

Original Research Article

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Hybrid Purity Testing of Chilli Hybrid (Pusa Jwala × Arka Lohit) through RAPD and ISSR Molecular Markers

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ABSTRACT

A fast and accurate genetic purity test of F₁ hybrid plants is essential for seed production and accelerating advanced breeding generations in breeding programmes. DNA technology has great potential for enhancing purity assessment of hybrids. Chilli F₁ hybrid (Pusa Jwala × Arka Lohit) and its parents Arka Lohit (male) and Pusa Jwala (female) were analyzed for the development of markers, identification of hybrids and ascertaining genetic purity in *Capsicum annuum* L. through PCR based Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSRs) DNA Markers. Twenty seven RAPD primers of Operon series and twenty ISSR primers were used for analysis. RAPD primer, OPA 15 and OPD 16 were found to be heteroallelic for parents. OPA 15 identified male specific amplicons of about 1200bp and OPD 16 produced male specific amplicon of about 1100bp in the hybrid (Pusa Jwala × Arka Lohit). The other repeats of different base pairs were homoallelic for both the parents. All twenty ISSR primers used were found to be homoallelic for both the parents. This was due to the RAPD marker system producing a greater number of markers than the ISSR system. OPD 16 identified a female-specific amplicon of about 800bp in the hybrid (Pusa Jwala × Arka Lohit). Comparison of the two RAPD banding pattern of the parents with respective hybrid clearly identified genuine hybrid. Thus RAPD markers individually have their own merits in the identification of parents and their hybrids. However, a combination of two PCR based markers can be used for testing the genetic purity of chilli seeds or seedlings, which will be more reliable substitute for GOT and a tool for seed certification. This study suggested that efficiency of RAPD assay to evaluate successfully hybridity determination, which would be a valuable genomic tool for the chilli breeders.

Keywords

Chilli, Hybridity test, RAPD, ISSR and GOT.

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Introduction

Chilli (*Capsicum annuum* L.) is a spice cum vegetable crop belonging to the family Solanaceae with chromosome number is $2n = 24$. Now a day hybrids are gaining much importance because of their high performance towards yield and resistant to pest and diseases. Hence, hybrid seed production is becoming a dominant in seed industries.

Hybrid seed purity is one of the important aspects in hybrid seed production technique. The primary method to check the hybrid seed purity has been the grow-out test (GOT). However it is environmental sensitive and may alter the expression of specific morphological or physiological traits. Besides that, the time required and space to grow and

evaluate a large number of seed lots will be a major limitation of GOT. Therefore, alternative reliable methods to assess the genetic purity of seed sample are needed to help seed producer to assure high quality standards.

Genetic purity test in chilli has been investigated in several studies using isozyme and protein polymorphism (Ballester and Vicente, 1998 and Sharmila *et al.*, 2011). However, this procedure may be limited by environmental conditions and tissue type, and may require selection of a suitable isozyme for purity testing.

Isozymes and restriction fragment length polymorphisms (RFLPs) have been used for testing hybrid purity (Tanksley and Jones, 1981; Arús *et al.*, 1985; Livneh *et al.*, 1990). Both techniques are useful but have different drawbacks: often not enough number of developed isozyme systems are available for a meticulous species and, on the other hand, RFLPs have the limitation of their cost and complexity for routine commercial testing.

The advent of the Polymerase Chain Reaction (Saiki *et al.*, 1987) had a direct impact on the development of new markers more suited to plant breeding related practices. In recent years, an increasing number of PCR-based techniques have emerged which are proving useful for breeding applications (Staub *et al.*, 1996). The use of these markers follows a simple and fast course of action, needs a low quantity of DNA and is easily automated.

RAPD (Williams *et al.*, 1992) uses the PCR with non-specific primers to amplify random DNA fragments. The single primer amplification reaction (SPAR) is a different approach that uses also one primer based on a simple sequence repeat (SSR) and amplifies inter-SSR DNA sequences (Gupta *et al.*, 1994).

