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Study of Gene Polymorphism in Litchi Chinensis Sonn. (Sapindaceae) using Various DNA Markers

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ABSTRACT

Litchi chinensis (Gaertn.) Sonn. is commercially an important plant much valued for its fruits belong to family Sapindaceae found both wild and introduced, in the tropical and sub-tropical regions of the world. Litchi is reported to have been introduced into India from China towards the end of 18th century. It is now cultivated in number of countries outside China including India, Burma, Thailand, South Japan, Australia, New Zealand, Hawaii, USA, Brazil, West Indies and South Africa. India and South Africa are the largest producers of Litchi outside China. In the present study RAPD markers were used to study genetic diversity in genotypes of the Litchi chinensis. A total 28 genotypes of Litchi chinensis collected from different localities of two states (Uttar Pradesh and Uttarakhand), were taken into consideration. Ten RAPD primers were screened with template DNA. Two RAPD primers (OPB-19 and OPC-08) were selected for further profiling based on distinct banding patterns obtained. A total of 28 bands ranging from 300bp -3000bp were obtained with two RAPD primers. Out of these, 18 bands were polymorphic revealing 64% polymorphism across all genotypes of Litchi chinensis. Similarity coefficient value varied from 0.51 to 1.00 across different genotypes. The results show that RAPD profiles are sufficiently informative and useful to unravel the genetic diversity in Litchi Chinensis. The information on genetic diversity of Litchi could further be utilized in the breeding of genetically divergent and geographically isolated genotypes to obtain better germplasm in terms of yield and quality.

1. Introduction

The genus *Litchi* belong to the family Sapindaceae, a soapberry family that has over 2000 species and 150 genera, mostly tree or shrubs but rarely herbs, widely distributed the warm tropic and subtropics. *Litchi* is a genus of tree comprising two species *Litchi phillipensis*Radlf and *Litchi Chinensis* (Gaertn) Sonn. (Anonymous, 1962) plant are evergreen woody trees with 10-12 m high with broad round topped crown of glossy green foliage. *Litchi* has been shown to possess variable diploid chromosome numbers where 2n = 28, 30, 32 (Chapman, 1984).

Litchi is reported to have been introduced into India from China towards, the end of 18th century. It is now cultivated in number of countries outside China including India, Burma, Thailand, South Japan, Australia, New Zealand, USA, Brazil, West Indies and South Africa. In India *Litchi* is cultivated in North Bihar, mostly in Muzzafarapur and Dharbanga districts. It is also successfully cultivated in Gorakhpur, Faizabad, Bareilly, Bahraich, Kheri, Pilibhit and Dehradun districts of Utter Pradesh and Uttarakhand respectively.

Litchi grows in the areas having short, dry and winter with long and hot summer. Winter frost and dry heat in summer considerably damage the growth of the plants. It causes fruit cracking and subsequently damages the pulp. Humidity is another important factor for the successful cultivation of *Litchi*. Although it can grow up to an altitude of 800m above the sea level, the best growth and yield is obtained at lower elevations. Well separate rainfall or adequate supply of irrigation water is essential for *Litchi* cultivation. *Litchi* can grow in a variety of soil types particularly in fairly deep, well drained loam rich in organic matter. A sandy loam or clayey loam with pH ranging from 5.5-7, with sufficient depth is an ideal soil for *Litchi* cultivation. The flowering season starts in the spring and fruiting season starts at the beginning of summer. Leaves are alternate, estipulate, and pinnate, leaflets 2-9 sub opposite, entire (rarely serrate). Flowers numerous, regular small polygamo-dioecious, in terminal axillary recemes or panicles, slightly imbricate in bud. Petals 4-6 or 0, yellow rarely with 2 scales, disc annular or swollen, glabrous or pubescent. Stamens 6-8 inserted within the discs long exserted, filaments filiform, usually pubescent, ovary pubescent often varicose 2-3 lobed; lobes one-celled, one ovulate style 2-3 fid or partite. Fruit globose or oblong to obviate 2-5 cm or more in diameter with a dark or light red or yellow rind faintly or sharply tubercled aril which can be readily separated from the seed is fleshy, soft juicy white translucent with a delicious flavor covering fully a large dark brown elliptic seed.

