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Research Article

RAPD markers for genetic variability studies in *Indigofera tinctoria*

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Abstract
Genetic relationship among twenty accessions of *Indigofera tinctoria* using PCR based random amplified polymorphic DNA (RAPD) markers was assessed. Forty decamer primers were screened for their efficiency using the isolated DNA, of which twenty two yielded amplification products. Based on the reproducibility of the bands, level of polymorphism, number and intensity of bands produced the primers OPA-10, OPB-3, OPB-5 and OPB-10 were selected for further PCR amplification and statistical analysis. A total of 26 RAPDs were obtained from the four selected primers, of which 17 were polymorphic. The overall similarity coefficients ranged from 0.28 to 1.0. At a similarity coefficient of 0.46, the twenty accessions grouped into two clusters, accession IT 13 with least similarity coefficient forming a separate cluster, and all the others grouped into a large cluster. The study demonstrated the usefulness of RAPD markers in effectively assessing the genetic relationship among the *Indigofera tinctoria* accessions.

Key words
Neelayamari, *Indigofera tincloria*, RAPD, genetic variability, medicinal plant

Introduction

Indian indigo (*Indigofera tincloria* L.) known as neelayamari in Malayalam, is a leguminous deciduous sub shrub of the south-eastern Asia. The shoot of the plant contains 'indigotin', a deep blue dye in the form of a glycoside namely ‘indican'. Plant derived dyes like indigotin have been shown to be useful in dying clothes which do not produce "dermatitis", an allergic condition of the skin often caused by synthetic dyes. Historically, indigo is the oldest blue dye utilized by man and was commercially much exploited till synthetic coloring materials were evolved. The plant besides utilized as a source of blue dye, is also valued in Ayurveda as an important ingredient of hair tonics like 'Neelibhringadi thailam' Neelayamari is also reported to be useful in the treatment of myelocytic leukemia, inflammatory skin conditions and in the glandular inflammation of the lymph nodes and tonsils. The root of the plant is used for the treatment of hepatitis. An infusion of the root is given as an antidote in case of snake bite and poisoning by arsenic. Lack of authentic varieties of medicinal plants is a major drawback that hinders quality standardization of pharmaceutical preparations made out of them. Screening of existing germplasm for evolving superior genotypes and releasing authentic varieties of medicinal plants would help in maintaining the uniformity of materials used in pharmaceutical industries. Natural variations in DNA sequence, that can be detected using marker systems, are nowadays reliably utilized for differentiating various accessions and their different traits. Molecular marker assisted breeding has proved to have the potential to enhance the pace and efficiency of genetic improvement of crop plants. Random Amplified Polymorphic DNA (RAPD) is a technique useful for genomic analysis as it detects high level of polymorphism in a much shorter time. The amount of DNA used in RAPD reactions is very low and hence needs only small amount of tissue. The simplicity, reliability and not affected nature by environmental and tissue sources, all add to the utility of this technique. Hence in this study, an attempt has been made to evaluate the genetic variability of twenty accessions of *Indigofera tinctoria* collected from different parts of Kerala using RAPD markers.

Materials and Methods

Seeds of twenty accessions of *Indigofera tinctoria* were collected from different parts of Kerala. Seeds were sown on earthen seed pan after subjecting to acid scarification. Seedlings were transplanted after three weeks at five leaf stage to 250guage polythene bags of size 40×20cm filled with potting mixture consisting of cow dung, sand and soil in the ratio 1:1:1. The experiment was laid out in CRD with three replications. The details of the accessions and sources are presented in Table 1.

