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Assessment of Genetic Diversity among Elite Cultivars of *Ricinus communis* L. Using RAPD Markers for Plant Breeding

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ABSTRACT

The present investigation screened the diversity among 19 elite cultivars of castor bean (*Ricinus communis* L.) genotypes based on RAPD markers together with morphological traits in an approach to mine genes governing commercially important traits for future crop improvement programmes. A total of six primers were selected from an initial screening of 64 RAPD primers. The frequency of polymorphic amplicons observed among cultivars varied greatly from 0.05 to 0.95 in all 19 cultivars. A dendrogram showing genetic similarities among the cultivars was constructed based on the polymorphic bands using UPGMA cluster analysis. The UPGMA clustering method revealed two major groups. The first one was made up of two cultivars, 'DCH 190' and 'RG 90' while the remaining cultivars were clustered into the second group. Cultivars belonging to the second group were grouped into five sub-clusters. Genetic differences ranging from 0 to 0.86 among the cultivars indicate a diverse gene pool in the castor bean genotypes studied. Morphological and RAPD tools to determine genetic diversity among *R. communis* cultivars were established.

Keywords: castor bean, cultivars, dendrogram diversity, polymorphism
Abbreviations: B, bloom; **bp**, basepair; **CAPS**, cleaved amplified polymorphic sequences; **cDNA**, complimentary DNA; **dNTP**, dinucleotide triphosphate; **DOR**, Directorate of Oilseed Research, Hyderabad; **FAO**, Food and Agriculture Organization; **GD**, genetic distance; **MAS**, marker-assisted selection; **NB**, non-bloom; **RAPD**, random amplified polymorphic DNA; **RFLP**, restriction fragment length polymorphism; **UPGMA**, unweighted pair-group method with arithmetical averages

INTRODUCTION

Castor bean (*Ricinus communis* L.; *Euphorbiaceae*) is a major oilseed crop plant in India. The crop holds notable place in the global oilseed market accounting for 19% of the total area cultivated and production of 9% of the total vegetative oil (Sengupta and Das 2003). India is a leading castor seed producer, followed by China and Brazil (FAO 2008). The United States is the largest importer and consumer of castor oil in the world due to its application in hundreds of products (Kammili and Jain 2004). Castor bean plants commonly escape cultivation and are found in disturbed sites such as roadsides, stream banks, abandoned lots, and the edges of agricultural fields, such that the species is considered to be an invasive weed. Castor bean populations show diverse morphological traits and hundreds of breeding lines are available in germplasm centers through the world. The diverse phenotypic characters of castor plants that are undergoing adaptive radiation are especially important for exploring the genetic basis of morphological diversification. India has castor bean improvement programmes under the Directorate of Oilseed Research (DOR), Hyderabad. Attempts are being made to utilize a diverse array of castor plant traits for generating disease- and pest-resistant high-yielding elite lineages. Monitoring genetic variability within breeding material is essential before a breeder looks for hybrid vigor since is the greater the genetic distance the better will be the hybrid vigor when the two genomes come within a single nucleo-cytoplasmic environment. The main requirement is the selection of parents. Therefore, knowing the genetic distance of two related or near-related parents is important. This would make crop improvement more efficient by the directed accumulation of favored alleles.

A number of approaches have been used for crop improvement programmes such as studying crop diversity – starting from morphological traits – through biogeography and biomolecules to modern molecular biology approaches. Each protocol has advantages and disadvantages if one tries to treat 'characters', including molecular characters, as markers. The generation of DNA markers and genetic maps are two very important tools for plant geneticists and breeders to understand marker-assisted selection (MAS). They are useful for studying genetic diversity within a group of plants. Earlier attempts have been made using morphological markers (Rao *et al*. 2000), resulting in low levels of genetic polymorphism among them. Unlike morphological and protein markers, DNA markers are not influenced by environmental fluctuations. Therefore, the major advantage of DNA markers is their presumed selective neutrality. A good number of DNA markers were developed for various purposes such as restriction fragments length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), cleaved amplified polymorphic sequences (CAPS), microsatellites and minisatellite sequences, cDNA fragments, etc. Since the early 1990s, PCR has been popular among molecular biologists due to its low cost, easy handling, and reduced time consumption. Amongst the previously mentioned DNA markers, RAPD is quick and less expensive and is therefore popular in many laboratories. To measure genetic diversity within a species, genetic variability has been extensively assessed through PCR markers. After the pioneering work of Williams *et al*. (1990), RAPD has become one of the essential tools for mark-assisted molecular breeding (Patra *et al*. 2011). Though numerous DNA markers linked to genes of interest were identified by RAPD mapping, problems could occur for the recovery of these markers in different laboratories (Purugganan and Robichaux 2005). These criticisms for RAPD-based trait selection and diver-

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Table 1 Phenotypic traits of the *Ricinus communis* L. cultivars.