RAPD methodology has found extensive use in plants as it offers the promise of virtually unlimited markers (Welsh and McClelland, 1990; Williams *et al.*, 1990). The advantages of RAPD markers include their technical simplicity, independence of any prior knowledge of DNA sequence information, no environmental or developmental influences, a random scattering throughout the genome, and it gives variation at suitable taxonomic ranks. Despite a number of drawbacks like dominant nature, reproducibility, allelic variation, product competition, RAPD markers have been widely used for assessment of genetic diversity, identification of cultivars,

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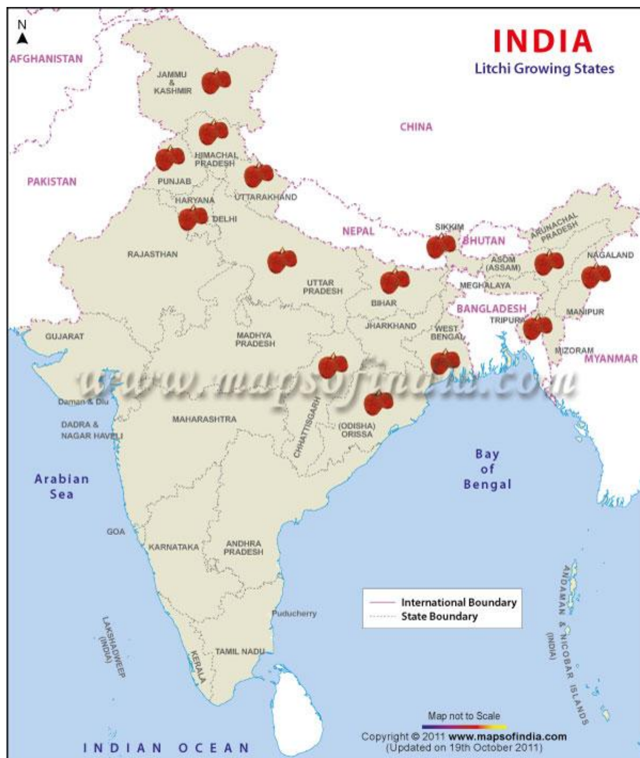
molecular characterization and population genetic structure in numbers of plant species like *Punica*(Narzary*et al*., 2009), *Chenopodium*(Rana*et al*., 2010), (*Pongamia* (Kesari and Rangan 2011).



2. Material Method

About sixty accessions of *Litchi* cultivars were collected for Uttarakhand Uttar Pradesh, Bihar and Punjab collected the plant sample These sixty accessions representing *Litchi* were collected from five different geographical selected for the molecular studies on the basis of their DNA quality and reproducibility in PCR reactions.Table3.1: Description of *Litchi* accessions collected from different geographical regions.

1) DNA EXTRACTRACTION 500 mg of dried leaf tissue was weighed and ground to make a fine powder adding liquid Nitrogen in a pre-chilled mortar and pestle. 100 mg of PVP was also added during grinding. Immediately the fine powder of tissue was transferred to a pre-warmed tube containing 10 ml of CTAB Buffer and 1% of β-mercaptoethanol (*i.e.*, 10 µl in 10 ml Buffer) at 65°C. It was mixed properly in buffer by vortexing the tube.The tube was incubated at 65°C for 1 hour and mixed 4 or 5 times during incubation by inverting the tube for homogenous digesti. Then, cooled to room temperature and 10 ml Chloroform/Isoamyl alcohol (CIA, 24:1, v/v) solution was added. Tube was gently inverted 20-30 times. After cooling at room temperature tubes were centrifuged at 8000 rpm for 15 min at room temperature. Upper aqueous phase was transferred to a fresh centrifuge tube and equal amount of CIA was added, and then mixed by gently inverting the tube. The pellet was washed by adding 1-2 ml of 70% ethanol and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was decanted off, retaining the pellet. This step was repeated for once agaThe pellet was air dried till the smell of ethanol goes of The pellet was then re-suspended in 500 µl of 1x TE Buffer (10 mMTris-HCl, pH 8.0, 1 mM EDTA), and then transferred to a fresh eppendorf tube.