To isolation of genomic DNA, tender leaves were chosen from the *Indigofera tinctoria*. The isolation was done following a modified method given by Murray and Thompson (1980). About l g of the plant leaves was taken as the base material for isolation after removing the midrib. The leaves were collected during early morning and washed thoroughly in running tap water and then twice rinsed with distilled water. The leaves were then placed in tissue paper for removing the entire water content. After this leaves were placed in pre-chilled porcelain container and liquid nitrogen was poured over the leaf material. The leaves were ground well to a fine powder and then transferred to 1ml extraction buffer (0.7 N NaCI, 1% CTAB, 50 mM Tris- HCI (pH 8.0), 10 µM EDTA) placed in 1.5 ml polypropylene centrifuge tube. To this 5µl 0.2% ß-mercaptoethanol and 1% polyvinyl pyrollidone (PVP) was added. PVP was added to remove the phenolic compounds from plant DNA extracts. This was mixed gently by tapping and is placed in the water bath preset at 60^{o}C for one hour. The tubes were then centrifuged at 15,000 rpm for 10 minutes. The supernatant was taken and to this equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added and again subjected to centrifugation at 15,000 rpm for 10 minutes at 4^{o}C. After collecting the upper phase the Phenol: Chloroform: Isoamyl alcohol (25:24:1) extraction was repeated until the interphase disappeared. To the aqueous phase collected equal volume of Chloroform: Isoamyl alcohol (24:1) solution was added and the two phases was mixed gently. Centrifugation was done at 15000 rpm for 10 minutes at 4^{o}C. To the supernatant collected 1/10th volume of 3.0 M sodium acetate and two volumes of cold absolute ethanol were added and were kept in refrigerated condition at 4^{o}C for 30 minutes. Centrifugation of the resultant was done at 10000 rpm for 10 minutes to pellet the DNA. The aqueous phase is drained out and the pellet was washed with 70 % cold ethanol and at 10,000 rpm for 5 minutes at 4^{o}C. The excess ethanol was drained out and kept in hybridization incubator for drying. It was then dissolved in 0. 5µl of 1x Tris EDTA buffer (10mM Tris-HCI, ImM EDTA, pH 8.0) and stored at 4^{o}C.

Reliable quantification of DNA concentration is important for many applications in molecular biology including amplification of target DNA chain reaction. DNA quantification was carried out with the help of UV-VIS Spectrophotometer (Spectronic Genesis 5). The buffer in which the DNA was already dissolved was taken in a cuvette and used for the calibration of the spectrophotometer at 260 nm as well as 280 nm wavelength. The optical density of the DNA sample dissolved in the buffer was recorded at both 260 nm and 280 nm. Since an OD value of 1.0 at 260 nm represents 50 µg / ml of DNA, the DNA concentration was estimated by employing the following formula:

Amount of DNA (µg / ml) = (A_{260} × 50 × dilution factor) / 1000

Where, A_{260} = absorbance at 260 nm

The quantity of DNA could be judged from the ratio of the OD values, recorded at 260 and 280 nm. The A_{260} / A_{280} ratio between 1.7 and 1.9 indicates best quality of DNA.

Agarose gel electrophoresis was carried out in a horizontal gel electrophoresis unit supplied by the Bangalore Genei^{TM}. Required amount of agarose was weighed out (0.8% for visualizing the genomic DNA and 1.5% for visualizing the amplified products) and melted in 1x TAE buffer (0.04M Tris acetate, 0.001M EDTA, pH 8.0) by boiling. After cooling to about 500^{o}C, ethidium bromide was added to a final concentration of 0.5 mg/ml. The mixture was poured immediately to a preset template with appropriate comb. After solidification, the comb and the sealing tapes were removed and the gel was mounted in an electrophoresis tank filled with 1xTAE buffer. The DNA sample was mixed with required volume of gel loading buffer (6.0x loading dye *viz*. 40% sucrose, 0.25% bromophenol blue). Each well was loaded with 20µl of sample. One of the wells was loaded with 5 µl of molecular weight marker along with required volume of gel loading buffer. Electrophoresis was carried at 50 volts until the loading dye reached 3/4th length of the gel. The gel was visualized using an ultra-visible (uv-vis) trans-illuminator (Appligene, Oncor, Trans).

RAPD analysis was performed by the method recommended by Williams *et al.*, (1990), with required modification. Forty arbitrarily designed decamer primers supplied by Operon Inc., CA, USA were used. Genomic DNA (40 ng) was amplified in a 25 µl reaction mixture volume containing 2.5 µl 10x PCR buffer, 2 µl $MgC1_2$ (Magnesium chloride), 1 µl primer, 2 µl each of deoxy nucleotides (dNTPs) and 0.6U of Taq DNA polymerase (Bangalore Genei Pvt. Ltd; Bangalore). Amplification was carried out in a programmable thermal controller (MJ Research Inc. USA) set for the following programme. An initial denaturation at 94^{o}C for 4 minutes followed by 40 cycles of denaturation at 94 ^{o}C for 1 minute, annealing at 35 ^{o}C for 1 minute and extended at 72^{o}C for 1 minute 30 seconds. The synthesis step of the final cycle was extended further by 10 minutes. Finally the products of amplification were cooled to 4^{o}C. The amplified products along with DNA molecular

weight marker supplied by Bangalore Genie were separated by electrophoresis using 1.5 per cent agarose gel, stained with ethidium bromide and visualized on a UV trans-illuminator. The number of monomorphic bands, number of polymorphic bands and intensity of bands were recorded. Those primers, which when used for amplification, produced the maximum number of bands were used to amplify the DNA of all the twenty *Indigofera tinctoria* accessions. The photograph of the amplified profile obtained in all the accessions using A-10, B-3, B-5, and B-10 primers, were taken with the help of a gel documentation system. The PCR was repeated in order to check the reproducibility.