The cultivated pepper is known as possessing very little genetic diversity; however, some authors have pointed out that it does not seem to be as narrow as for other crops (Heiser, 1979). In a recent publication, 17% of the probes used in a RFLP study (with one enzyme) of the genus *Capsicum* succeeded to differentiate four *C. annuum* varieties, and the same did 12% of RAPD PCR primers (Prince *et al.*, 1995). In a different report (Lefebvre *et al.*, 1993), 14% of RFLP probes (with 10 restriction enzymes) distinguished six out of 13 varieties of *C. annuum*. In spite of that, it is general knowledge that commercial varieties, and particularly hybrid varieties of vegetable crops, are based on increasingly narrower variation at the genetic level.

In consequence, discriminating between elite lines and their hybrids is often difficult. The present work reports the results of applying both methods, RAPDs and ISSR, to assess the genetic purity of hybrid seeds.

Materials and Methods

Plant material and DNA extraction

For the experiment we used two parents *i.e.*, Pusa Jwala as female parent (which are released by IIHR) and Arka Lohit as male parent (IARI, New Delhi). Both the parental seed materials were available in department germplasm collection. Crossing was done in protected structures of mesh/net house and buds which are to be crossed are covered with butter paper bag to confirm only designated cross has to occur. Sufficient DNA was extracted from both the parents and hybrid seedlings by modified CTAB method (Saiki *et al.*, 1998). Then quality of DNA was checked through UV spectrophotometer. After which dilution of extracted DNA to 100 ng/ μ l concentration which further diluted into 25 ng/ μ l and 10 ng/ μ l for RAPD and ISSR primers respectively

Primers

RAPD and ISSR primers were used for the screening of hybrid purity of F₁ under test. In the present investigation, RAPD ten-mer primers of Operon tech were used which consisted of A, B, C, D, F, G, J and K series. Ten randomly selected primers from each series were employed for the parental survey (Pusa Jwala and Arka Lohit). Similarly 15, UBC series (UBC808, UBC810, UBC811, UBC825, UBC826, UBC840, UBC841, UBC842, UBC848, UBC855, UBC856, UBC861, UBC880, UBC881 and UBC889) ISSR primers were used for screening of F₁ hybrid purity test. The sequence details of primers used in the experiment are given in table 1.

Thermo profile for PCR

RAPD

A master mixture of 20 µl/tube was consisted of mixture of assay buffer 2µl of 10x concentration, dNTPs- 2µl of 2mM conc., 1.8 MgCl₂ of 25mM conc., 2µl primer of 3µM conc., 0.3µl of taq polymerase of 5U/µl, 2µl of template DNA of 25ng/µl dilution and remaining with quantity was made upto 20µl/PCR tube with sterile distilled water. The thermal cycling of master mix was done as follows: 95°C for 5 minutes for initial denaturation. Later 35 cycles of denaturation at 94°C for one minute, annealing at 38°C for one minute and extension at 72°C for 1:30 minutes. After which final extension was done at 72°C for 10 minutes and dump at 4°C for one minute.

ISSR

A master mixture of 20 µl/tube was consisted very similar PCR components of RAPD with same concentration except for MgCl₂ (2µl of 25mM conc instead of 1.8µl), taq polymerase

(0.2µl of 5U/µl conc. instead of 0.3µl) and template DNA (3µl of 10ng/µl dilution, instead of 2µl of 25ng/µl conc.) and remaining with quantity was made upto 20µl/PCR tube with sterile distilled water. After completion of 35 cycles, the samples were stored at 4°C in thermocycler and all the contents were loaded on to the gel for electrophoresis. Separation of PCR amplified products by agarose gel electrophoresis.

Gel electrophoresis

2% gel which was prepared out of 1x TAE buffer was used for gel running of PCR products were used where electrodes were connected to the power supply and electrophoresis was carried out at 100 volts for 1 hr or till the dye migrated to the end of the gel. The DNA was visualized on a UV transilluminator and documented in a gel documentation system.

Gel scoring

To identify male specific band (band which is present in male parent and absent in female parent) and for hybrid purity, it was expected that the male specific band should be present in hybrid.