2) POLYMARASE CHAIN RECTION Minor variations in PCR amplification reaction parameters and or cycling conditions can result in large differences in the overall product pattern and yield. These variations may include undetectable PCR product, low yield of the desired product, presence of nonspecific background bands due to mispriming and misextension of the primers, formation of primer- dimers artifacts etc. It is therefore essential to optimize the PCR reaction conditions to obtain reproducible and interpretable results. There are number of reports available on RAPD optimization (Munthaly et al., 1992; Benter et al., 1995). In the present investigation, the reaction conditions as described by William et al., (1990) were followed which includes 25ng of genomic DNA, 2mM $MgCl_2$, 0.2µM Primer, 100µM each of dATP, dCTP, dGTP and dTTP and 0.5 unit of *Taq* DNA polymerase, containing suitable buffer (10mM Tris- HCl pH 8.3, 50mM KCl, 2mM $MgCl_2$, 0.001% gelatin), in a volume of 25µl. These reaction conditions were optimized for template DNA of *Litchi* cultivar. Optimization were carried out for each PCR component by varying one component from low to high and keeping other components constant. PCR cycling parameters included 45 cycles of denaturation at 94ºC for 1 min, annealing at 36ºC for 1 min and extension at 72^0C for 2 min. using optimum transition paired between each temperature.

3. Data Analysis

Amplified fragments of RAPD were scored for each individual. Data analysis was carried out only for those genotypes that resulted consistent and reproducible profiles. For each primer, the molecular sizes of each fragment were estimated on the basis of the corresponding marker lane. Distinct and well separated bands were coded in a binary form by denoting '0' for absence and '1' for presence of the bands in each genotype and binary data generated was used as input for further calculations. In order to estimate genetic relationships in *Litchi* cultivars RAPD were analyzed separately and then in combination for *Litchi* using the following statistical methods.

The pairwise distances were computed by using Jaccard's coefficient for neighbour joining (NJ) method in the FreeTree program (version 0.9.1.5; Pavlicek et al., 1999). Jaccard's coefficient of similarity (J_{sim}) and dissimilarity (J_{dist}) between two individuals i_1 and i_2, are computed using the expressions

4. Result

The Collected leaf samples of *Litchi* were subjected to DNA isolation and Analysis of genetic diversity had been done with the use of RAPD and ISSR Markers. The analysis of die pre-screening data using 27 accessions of Indian *Litchi* collected front different locations and 14 primers out of 22 RAPD operon primers (OPA-1, OPA-2, OPA-3, OPA-4, OPA-5,

OPA-6, OPA-7, OPA-8 OPA-9, OPA-10, OPB-4, OPB-7, OPG-I and OPG-3) detected the diversity among the cultivars used. Out of 14, 7 primers (OPA-1, OPA-4, OPA-6, OPA-7, OPI3-4, OPB-7 and OPG-1) were chosen that had more than 5 scorable bands and 7 primer (OPA-2, OPA-3, OPA-5, OPA-8, OPA-9, OPA-10 and OPG-3) generated less than five bands (Table 4.1) Using die DNA purification strategies, good and clear amplification pattern could be obtained for the various accessions of *Litchi* cultivars (Fig 4.1 a, b and 2).

From the integration of data obtained from the RAPD technique using 14 selected primers for all the 27 accessions, 12-77 amplicons per selected RAPD operon primer were scored, originating a total of 538 fragments. Of these, an average of only 15.8% were polymorphic and a least percentage (0.10%) of monomorphic bands (data was not shown) were found (Table4.2). The lower level (15.8%) of polymorphic bands generated by the various primers enabled narrow genetic discrimination of all cultivars except LI and L8. RAPD analysis also revealed putative accession-specific amplified products. A DNA banding profile using primers OPA-1, OPA-4 and OPA-7 as shown in Fig.4.1 a,b and 2 indicated the specific markers of 200, 1031, 1200, 400, 2000 and 1200 Up at 40 ng DNA templates of accessions L15 and L21, respectively.