The data obtained were subjected to cluster analysis by UPGMA method using NTSYS software (Version 2.2) to estimate the similarity indices and genetic relatedness among the accessions. The reproducible bands were scored for the presence '+' or absence for all the accessions studied. A genetic similarity matrix was constructed using the Jaccard's coefficient method (Jaccard, 1908). Jaccard's coefficient Sj = a / (a + b + c) where, a is the number of bands present in both accessions, b is the number of bands present in 1st accession but not in the 2nd and, c is number of bands present in the 2nd accession but not in the 1st. Association between the various accessions was found out from the dendrogram.

Results and Discussion

In the present study, the DNA yields from 20 accessions of *Indigofera tinctoria* estimated using UV-Vis spectrophotometer ranged from 0.51 (IT 5) to 5.85 µg/µl (IT 3). The A_{260}/A_{280} ratio ranged from 1.53 (IT 3) to 2.01 (IT 10). The results are detailed in Table 2. The quality of the isolated DNA was assessed by gel electrophoresis. For RAPD analysis, forty decamer primers were screened for their efficiency using the DNA isolated from IT 15 as the representative sample. The use of RAPD markers to genetically fingerprint plants which are morphologically similar or indistinguishable has been established as a reliable, efficient and informative tool. RAPD analysis has been successfully used to analyze genetic diversity in different medicinal plant as reported by Wachira *et al* (1995), Padmesh *et al*.(1999), Nybom *et al.* (2000), Raimondi *et al.* (2001), Nybom (2004), Canter *et al*. (2005), Guo *et al.* (2006), Tejesvi *et al.* (2007), Sarwat *et al.* (2008) and Rahimmalek *et al* (2009). The primer associated banding pattern in DNA sample of accession IT 15 is shown in Table 3. Out of this, twenty-two yielded amplification products. The primers OPA-2, OPA-3, OPA-5, OPA-7, OPA-8, OPA-13, OPA-14, OPA-16, OPA-18, OPA-19, OPB-1, OPB-6, OPB-7, OPB-13, OPB-15, OPB-16, OPB-18 and OPB-20 yielded no amplification. The primers produced a total of 44 bands, of which the highest number of RAPDs was produced by the primers OPA-10 (6 bands), OPB-3 and OPB-5 (4 bands) and OPB-10 (3 bands). Based on the reproducibility of the bands, level of polymorphism, number and intensity of bands produced the primers OPA-10, OPB-3, OPB-5 and OPB-10 were selected for further PCR amplification of twenty accessions of *Indigofera tinctoria*. The reproducibility of the selected primers was confirmed, by repeating the PCR reaction at least twice and the data obtained from these primers were used for further statistical analysis. Nucleotide sequence of the selected primers used in the study is given in Table 4.

The highest number of scorable bands (8) was obtained from the primer opA-10. Four bands were monomorphic for all the accession. The accessions IT 3, IT 6, IT 8, IT 10, IT 16, IT 18 and IT 20 produced seven bands each. Six bands were produced by the accessions IT 7, IT 13, IT 14 and IT 15. A total of five bands were produced by the accessions IT 9, IT 12, IT 17 and IT 19. The accessions IT l, IT 3, IT 5 and IT I I produced four bands each (Fig. 1). The primer OPB-5 produced a total of seven scorable bands, of which one band was monomorphic to all the accessions. Almost all the accessions produced a total of four bands each except IT 8 and IT 20 which produced a total of three bands and IT 6, IT 18 and IT 20 which produced two bands each. A polymorphic banding pattern was observed in the accession IT 13 (Fig. 2). Primer OPB-10 could amplify a total of six scorable bands for twenty accessions of *Indigofera tinctoria.* The accessions IT 3, IT 7, IT 10, IT 15 and IT 18 produced three bands each, while the accessions IT l, IT 2 and IT 20 produced four bands each. All the other accessions produced five bands each. Of the six scorable bands, three were monomorphic and the rest were polymorphic (Fig. 3). The primer OPB 3, from a total of five scorable bands produced three monomorphic bands. The accession IT 20 produced five bands, whereas the accessions IT 1, IT 2, IT 3, IT 12, IT 18 and IT 19 produced three bands each. All the other accessions gave four bands each (Fig. 4). Reproducible bands were scored for their presence or absence for all the accessions studied. The data obtained were subjected to cluster analysis by UPGMA method using NTSYS software, (Version 2.2) to estimate the similarity indices and genetic relatedness among the accessions. A genetic similarity matrix (Fig. 5) was constructed using the Jaccard's coefficient method (Jaccard 1908). The cluster