Results and Discussion

DNA quantity and quality

The isolated DNA was analyzed quantitatively by using UV Spectrophotometer. DNA had an A₂₆₀/A₂₈₀ ratio of 1.94 to 2.01 showing that it was relatively pure and free from impurities. The quality of DNA isolated was very good. The DNA yielded for both parents and the hybrid individual are, Arka Lohit (Male parent) yielded highest quantity of DNA (2038.20 ng/µl) and Pusa Jwala (female parent) yielded optimum quantity of DNA (811.10 ng/µL).

Table.1 List of primers used for hybrid purity testing of chilli hybrid (Pusa Jwala × Arka Lohit)

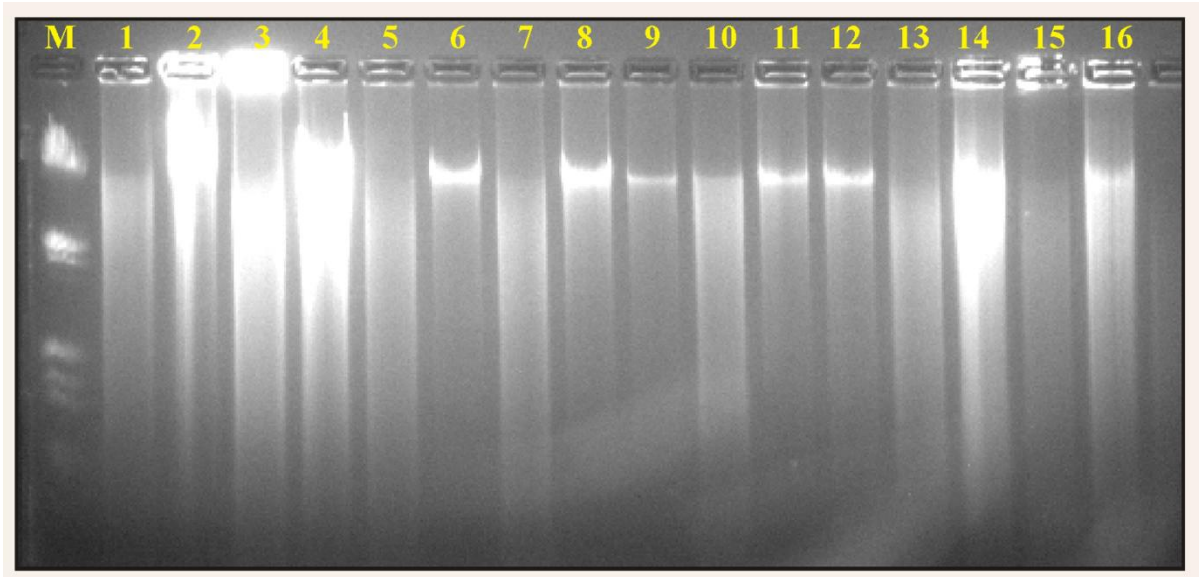
Sl. No.	Primer	Sequence (5'-3')
RAPD Primers		
1	OPA 01	CAG GCC CTT C
2	OPA 02	TGC CGA GCT G
3	OPA 04	AAT CGG GCT G
4	OPA 07	CCG ATA TCC C
5	OPA 12	TCG GCG ATA G
6	OPA 15	TGC CGA GCT A
7	OPB 02	TGA TCC CTG G
8	OPB 03	CAT CCC CCT G
9	OPB 06	TGC TCT GCC C
10	OPB 08	GTC CAC ACG G
11	OPB 11	GTA GAC CCG T
12	OPC 02	GTG AGG CGT C
13	OPC 03	GGG GGT CTT T
14	OPC 05	GAT GAC CGC C
15	OPC 08	TGG ACC CGT G
16	OPC 13	AAG CCT CGT C
17	OPD 02	GGA CCC AAC C
18	OPD 04	TCT GGT GAG G
19	OPD 06	ACC TGA ACG G
20	OPD 16	AGG GCG TAA G
21	OPD 18	GAG AGC CAA C
22	OPF 11	TTG GTA CCC C
23	OPF 15	CCA GTA CTC C
24	OPG 05	CTG AGA CGG A
25	OPG 15	ACT GGG ACT C
26	OPJ 01	CCC GGC ATA A
27	OPK 16	GAG CGT CGA A
ISSR Primers		
1	UBC808	AGAGAGAGAGAGAGAGC
2	UBC810	GAGAGAGAGAGAGAGAT
3	UBC811	GAGAGAGAGAGAGAGAC
4	UBC825	ACACACACACACAACCT
5	UBC826	ACACACACACACAACC
6	UBC840	GAGAGAGAGAGAGAGATCT
7	UBC841	GAGAGAGAGAGAGAGATAG
8	UBC842	GAGAGAGAGAGAGAGATAG
9	UBC848	CACACACACACACAAGG
10	UBC855	ACACACACACACACCTT
11	UBC856	ACACACACACACACCTA
12	UBC861	ACCACCACCACCACCACC
13	UBC880	GGAGAGGAGAGGAGA
14	UBC881	GGGTGGGGTGGGGTG
15	UBC889	CGTAGTCACACACACACA