Table 4.1: RAPD markers produced by 14 selected primers (out of 22 tested primers) In 27 types of *Litchi* cultivars studied.

	Sequences (5'-3)	**Total No. of fragments**	**Poly-morphic bands**	**Poly-morphism (%)**	**Fragment size (base pair)**
OPA 01	CAG GCCCTT C	38	6	10.5	>800, 800,>700, 600, 500,400
OPA 02	TGCCGAGCT G	35	5	14.3	>1200,1200,1031,<900, 900
OPA 03	AGT CAG CCA C	25	2	8.0	2500,2000
OPA 04	AATCGCGCT G	68	7	10.2	2500,<2500,2000,1500,1200,800,600
OPA 05	AGGGGTCTT G	29	3	10.4	800,450,600
OPA 06	GGT CCC TGA C	35	7	20.0	2000,2000,1500,1200,1031,900,<900
OPA 07	GAAACGGGT G	77	7	9.1	1200,1031,>900,900,800,700, 600
OPA 08	GTGACG TAG G	42	3	7.1	>700, 600,500
OPA 09	GGGTAACGC C	35	4	14.2	2500,2250,1500,1200
OPA 10	GTGATCGCA G	26	3	11.5	600,500,200
OPB 01	GTTTCG CTC C	00	0	0.0	-
OPB 02	TGATCCCTG G	00	0	0.0	-
OPB 04	GGACTG GAG T	12	6	50.0	>500,<500,450,400,200,150
OPB 07	GGTGACGCA G	51	7	7.4	1200,1031,900,700,650,400
OPB 08	GTCCACACG G	00	0	0.0	-
OPB 09	TGGGGG ACT C	00	0	0.0	-
OPB 16	TTTGCCCGG A	00	0	0.0	-
OPB 17	AGGGAACGA G	00	0	0.0	-
OPG 01	CTA CGGAGG A	32	7	40.5	1350,1200,1031,800,600,500
OPG 02	GGC ACT GAG G	00	0	0.0	-
OPG 03	GAG CCC TCC A	33	4	7.7	900,700,500,400
OPG 04	AGCGTGTCT G	00	0	0.0	-

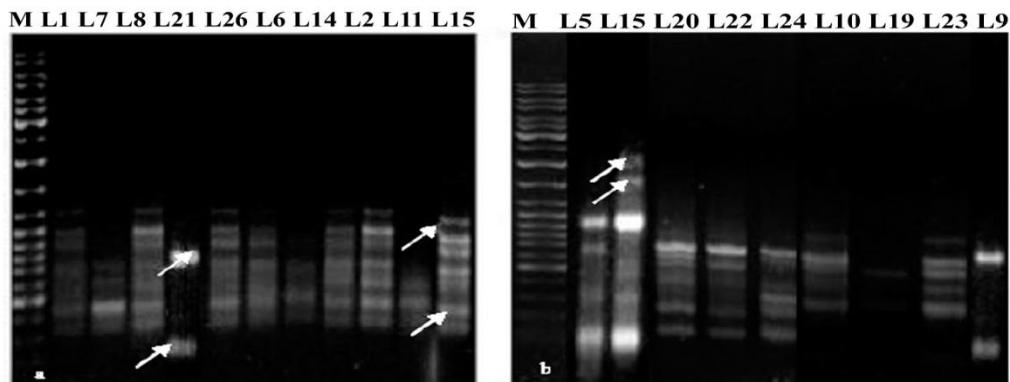


Fig.4.1: RAPDagarosc gel elcctropherosis profiles of the litlu accessions using primers OPA-1 (a) OPA-4 (b) Lanes indicated by M represents molecular mass marker (gene ruler 1K6 ladder, MBI, Fermentas). The accessions numbers (L1-L15 and 5-L9) indicated the template DNA from appropriate cultivars as shoen in Table 3. Arrows indicate putative accession specific marker