based on RAPD analysis using four primers shows that the similarity indices range from 0.46 to 1.00. The twenty accessions that were used for RAPD analysis formed three main clusters in the UPGMA cluster analysis (Fig. 6). At a similarity coefficient of 0.46, the twenty accessions grouped into two clusters. At 0.46, the accession IT 13 formed a separate cluster, while all the other accession grouped into a larger cluster. The accessions IT3, IT 10, IT 12 and IT 15 showed a similarity coefficient of 1.00 with each other. Similarly, the accessions IT6 and IT 17 showed a similarity coefficient of 1.00 with each other. Same is the case with accessions IT1, IT2, IT4 and IT5. At similarity coefficient of 0.784, the dendrogram got divided into 5 clusters, of which the accessions IT3, IT 10, IT 12, IT 15, IT 18 and IT 19 formed the first cluster. The second cluster was formed from the accessions IT17, IT6, IT7, IT16, IT5, IT4, IT2 and IT1. The three accessions, IT20, IT 8and IT14 grouped together to form the third cluster. The fourth cluster had two accessions, namely IT 9 and IT 11. The accession IT13 formed an individual cluster. The molecular variability analysis of twenty accessions of Indigofera tinctoria revealed that the accession IT 13 was found to be distinct from all the other accessions. This finding was in par with the study conducted by the same group, where genetic variability analysis of *Indigofera tinctoria* was carried out by morphological characterization (Neema and Reghunath, 2016). The study asserts the fact that RAPD technique can detect sufficient polymorphisms for germplasm characterization and genetic distance studies.

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Table 1. Accession number and source of *Indigofera tinctoria* accessions used for the study

Sl. No	Accession No.	Source
1.	IT 1	Palode, Thiruvananthapuram
2.	IT 2	Mundakkayam , Kottayam
3.	IT 3	Vellanikkara , Thrissur
4.	IT 4	Pala , Kottayam
5.	IT 5	Odakkali , Eranakulam
6.	IT 6	Ollur , Thrissur
7.	IT 7	COH, Thrissur
8.	IT 8	Vellayani, Thiruvananthapuram
9.	IT 9	Nellimoodu , Thiruvananthapuram
10.	IT 10	Kazhivoer road, Thiruvananthapuram
11.	IT 11	Ammanada, Kollam
12.	IT 12	Chalai, Thiruvananthapuram
13.	IT 13	Ayurveda College, Thiruvananthapuram
14.	IT 14	Nagaroor Thiruvananthapuram
15.	IT 15	Varkala, Thiruvananthapuram
16.	IT 16	Kottakkal, Malapuram
17.	IT 17	Palapur, Thiruvananthapuram
18.	IT 18	Vellanadu, Thiruvananthapuram
19.	IT 19	Pattambi, Palakkad
20.	IT 20	Neyyattinkara Thiruvananthapuram

Table 2. Quantitative and qualitative characters of DNA isolated from 20 accessions of *Indigofera tinctoria*

Accession No.	$A_{260\ nm}$	$A_{280\ nm}$	Ratio 260 /280	DNA yield (µg/µl)
IT1	0.032	0.018	1.78	0.96
IT2	0.143	0.075	1.92	4.29
IT3	0.195	0.128	1.53	5.85
IT4	0.077	0.046	1.68	2.31
IT5	0.017	0.01	1.72	0.51
IT6	0.169	0.088	1.92	5.07
IT7	0.094	0.05	1.86	2.82
IT8	0.13	0.079	1.65	3.9
IT9	0.04	0.021	1.87	1.2
IT10	0.038	0.019	2.01	1.14
IT11	0.096	0.058	1.65	2.88
IT12	0.029	0.016	1.79	0.87
IT13	0.042	0.022	1.95	1.26
IT14	0.083	0.053	1.57	2.49
IT15	0.02	0.012	1.69	0.6
IT16	0.056	0.032	1.75	1.68
IT17	0.072	0.039	1.83	2.16
IT18	0.056	0.029	1.94	1.68
IT19	0.073	0.046	1.58	2.19
IT20	0.08	0.048	1.68	2.4