Cultivar	Stem colour	Morphological traits			
		Nature of spike	Bloom	Internodes	Capsule
GCH-5	Pink	Loose	Triple	Medium	Spiny
GCH-4	Light red	Loose	Triple	Elongated	Partially spiny
48-1	Red	Loose	Double	Elongated	Non-spiny
VP-1	Green	Semi-compact	Triple	Condensed	Spiny
M-619	Green	Semi-compact	Triple	Condensed	Spiny
DCH-178	Red	Semi-compact	Single	Elongated	Semi-spiny
DCH-190	Red	NA	Single	NA	Spiny
DCH-177	Red	Compact	Single	Elongated	Spiny
DCS-9	Red	Compact	Double	Elongated	Spiny
DPC-9	Green	Compact	Zero	Elongated	Spiny
Aruna	Red	Compact	Double	Elongated	Spiny
Kranti	Red	Compact	Double	Elongated	Spiny
RG-954	Red	NA	Zero	NA	Spiny
Local Red NB	Red	Loose	Zero	Elongated	NA
ER-2	Green	Semi-compact	Triple	Condensed	Spiny
ER-5	Green	Semi-compact	Triple	Condensed	Spiny
GC-2	Green	Semi-compact	Triple	Condensed	Spiny
Local Red B	Red	Loose	Triple	Elongated	Spiny
RG 90	Purple	NA	Zero	NA	Spiny

NA, Data not available

sity study based on its reproducibility of producing amplicons has been restricted in the past few years by stringent and improved laboratory practices and development of more accurate scoring procedures (Skaria *et al*. 2011).

As no genetic map of *Ricinus* species is available to date, generation of a sufficient number of polymorphic DNA markers in cultivated *R. communis* shall be needed for developing a saturated castor genome molecular map. This map will accelerate breeding programs in castor bean. Here, the use of DNA marker (RAPD) was practiced to evaluate and study the diversity of 19 *Ricinus* genotypes. The objectives of the present study, therefore, were (1) to analyze the genetic diversity in 19 cultivated castor bean lines, based on studied morphological traits and RAPD data and (2) to distinguish the phylogeny of *Ricinus* using RAPD.

MATERIALS AND METHODS

Plant materials

Nineteen castor bean genotypes brought from DOR, Hyderabad, India, were used for the present study (**Table 1**). Morphological data were collected from a DOR study field. Samples were collected from 15-days-old seedlings of each cultivar grown in a glasshouse in the Calcutta University experimental garden. Entire seedlings were surface sterilized by liquid detergent and 0.1% $HgCl_2$ for 10 min each and finally rinsed 3-4 times in sterilized double distilled water. The samples were then wrapped in aluminium foil and kept at -20°C until needed for DNA extraction. The leaves of a few cultivars ('ER 2', 'RG 90', 'RG 954'), were dried with silica gel for DNA extraction.