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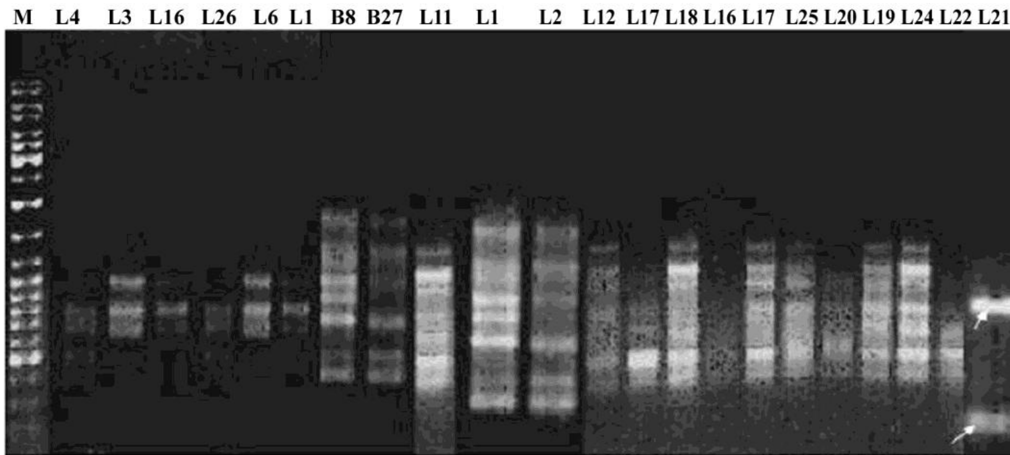


Fig.4.2: RAPD agarose gel electrophoresis profiles of the *Litchi* accessions using primers OPA-7 Lane indicated by M contains molecular mass marker (Gene ruler 1K6 ladder, MBI, Fermentas). The accessions numbers (L1-L15 and 5-L9) indicated the template DNA from appropriate cultivars as shown in Table 3. Arrows indicate putative accession- specific marke

	L 10	L 11	L 12	L 13	L 14	L 15	L 16	L 17	L 18
L 1									
L 2									
L 3									
L 4									
L 5									
L 6									
L 7									
L 8									
L 9									
L 10	1.000								
L 11	0.027	1.000							
L 12	0.000	0.149	1.000						
L 13	0.174	0.104	0.132	1.000					
L 14	0.000	0.136	0.326	0.077	1.000				
L 15	0.000	0.121	0.000	0.000	0.026	1.000			
L 16	0.000	0.125	0.061	0.097	0.143	0.000	1.000		
L 17	0.000	0.025	0.033	0.000	0.048	0.077	0.000	1.000	
L 18	0.000	0.081	0.200	0.074	0.103	0.000	0.000	0.133	1.000
L 19	0.080	0.152	0.229	0.000	0.302	0.000	0.030	0.071	0.074
L 20	0.167	0.111	0.071	0.000	0.049	0.083	0.000	0.214	0.143
L 21	0.000	0.063	0.000	0.000	0.000	0.000	0.000	0.000	0.000
L 22	0.222	0.057	0.080	0.083	0.053	0.000	0.059	0.077	0.083
L 23	0.154	0.051	0.033	0.000	0.073	0.000	0.000	0.125	0.133
L 24	0.000	0.093	0.156	0.000	0.190	0.000	0.077	0.091	0.045
L 25	0.000	0.195	0.219	0.027	0.182	0.000	0.154	0.238	0.042
L 26	0.057	0.286	0.262	0.106	0.245	0.000	0.048	0.000	0.054
L 27	0.133	0.132	0.114	0.114	0.189	0.065	0.051	0.121	0.200

The similarity matrix obtained using Jaccard's coefficient is shown in Table 4.3 Similarity coefficients ranged from 0.11 to 0.47 in 27 accessions of *Litchi* tested in the present investigation. These similarity coefficients were used to generate a tree for cluster analysis using UPGMA and SAHNmethod (Fig.4.3). Two types of comparisons were carried out to evaluate the degree of relationship in ale *Litchi* cultivars 1) among (different accessions collected from the same locations and 2) among common accessions collected from different locations. The cluster analysis indicates that 27 accession.