Table 3. The primer associated banding pattern in DNA sample of accession IT 15

Sl. No.	Primers	Total number of bands	Number of intense bands	number of faint bands
1	OPA-1	2	1	1
2	OPA-2	0	0	0
3	OPA-3	0	0	0
4	OPA-4	1	1	0
5	OPA-5	0	0	0
6	OPA-6	1	1	0
7	OPA-7	0	0	0
8	OPA-8	0	0	0
9	OPA-9	1	0	1
10	OPA-10	6	4	2
11	OPA-11	1	0	1
12	OPA-12	1	1	0
13	OPA-13	0	0	0
14	OPA-14	0	0	0
15	OPA-15	2	1	1
16	OPA-16	0	0	0
17	OPA-17	1	0	1
18	OPA-18	0	0	0
19	OPA-19	0	0	0
20	OPA-20	2	1	1
21	OPB-1	0	0	0
22	OPB-2	2	0	2
23	OPB-3	4	2	2
24	OPB-4	2	1	1
25	OPB-5	4	3	1
26	OPB-6	0	0	0
27	OPB-7	0	0	0
28	OPB-8	2	0	2
29	OPB-9	2	1	1
30	OPB-10	3	3	0
31	OPB-11	2	1	1
32	OPB-12	1	0	1
33	OPB-13	0	0	0
34	OPB-14	1	1	0
35	OPB-15	0	0	0
36	OPB-16	0	0	0
37	OPB-17	1	1	0
38	OPB-18	0	0	0
39	OPB-19	2	0	2
40	OPB-20	0	0	0

Table 4. Nucleotide sequence of primers selected for RAPD analysis

Sl. No.	Primer	Sequence
1	OPA-10	GTGATCGCAG
2	OPB-3	CATCCCCCTG
3	OPB-5	TGCGCCCTTC
4	OPB-10	CTGCTGGGAC

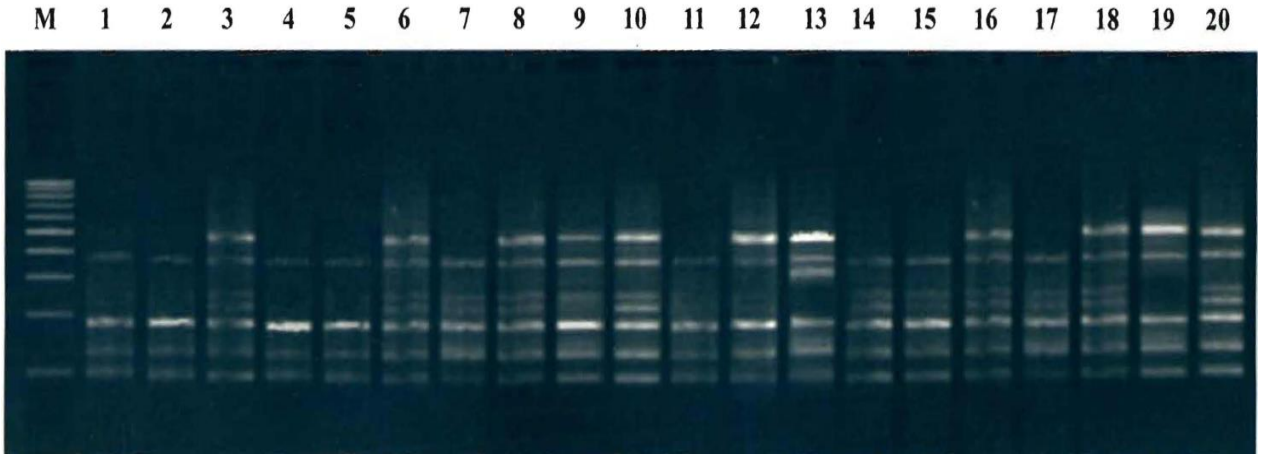


Fig. 1. Amplification profiles of the DNA of twenty accessions of *Indigofera tinctoria* (1-20) using the primer OPA-10; M: Molecular marker