Genomic DNA extraction

The DNA was isolated from fresh leaf tissue of the remaining 16 castor cultivars according to Walbot (1988) with some modifications. Castor leaves were harvested from one-month-old castor plants and thoroughly washed in autoclaved distilled water, wrapped in aluminum foil and stored at –70°C until further use. Pre-weighed castor leaves were ground in a homogenization buffer (15 mM sucrose, 50 mM Tris-HCl (pH 8.0), 50 mM Na_2EDTA and 250 mM $NaCl_2$, all purchased from Himedia, Mumbai, India). The homogenate was centrifuge at 5000 rpm for 5 min to collect the crude pellet of nuclei. This pellet was resuspended in Tris-EDTA buffer (10 mM Tris, pH 8.0; 1 mm EDTA, pH 8.0). The nuclei were lysed at 70°C for 25 min with 20% (w/v) sodium lauryl sulphate (20 µl/g leaf tissues) with intermittent shaking. To the sample, 150 µl of 7.5 M ammonium acetate was added after incubation at 70°C was complete, the suspension was thoroughly mixed, incubated on ice for 30 min, and then centrifuged at 12,000 rpm for 10 min at 4°C. To the supernatant, two volumes of chilled absolute ethanol were added and the DNA was spooled out using a sterile glass rod. The DNA was washed twice in 80% ethanol, vacuum dried, and dissolved in an appropriated volume of TE. To remove RNA, the DNA solution was treated with RNaseA (Himedia) (20 µg/ml final conc.) for 1 h at 37°C. To remove proteins, Proteinase K (Himedia) treated DNA solution was extracted with an equal volume of Tris-saturated phenol: chloroform: isoamyl alcohol (25: 24: 1). The supernatant was extracted twice with chloroform: isoamyl alcohol (24: 1) to remove traces of phenol and the DNA was treated with 1/10 volume of 3M Na-acetate (pH 5.2). An equal volume of isopropanol was added to the supernatant to precipitate DNA, which was then dried and rinsed with 80% alcohol, vacuum dried and dissolved in appropriate volume of TE. The purified DNA was quantified on a Hitachi U-2000 spectrophotometer and stored at – 20°C.

The genomic DNA from dried leaves samples of three cultivars (ER 2, RG 90 and RG 954) was isolated using a modified method of Wang *et al*. (1996). Due to the lack of fresh samples of three cultivars, their dried samples, which had been preserved in the previous year, were used. In brief, to dry the leaf samples, leaves that were collected from the field were washed with double distilled water and surface dried at room temperature for 2 h. Then the sample was kept in an air tight container with silica gel. Nuclear DNA was then isolated from these dried samples using the methodology described above for fresh leaf samples. The quality and quantity of DNA was estimated using a Hitachi U-2000 spectrophotometer and also checked visually by ethidium bromide staining of 0.9% agarose gels. The DNA samples were diluted with double distilled sterile water to 25 ng/µl.

RAPD protocol and gel electrophoresis

A total of 64 decamer oligonucleotide primers, 20 each of the OPA, OPJ, and OPBE kits from Operon Technologies, USA, and four self-designed primers (RKC 20 to RKC 23) (Huettel *et al*. 2002), were used individually to screen all 19 genotypes. PCR was carried out in a DNA thermal cycler (Perkin Elmer 2400). Each 25-µl reaction mixture contained 10 mM Tris-HCl, pH 9, 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM each of the four dNTPs (Bangalore Genei, Pvt Ltd., Bangalore, India), 1U *Taq* DNA polymerase, 50 pM primer and 25 ng of template DNA. PCR amplification conditions were as follows: initial denaturation at 95°C for 5 min followed by 45 cycles of denaturation at 94°C for 45 s, primer annealing at 36°C for 1 min, extension step at 72°C for 90 s, followed by a final elongation step at 72°C for 7 min. The annealing temperature of each primer was adjusted according to their Tm value. The RAPD products were separated by agarose (1.5% w/v) gel electrophoresis at 100 V for 4 h using 1X TAE buffer, pH 8.0. The gels were stained with 0.5 µg/µl ethidium bromide and photographed

Table 2 The number and percentage of polymorphic bands obtained by 6 primers in 19 cultivars of *Ricinus communis* L.

Primer	Sequence	Total bands	Polymorphic bands detected			% Polymorphic bands
			Unique	Shared	Total	
OPA 6	GGTCCCTGAC	20	0	20	20	100
OPA 11	CAATCGCCGT	21	6	15	21	100
OPJ 6	TCGTTCCGCA	14	1	13	14	100
OPBE 9	CCCGCTTTCC	18	4	14	18	100
OPBE 18	CCAAGCCGTC	17	1	16	17	100
RKC 23	GAGAGAAGAC	10	2	8	10	100
Total		100	14	86	100	100
Average		16.2	2.3	14	16.7	
% Polymorphism			14	86	100	

under UV light (Fotodyne, 302 nm) with a 35 mm SLR camera using a red filter on black and white 'Ilford Pan 100' photographic film.