Table 4.3: Distance matrix values based on RAPD data among 27 accessions of *Litchi*.

	L 1	L 2	L 3	L 4	L 5	L 6	L 7	L 8	L 9
L 1	1.000								
L 2	0.276	1.000							
L 3	0.346	0.263	1.000						
L 4	0.119	0.140	0.125	1.000					
L 5	0.000	0.000	0.000	0.000	1.000				
L 6	0.224	0.192	0.261	0.167	0.077	1.000			
L 7	0.308	0.186	0.200	0.103	0.000	0.191	1.000		

L 8	0.477	0.196	0.260	0.143	0.000	0.293	0.452	1.000	
L 9	0.000	0.025	0.000	0.000	0.000	0.000	0.061	0.000	1.000
L 10	0.077	0.128	0.081	0.000	0.000	0.000	0.027	0.029	0.125
L 11	0.172	0.148	0.179	0.103	0.000	0.244	0.255	0.271	0.000
L 12	0.160	0.053	0.244	0.000	0.000	0.122	0.149	0.133	0.000
L 13	0.295	0.157	0.222	0.000	0.000	0.000	0.128	0.163	0.000
L 14	0.246	0.217	0.278	0.122	0.114	0.341	0.196	0.208	0.000
L 15	0.025	0.024	0.054	0.067	0.000	0.115	0.057	0.000	0.000
L 16	0.140	0.063	0.068	0.143	0.000	0.156	0.125	0.135	0.000
L 17	0.125	0.022	0.049	0.053	0.000	0.065	0.171	0.056	0.200
L 18	0.048	0.054	0.050	0.000	0.000	0.067	0.053	0.057	0.000
L 19	0.075	0.093	0.122	0.032	0.190	0.216	0.128	0.111	0.091
L 20	0.048	0.070	0.050	0.000	0.000	0.185	0.081	0.057	0.222
L 21	0.000	0.000	0.000	0.000	0.000	0.000	0.063	0.069	0.000
L 22	0.051	0.049	0.054	0.000	0.000	0.074	0.088	0.063	0.000
L 23	0.098	0.146	0.049	0.111	0.000	0.100	0.079	0.086	0.200
L 24	0.063	0.104	0.043	0.040	0.188	0.147	0.093	0.073	0.059
L 25	0.152	0.058	0.109	0.077	0.000	0.139	0.195	0.150	0.111
L 26	0.264	0.255	0.327	0.105	0.000	0.222	0.260	0.666	0.000
L 27	0.231	0.179	0.148	0.147	0.032	0.268	0.091	0.188	0.033

Table 4.4: Contined

	L 19	L 20	L 21	L 22	L 23	L 24	L 25	L 26	L 27
L 1									
L 2									
L 3									
L 4									
L 5									
L 6									
L 7									
L 8									
L 9									
L 10									
L 11									
L 12									
L 13									
L 14									
L 15									
L 16									
L 17									
L 18									
L 19	1.000								
L 20	0.208	1.000							
L 21	0.000	0.000	1.000						
L 22	0.300	0.000	0.000	1.000					
L 23	0.071	0.214	0.000	0.077	1.000				
L 24	0.286	0.150	0.000	0.053	0.043	1.000			
L 25	0.188	0.087	0.000	0.048	0.083	0.143	1.000		
L 26	0.182	0.000	0.065	0.000	0.111	0.045	0.231	1.000	
L 27	0.167	0.125	0.000	0.065	0.156	0.075	0.098	0.157	1.000