Table.2 Banding pattern of amplified 27 RAPD primers

Sl. No.	Amplified Primers	Monomorphic	Polymorphic	
			Male Specific	Female Specific
1	OPA 01	+	-	-
2	OPA 02	+	-	-
3	OPA 04	+	-	-
4	OPA 07	-	-	+
5	OPA 12	+	-	-
6	OPA 15	-	+	-
7	OPB 02	+	-	-
8	OPB 03	+	-	-
9	OPB 06	+	-	-
10	OPB 08	+	-	-
11	OPB 11	-	-	+
12	OPC 02	+	-	-
13	OPC 03	-	-	+
14	OPC 05	+	-	-
15	OPC 08	+	-	-
16	OPC 13	-	-	+
17	OPD 02	+	-	-
18	OPD 04	+	-	-
19	OPD 06	+	-	-
20	OPD 16	-	+	-
21	OPD 18	+	-	-
22	OPF 11	-	-	+
23	OPF 15	+	-	-
24	OPG 05	-	-	+
25	OPG 15	+	-	-
26	OPJ 01	+	-	-
27	OPK 16	+	-	-

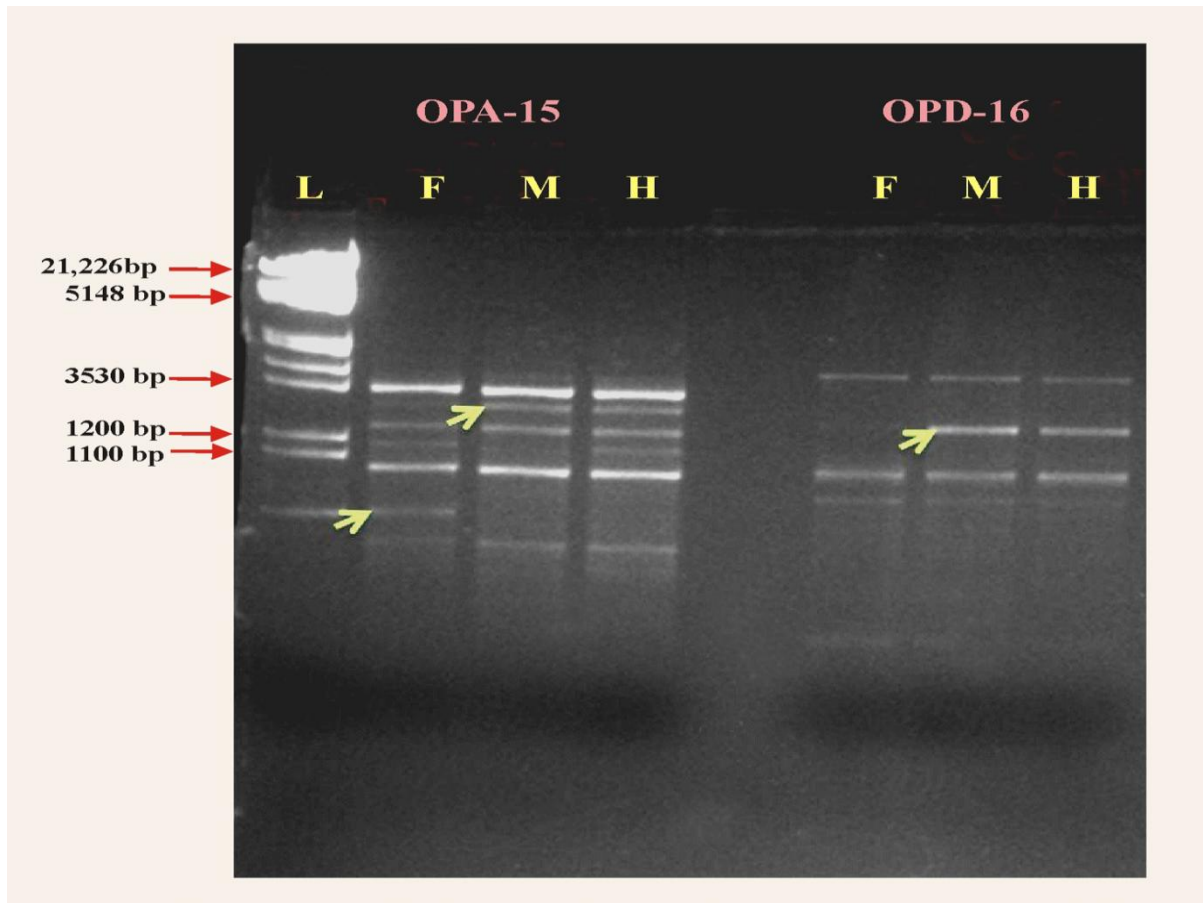
+ Present - Absent

Fig.1 Quality DNA of parents and hybrids



Lane 1-6: Arka Lohit, Lane 8-9: Pusa Jwala and Lane 10-16: Hybrids

Fig.2 Bulk hybrid DNA purity testing through two RAPD primers



L: Lane, F: Female, M: Male, H: Hybrid

Hybrids (1-4), yielded optimum range of DNA quantity (922.10, 820.50, 885.90 and 480.20 ng/ μ L) respectively. Qualities of both parental and individual hybrids were checked through gel electrophoresis which resulted in integrated and good quality of DNA was observed (Figure 1).

Gel scoring

Results of the study confirm that, hybrid produced was true cross between Pusa Jwala \times Arka Lohit. In the investigation carried out, hybrid purity was proved through two RAPD primers OPA 15 and OPD 16.

Among the series of 10-mer RAPD primers tested in this study, 27 primers (Table 2) were amplified for both parents; other primers failed to amplify. Among amplified 27, 10-mer primers, 20 primers produced monomorphic band pattern for both parents (Table 2). This suggests that both parents used in the study were of similar genetic background. After continuous screening of primers to get polymorphism between both parents, particularly for male specific band, five primers showed polymorphism but female specific which were not very useful for the study. After all, two RAPD primers (OPA 15 and OPD 16), showed male specific bands and were used for the purity testing, after confirming its reproducibility.