Data analysis

The RAPD bands were scored as discrete variables, using 1 for the presence and 0 for the absence of a band. Genetic distances (Nei and Li 1979) between two cultivars were computed as:

$$GD = 1 - \{2N / (N_i + N_j)\}$$

where N is the number of shared bands for cultivars i and j. Distances were used to construct a cluster diagram by the UPGMA (unweighted pair-group method with arithmetical averages) at Bioinformatics Information centre (BIC) (Sneath and Sokal 1973), Bose Institute, Kolkata, India.

RESULTS AND DISCUSSION

The genetic diversity of 19 *Ricinus* cultivars (**Table 1**) was analyzed with DNA (RAPD) markers using arbitrary primers. Fresh or dried leaves were used to isolate DNA, producing good quality DNAs (A_{260}/A_{280} > 1.8). Successful amplification of DNA extracted from desiccated plant tissues was reported earlier (Rajaseger *et al*. 1997). Use of dry-leaf samples would be an attractive alternative method for handling a large number of samples or fossilized samples.

For primarily selection of RAPD primers revealing a high level of polymorphism, between the cultivars, 64 primers were used. After initial screening of the primers in all cultivars, only six (OPA 6, OPA 11, OPJ 6, OPBE 9, OPBE 18 and RKC 23) were selected for further studies. A representative agarose gel profile revealed by RAPD markers with 2 primers (OPA 6 and OPA 11) is shown in **Fig. 1**. Analyses of the 19 *R. communis* lineages with six RAPD primers revealed a total of 100 loci, all polymorphic, between two or more cultivars. The 100 RAPD fragments were scored according to the presence (1) or absence (0) in the 19 cultivars tested. Polymorphic fragments (amplicons) were generated by each of the six primers. Each primer generated 100% polymorphic amplicons. The average number of fragments detected by individual primer ranged from 10 (for primer RKC 23) to 21 (OPA 11). The number of polymorphic amplicons for each primer varied between 10 and 21, with an average of 16.7/primer (**Table 2**). The frequency of individual polymorphic amplicons observed among the 19 cultivars varied greatly from 0.05 (present in only one cultivar) to 0.95 (polymorphism present in all 19 cultivars). The major portion (14%) of the polymorphic amplicons were detected in only one cultivars, 11% in two cultivars, 10% in seven cultivars, 8% in three cultivars, and 7% in four to five cultivars. The remaining RAPD fragments were detected in 8 to 18 cultivars (**Fig. 2**).

Computation of the dissimilarity matrix based on the RAPD profiles of six primers revealed that genetic distance between the cultivars ranged from 0 to 0.86 (**Table 3**). The lowest genetic distance (0) was observed between 'VP-1' and 'M 619', 'Kranti' and 'LRES', and 'Aruna' and 'ER 2',

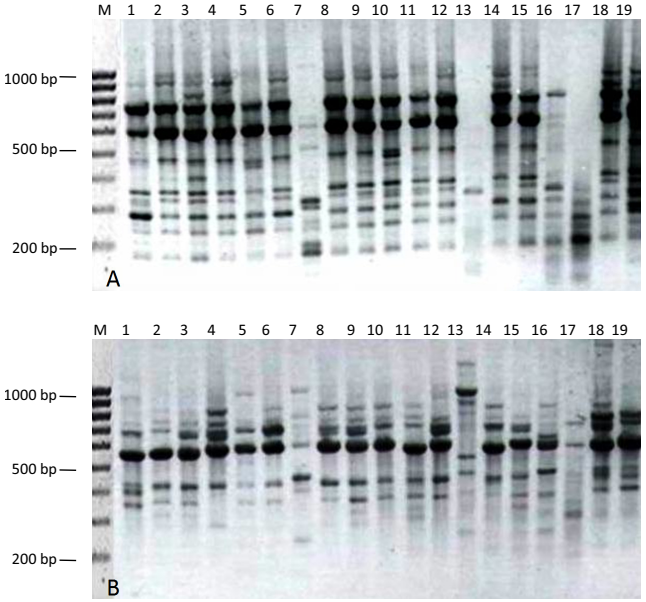

Fig. 1 PCR amplification of DNA fragments with RAPD primer (**A**) OPA 6 and (**B**) OPA 11 of *Ricinus communis* L. 19 cultivars by 1% agarose gel electrophoresis. Lane M, molecular weight marker (100-1000 bp); 1-19 represents 19 cultivars of castor bean. *Ricinus* cultivars 1-19 are listed in **Table 1**.