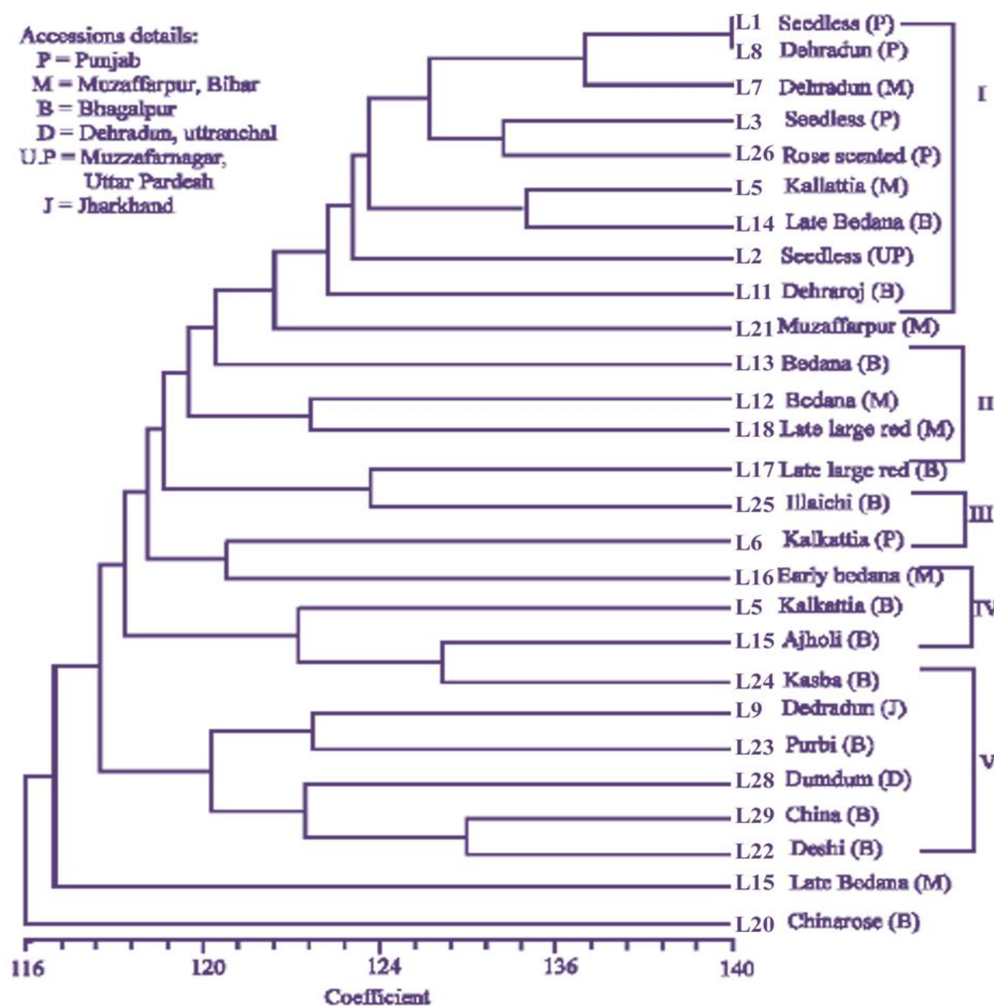
c (B), L26 Rose scented (M),L27Muzaffarpur (M), B=Bhagalpur, Bihar, M = Muzaffarpur, Bihar, P = Punjab, UP Uttar Pradesh, D= Dehradun, Uttaranchal, J=Jharkhand of*Litchi* formed five groups based on similarity coefficients (Table 3) except the accessions LI 5 [Late Bedana (M)J and L21 [Chinarose (B)] which exhibited only 0 and 3% genetic similarity to others. Therefore both of them are kept independent.