Observing to the banding pattern of RAPD primer OPA 15, it was clear that 1200bp band size was found to be male specific since it was absent in female parent but present in both male parent and hybrids (Figure 2). However, 600bp faint band was observed in female parent, which was supposed to be present in hybrid. This might be due to recombination between male and female gametes. Similar results were reported by Ballester and Vicente (1998), Inok *et al.*, (2004) in capsicum and Paran *et al.*, (1995),

Crockett *et al.*, (2000) and Liwang *et al.*, (2007) in tomato.

Observing to the banding pattern of another RAPD primer OPD 16, it was clear that 1100 bp size was found to be male specific (Figure 2). However, another faint band (600 bp) was observed to be male specific, which also found in hybrids. This result was supported by reports of Ballester and Vicente (1998), Sharmila *et al.*, (2011) and Ashok *et al.*, (2012) in cotton.

Two primers (OPA 15 and OPD 16) with male specific band, present in hybrid confirms that hybrids were true cross between Pusa Jwala \times Arka Lohit. Since there is no reliable morphological marker for testing hybrid (Pusa Jwala \times Arka Lohit) purity, use of both primers together may serve as an authentic tool for testing hybrid purity. Moreover, at seedling stage itself we can detect the off types without any environmental effect.

The simplicity and speed of molecular methods for testing purity of a hybrid are most interesting attributes and could help companies to assess hybrid seed purity with efficacy in very reduced time compared to the classical *in vivo* methods.

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