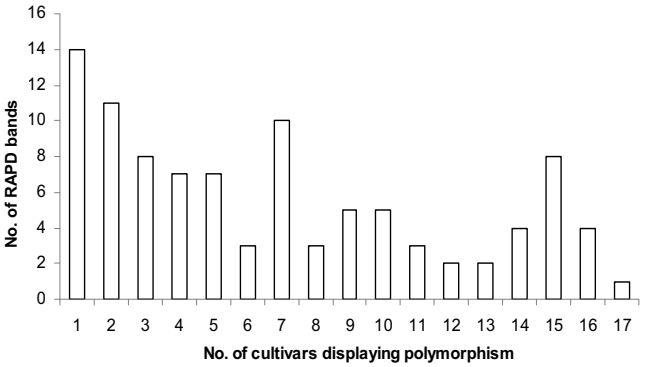

Fig. 2 Frequency of polymorphic RAPD fragments observed in the 19 castor bean cultivars studied. Polymorphic RAPD fragments are categorized by the number of polymorphic castor bean cultivars.

and the highest distance (0.86) was between 'RG 954' and 'Local Red-NB'. The UPGMA cluster diagram (**Fig. 3**) showed two major groups. The first one was made up of two cultivars ('DCH 190' and 'RG 90') while the remaining cultivars were clustered into the second group. Cultivars belonging to the second main group were grouped into five sub-clusters.

Subgroup 1: This subgroup consists of 3 cultivars, namely 'DCH-177', 'DCS-9' and 'DPC-9', all of them have common morphological features. Their common morphological traits are normal to medium growth habit, divergent branching, elongated internodes, flat leaf shape, compact

Table 3 Dissimilarity matrix of 19 cultivars based on the RAPD markers generated by the 6 RAPD primers. *Ricinus communis* L. cultivars 1-19 are listed in **Table 1**.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	0	0.58	0.23	0.36	0.3	0.34	0.5	0.46	0.5	0.5	0.43	0.46	0.5	0.46	0.43	0.45	0.63	0.34	0.5
2		0	0.07	0.19	0.29	0.34	0.31	0.58	0.48	0.48	0.56	0.58	0.7	0.58	0.58	0.34	0.31	0.34	0.42
3			0	0.1	0.24	0.29	0.25	0.56	0.44	0.44	0.53	0.56	0.67	0.56	0.53	0.29	0.25	0.3	0.38
4				0	0	0.1	0.75	0.58	0.5	0.5	0.54	0.58	0	0.58	0.54	0.1	0.5	0.54	0.5
5					0	0.08	0.64	0.42	0.47	0.47	0.38	0.42	0.46	0.42	0.38	0.39	0.46	0.5	0.47
6						0	0.5	0.45	0.38	0.38	0.3	0.34	0.5	0.34	0.3	0.29	0.34	0.42	0.38
7							0	0.75	0.72	0.72	0.6	0.63	0.8	0.63	0.6	0.34	0.4	0.47	0.43
8								0	0.1	0.1	0.15	0.1	0.63	0.1	0.15	0.45	0.5	0.34	0.3
9									0	0	0.06	0.1	0.72	0.1	0.06	0.38	0.43	0.27	0.34
10										0	0.06	0.1	0.72	0.1	0.06	0.38	0.43	0.27	0.34
11											0	0.05	0.6	0.05	0	0.3	0.34	0.2	0.27
12												0	0.63	0	0.05	0.34	0.38	0.24	0.2
13													0	0.63	0.6	0.84	0.8	0.74	0.86
14														0	0.05	0.34	0.38	0.24	0.2
15															0	0.3	0.34	0.2	0.27
16																0	0.17	0.18	0.13
17																	0	0.34	0.29
18																		0	0.16
19																			0

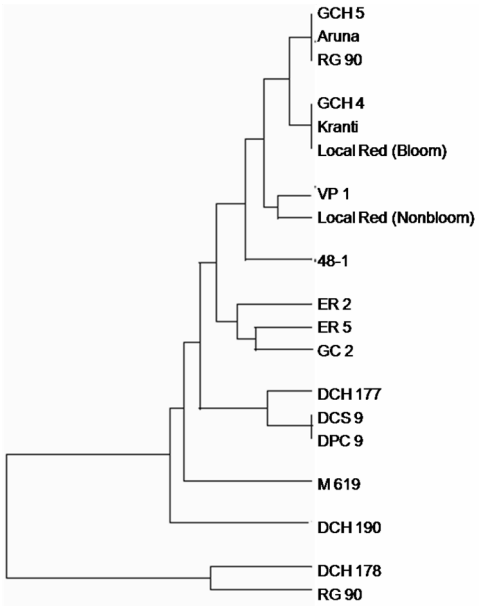

Fig. 3 Dendogram obtained by cluster analysis (UPGMA) showing genetic relationship among 19 cultivars of *Ricinus communis*.

spike, and spiny capsule.