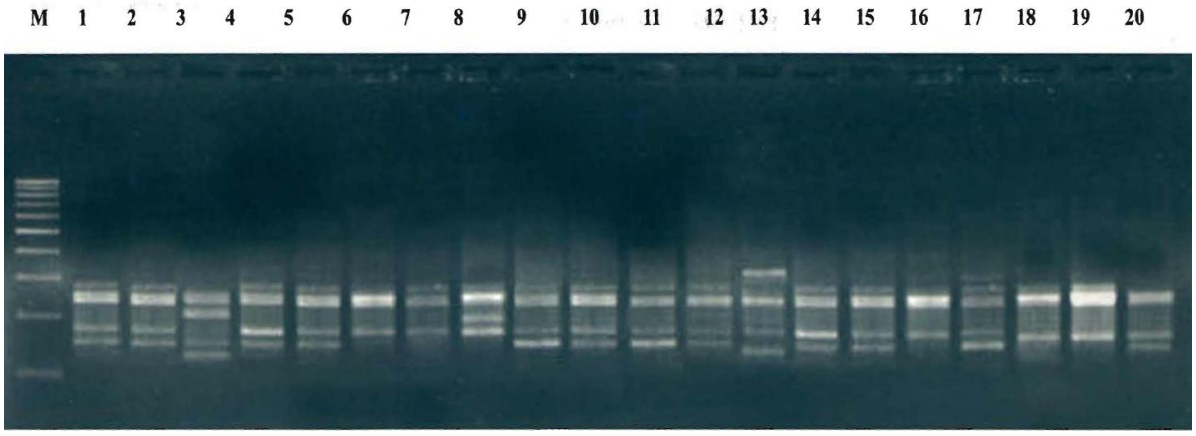


Fig. 2. Amplification profiles of the DNA of twenty accessions of *Indigofera tinctoria* (1-20) using the primer OPB-5; M: Molecular marker

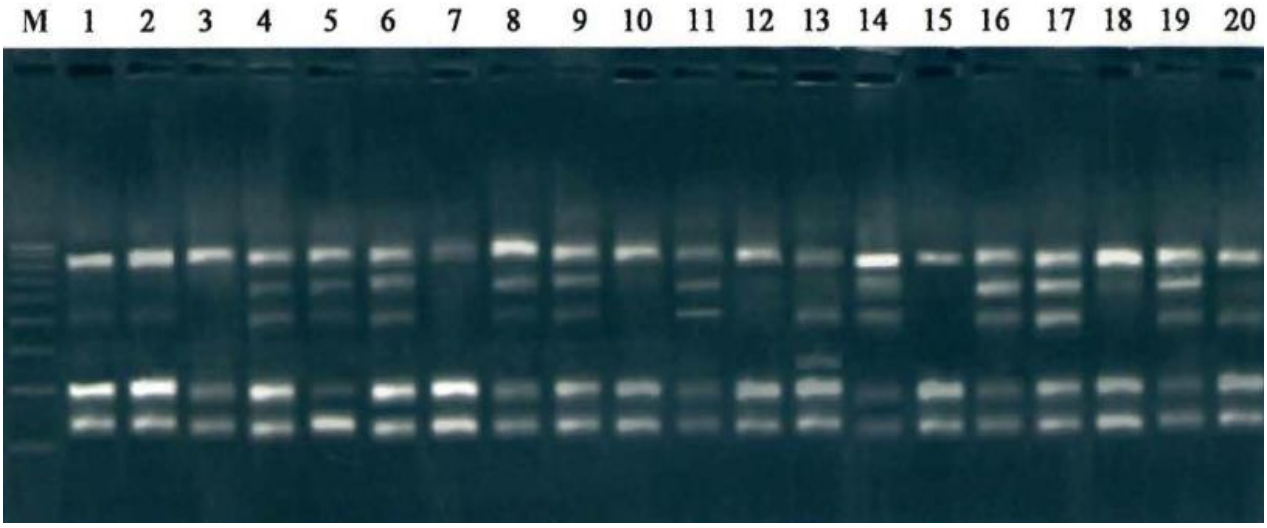


Fig. 3. Amplification profiles of the DNA of twenty accessions of *Indigofera tinctoria* (1-20) using the primer OPB-10; M: Molecular marker