Group I

Large clusters were formed which contained a mixture of 27 *Litchi* accessions obtained from different localities. Cluster analysis shows a close relation within them. Accession LI (Seedless) and L8 (Dehradun) obtained from Punjab were found to have the highest similarity (SI = 0.47, Table 3) while the other accessions belonging to this group also exhibited a co- similarity based on similarity coefficient (range of SI = Q.1 1 to 0.45, Table 4.3). As per the cluster analysis, Dehradun (M) was closely related (about 75%) with Seedless (P) and Dehradun (P). Similarly, accessions Seedless (J), Rose scented (M), Kalkattia (M), Late Bedana (B), Seedless (P), Dehra Rose (B), Mtizaffarpur (M) and Bedana (B) were found to be very close (27 to 75%) with Seedless (P) and Dehradun (P) respectively. The genetic similarity was high as per the coefficient similarity and it was possible to correlate them with each other despite their different geographical location. This is a good indication of the fitness of the result that was obtained, which is particularly important for analysis of Indian *Litchi* cultivars, since their genetic diversity and parental relations are unknown.

Group II

In this group, all four accessions were found partially close to each other. Between Late Large Red (B) and Illaichi (B) the similarity was high (SI = D.15, Table4.3) as compared to the



Late Fig.4.3: Dendrogram derived from UPGMA cluster analysis usoingJaccard's coefficient of RAPID markers.

Similarity (SI = 0.04, Table4.3) between the accessions Bedana (M) and Late Large Red (M). Late Large Red (B) and Illaichi (B) exhibited about 48% similarity with Badana (M) and LatBedna.

Group III and IV

Group III contains only two accessions Kalkalttia (P) and Early Badana (M). Dendrogram reveals the similarity (SI=0.14, Table4.3) between two accessions which reflects a lower level of genetic diversity despite their different geographical locations. Cluster analysis indicated that these two accessions were close (27%) with Late Large Red (B) and Illaichi (B). While group IV contains three accessions Kalkattia (B), Ajholi (B) and Kasba (B) which belong to the same place, these three accessions were found to be associated (35 to 60%) with each other according to their origin and habitat relatedness.

5. Discussion

Genetic diversity among 27 selected accessions of Indian *Litchi* was assessed with 77 RAPD polymorphic bands generated by 14 selected operon primers. In the recent past with die same objective, RAPD analyses were carried out by Paran et al. (1998) in Capsicum and by Tongpamnak el al., 2002 in *Litchichinensis* (Thai *Litchi* accession). The percentage of polymorphic hands (PPB) in each accession ranged from 7.1 to 50.0 % 'these results more or less agreed with RAPD and AFLP analysis in exotic *Litchi* accessions (Tongpamnak et al., 2002) and other species e.g., Japanese plum cultivars (Bellini et al., 1998), chilli (Paran et a1.,1998) mid soybean (Choudhary et al., 2001).

There was no higher level of similarity observed among the cultivars originality from the same or nearby geographical locations supporting both the hypothesis of authoctonal origin (Tongpamnak et al. 2002) as well as the limited diffusion of *Litchi* cultivars from their zones of cultivation (Barile1 al., 1997).

Estimation of genetic diversity is highly influenced by the genome selected for evaluation and by the number of markers assayed. Since fruit tree cultivars arc maintained by vegetative propagation, accurate identification of vegetative materials is crucial for growers arid is required accessions in germplasm holdings. for plant breeder's rights. This DNA marker technique can be used to identify genetic variation and detect the relationship between DNA markers and horticultural traits of interest. For this reason, RAPD technique has been employed to screen die germplasm in case of several higher plants. Most

of these studies have been carried out in case of cross-pollinated plants and consequently, relatively higher estimates of genetic variability were obtained. In die case of tissue culture, RAPD technique has enabled the testing of fidelity of micropropagated plants (Rani et al., 1995). The RAPD profiles, however, could reveal relative variability as well as similarity within the 27 accessions of 19 *Litchi* cultivars. Clearly there is scope for large-scale application of RAPD for analysis of such cross-pollinated/heterozygous plants. The present study reveals that PCR based fingerprinting technique-,RAPD is informative for estimating the extent of genetic diversity as well as to determine the pattern of genetic relationships among different accessions of Indian *Litchi* cultivars. The information obtained from die present study could be of practical use in mapping die *Litchi* genome as well as for classical breeding. The markers identified in our studies will be useful in genetic analysis of *Litchi.*

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