

S.No	Genotypes	No. of unique allele	Name of marker
1.	48-1	6	mRcDOR-24, 29, 90, 92, 206 and RCM13531
2.	DCS-107	3	mRcDOR-54,159 and RCM-13315
3.	DCS-9	1	mRcDOR-151
4.	DPC-9	4	mRcDOR-8, 203 and RCM-13360, 13405
5.	M-574	1	mRcDOR-206

Table 4: SSR primers polymorphic in parents of hybrids

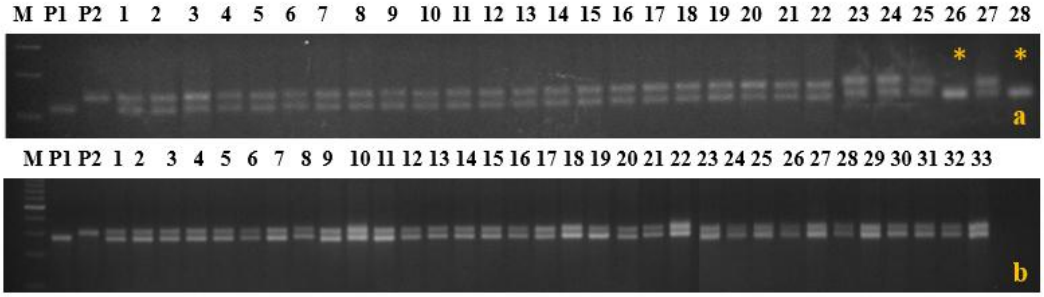
Hybrids	No. of markers polymorphic	Marker name
DCH-177 (DPC-9 x DCS-9)	24	mRcDOR-3, 7, 8, 9, 29, 49, 55, 82, 92, 103, 113, 122, 130, 144, 150, 151, 153, 159, 166, 176, 203, 223, 225, 226
DCH-519 (M-574 x DCS-78)	13	mRcDOR-13, 49, 55, 69, 76, 82, 92, 113, 144, 181, 206, 225

Fig. 1: Unique alleles identified for castor parental lines using SSR markers



a) mRcDOR-24 b) RCM-13315 c) mRcDOR-151 d) RCM-13405 e) RCM-12532

Fig. 2: Hybrid purity assessment of castor hybrids with SSR markers



a). Single seedling assay of DCH177 castor hybrid using mRcDOR103; M-Marker, P1 (DPC-9), P2 (DCS-9), 1-28 individual hybrid seed, the asterisk indicates offtypes b). Single seedling assay of DCH519 castor hybrid using mRcDOR181; M-Marker, P1 (M-574), P2 (DCS-78), 1-33 individual hybrid seed.

CONCLUSION

In this study, DNA fingerprinting of the six main commercial parental lines of varieties and hybrids under cultivation in India was built using 56 polymorphic SSR primer pairs. The present study clearly proven the ability of SSR markers to generate locus specific allelic information and the utility of highly informative SSR markers in seed genetic purity assessments has been validated and compared with GOT.

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