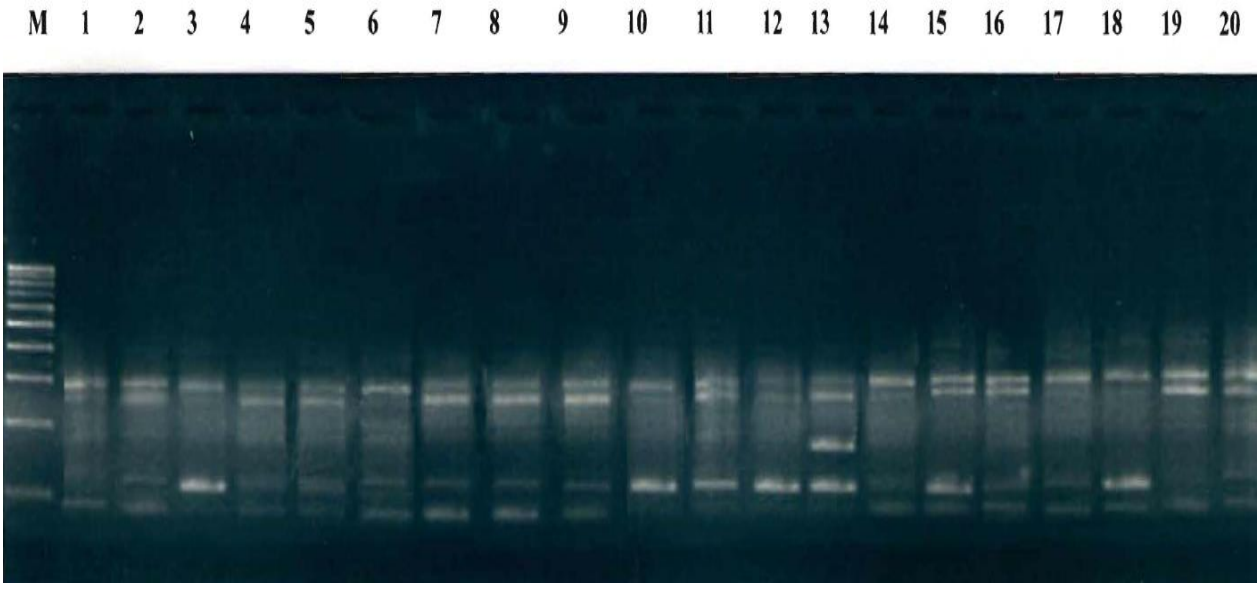


Fig. 4. Amplification profiles of the DNA of twenty accessions of *Indigofera tinctoria* (1-20) using the primer OPB-3; M: Molecular marker

	IT1	IT2	IT3	IT4	IT5	IT6	IT7	IT8	IT9	IT10	IT11	IT12	IT13	IT14	IT15	IT16	IT17	IT18	IT19	IT20
IT1	1.00																			
IT2	1.00	1.00																		
IT3	0.69	0.69	1.00																	
IT4	1.00	1.00	0.69	1.00																
IT5	1.00	1.00	0.69	1.00	1.00															
IT6	0.85	0.85	0.71	0.85	0.85	1.00														
IT7	0.88	0.88	0.42	0.88	0.88	0.42	1.00													
IT8	0.60	0.60	0.52	0.60	0.60	0.54	0.55	1.00												
IT9	0.65	0.65	0.30	0.65	0.65	0.40	0.40	0.69	1.00											
IT10	0.69	0.69	1.00	0.69	0.69	0.50	0.44	0.52	0.42	1.00										
IT11	0.50	0.50	0.85	0.50	0.50	0.62	0.37	0.69	0.86	0.60	1.00									
IT12	0.69	0.69	1.00	0.69	0.69	0.55	0.33	0.52	0.43	1.00	0.50	1.00								
IT13	0.46	0.46	0.55	0.46	0.46	0.50	0.45	0.39	0.42	0.55	0.42	0.55	1.00							
IT14	0.55	0.55	0.50	0.55	0.55	0.50	0.44	0.90	0.69	0.80	0.69	0.54	0.40	1.00						
IT15	0.69	0.69	1.00	0.69	0.69	0.28	0.16	0.52	0.40	1.00	0.42	1.00	0.55	0.33	1.00					
IT16	0.83	0.83	0.30	0.83	0.83	0.45	0.90	0.57	0.46	0.72	0.54	0.80	0.49	0.58	0.18	1.00				
IT17	0.85	0.85	0.55	0.85	0.85	1.00	0.70	0.54	0.40	0.70	0.50	0.77	0.50	0.70	0.22	0.80	1.00			
IT18	0.66	0.66	0.50	0.66	0.66	0.50	0.44	0.51	0.42	0.80	0.45	0.54	0.43	0.70	0.33	0.58	0.70	1.00		
IT19	0.69	0.69	0.47	0.69	0.69	0.37	0.50	0.53	0.56	0.40	0.50	0.44	0.51	0.55	0.33	0.36	0.44	0.87	1.00	
IT20	0.71	0.71	0.56	0.71	0.71	0.65	0.66	0.82	0.69	0.56	0.69	0.56	0.59	0.82	0.56	0.63	0.65	0.88	0.44	1.00

Fig. 5. Similarity matrix among twenty accessions of *Indigofera tinctoria* obtained by RAPD analysis using four primer combinations