Subgroup 2: Since the three cultivars that formed this subgroup were isogenic lines of 'VP-1', it is therefore a homogenous group. Morphologically they are all alike except that they differ in their sex expression pattern.

Subgroup 3: This subgroup consists of two cultivars, namely 'VP-1' and 'M 619'. 'M 619' is the mutant form of 'VP 1' and its morphological traits are similar to those of 'VP-1'. The genetic distance between cultivars is 0 (**Table 2**) which correlates positively with the clustering pattern.

Subgroup 4: This subgroup consists of three cultivars, namely 'GCH-4', 'Kranti', and 'Local Red-NB'. 'GCH-4', a high yielding wilt disease resistant hybrid, was developed in Gujarat by crossing '48-1' (wilt-resistant) and 'VP-1' (wilt-susceptible) genotypes. The two parents possess a wide genetic base, and therefore they were not featured in this group. The subgroup's common morphological traits are: a red stem, elongated internodes, a spiny capsule, flat leaf shape, and they have blooms.

Subgroup 5: The subgroup is composed of three cultivars, 'GCH-5', 'Aruna', and 'RG 954' which have a red stem, divergent branching, elongated internodes, a flat leaf shape, and a spiny capsule.

A few cultivars of the second group did not form a cluster with the existing cultivars within the group because they are phenotypically very diverse due to variability present in their genotypic constituent. '48-1', the parent of hybrid 'GCH-4', is highly resistant to Fusarium wilt and its morphological traits differ widely from its counter partner, 'VP-1' and its hybrid 'GCH-4'. Thus, this genotype did not feature in either subgroup in which 'VP-1' and 'GCH-4' were present. 'DCH-190' and 'LR B' are the other two cultivars that did not form a cluster.

The use of RAPD markers in DNA fingerprinting of castor bean cultivars is feasible. For example to find markers linked to Fusarium wilt resistance (Singh *et al*. 2011). Although RAPD markers do not appear to be more polymorphic than RFLPs on a per locus basis (McCouch 1988), their abundance may allow then to be used for genetic studies within *R. communis* cultivars. Considerable interest exists in using molecular markers to assign crop cultivars to heterotic groups, and to predict heterotic hybrids (Mackill 1995). The heterotic hybrid 'GCH-4' is the outcome of crosses between two homozygous cultivars, viz. '48-1' and 'VP-1' with wide genetic bases. The two parents have highly dissimilar values and are not in the same cluster (**Fig. 3**). The results presented indicate that extremes within cultivars would provide a heterotic combination.

The cultivars, which did not form a cluster with the existing cultivars, are very diverse in morphology due to variability in their genotypes. For example, '48-1', the parent of 'GCH-4', is highly resistant to Fusarium wilt. Its morphological traits differ widely from its counter partner, 'VP-1', and its hybrid 'GCH-4' (**Table 1**). Thus, '48-1' was not featured in either of the groups to which 'VP-1' and 'GCH-4' belonged.

Grouping of 'GCH-4' with RAPD primers substantiates an earlier observation with RAPD primers (Khush 2002). There is much evidence that markers can be used to assign heterosis to cultivars for specific combinations (Dubley 1991; Stuber 1992). Here, RAPD markers predicted the genetic distances between cultivars. It used to be presumed that finding genetic polymorphisms in monotonous genomes like common bean (Sonnate 1994) and tomato (Miller 1990) would make it difficult to generate molecular linkage maps for these crops, although newer molecular studies have disproved this. Our findings show that the use of RAPD markers for DNA fingerprinting of castor crop is feasible. Our results will be useful to castor bean breeders as a quick tool for selecting suitable parents with a wide genetic distance for developing desirable hybrids.

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