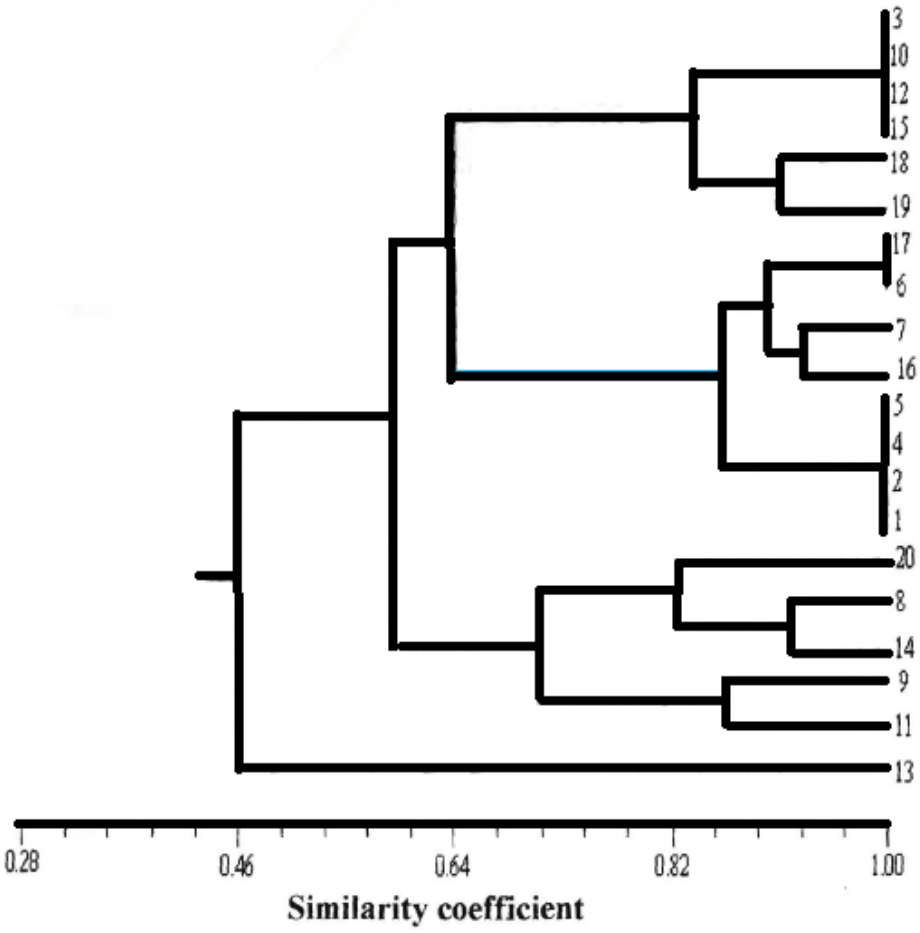
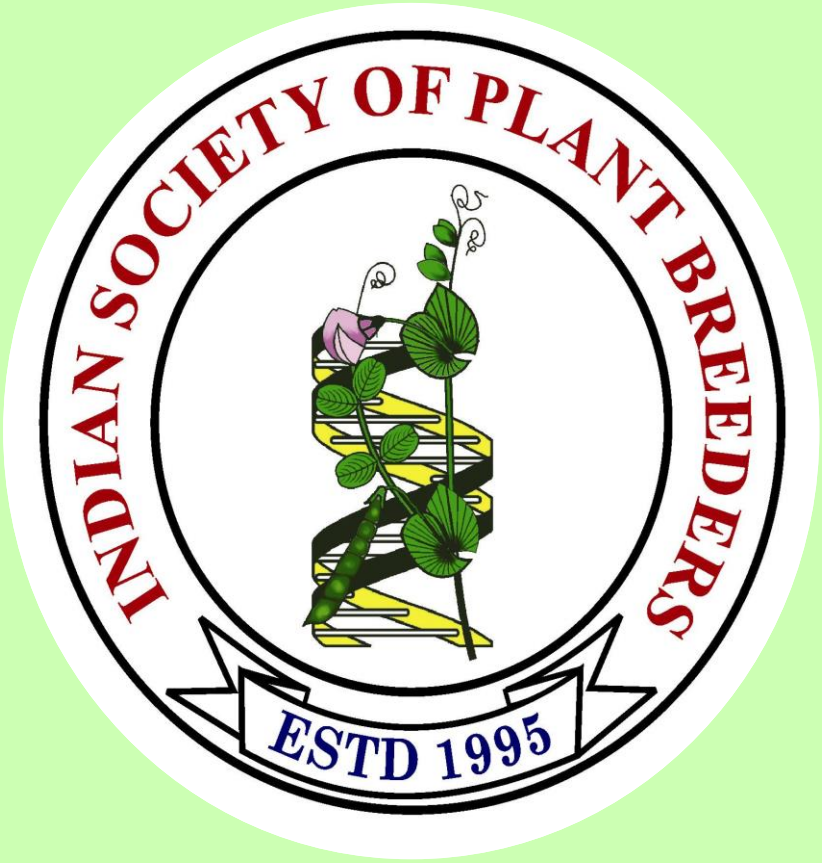


Fig. 6. Dendrogram of RAPD markes for twenty accessions of *Indigofera tinctoria*



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