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Narrow genetic base of Indian litchi (*Litchi chinensis*) cultivars based on molecular markers

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ABSTRACT

Litchi (*Litchi chinensis* Sonn.) is an introduced crop in India and has limited genetic variability characterized by differences in flushing pattern, leaf, panicle and fruit traits. Molecular markers were employed to expose the genetic diversity of 20 litchi cultivars from the Indian peninsula and facilitate documentation of the native germplasm diversity. Efficiency of individual primers was evaluated on the basis of average band informativeness and resolving power, where random oligonucleotide markers OPA-5 and OPA-3 scored best. Among tested microsatellite markers, ISSR 01 and 13 had high values for primer efficiency and these were found to supplement simple sequence repeats for generation of cultivar barcode and clustering analyses. Efficiency of microsatellites (Simple Sequence Repeats and Inter Simple Sequence Repeats) was established by high values for polymorphism (0.691), diversity index (0.264), effective multiplex ratio (48.8470) and marker index (12.896), thus reiterating its potential as for developing barcodes for cultivar identification and conservation. Phylogenetic analysis based on RAPD and microsatellites revealed clustering of the cultivars into four major groups, although within a very narrow range (0.63 - 0.90) of similarity, viz. Seedless (i.e. Bedana), Mandarji, Shahi and China groups. The clustering followed grouping based on fruit morphology, leaf and panicle attributes disagreeing with earlier views regarding incongruity of clustering pattern with morphological, ecological and climatic adaptations. Discrimination of cultivars like Dehrarose and Dehradun, being often labeled as synonyms, was also done. Interestingly high polymorphism and low gene diversity have been exposed by molecular markers, commenting on narrow genetic background of litchi cultivars from India.

Key words: Genetic diversity, ISSR, Phylogenetic analysis, RAPD, SSR barcode

In litchi (*Litchi chinensis* Sonn.) there exist variations for vegetative flushing pattern, flush colour and flowering ability. The shape of skin segments and protuberances are the reliable and stable genetic characteristics used for discriminating the varieties (Singh and Babita 2002). Earlier the Chinese litchi cultivars were separated into 3 types: smooth pericarp, somewhat warty pericarp, and pericarp with sharp protuberances (Wu *et al.* 2007). In India and abroad, litchi cultivar nomenclature is confusing with presence of homonyms and synonyms. The confusion emanates due to derivation by translation from Cantonese and Mandarin to English. This has been identified as one of the main bottlenecks for germplasm exchange among different producing countries and germplasm collections made worldwide and its management in the species (Viruel and Hormaza 2004, Madhou *et al*. 2013).

Characterization of Indian genetic resources using suitable molecular markers are worth exploring, to unravel the problems in its nomenclature, evaluating genetic relationships, and establishing the need for broadening of genetic base for increasing its cross breeding efficiency by aiding international germplasm exchange, which was used in other fruit crops besides litchi and longan (Viruel and Hormaza 2004, Fenggang *et al*. 2009, Sun *et al*. 2011, Madhou *et al*. 2013). The native genetic diversity of litchi in India was explored in a selected group of 20 litchi cultivars sourced from the country and growing in North west Alluvial plains of the Bihar state in India utilizing available polymorphic marker resources.

MATERIALS AND METHODS

Twenty economically important litchi cultivars, collected from germplasm repository of National Research Centre for Litchi located at Mushahari, Bihar state in eastern part of the country (26.25^0 N 85.16^0 E), formed the material in the study. The fresh young leaves were collected from plants of varieties Dehradun, Shahi, Seedless No.2, Lal Bombay, Dehrarose, Purbi, China, Yogda Selection, Trikolia, Bedana Selection Plant, Calcuttia, Calcuttia Late, Mandarji, Swarnaroopa, Seedless late, Bedana, Late Bedana, Bedana Sabour, Late Large Red, Kasba, and used for DNA extraction

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by the method standardized for litchi nucleic acid isolation (modified CTAB method with double extraction and spin column purification, Kumar *et al*. (2012).

RAPD-PCR was performed using RAPD primers of OPA, OPB, OPD and OPX series, ISSR-PCR was performed with 20 ng DNA template as per protocol standardized for mango (Bajpai *et al*. 2008) and SSR was performed using primers described and analyzed by Li *et al*. (2006). Allele size determination was performed in ABI systems fragment analyzer post amplification using FAM and HEX labeled forward primers.

The clustering analysis was carried out based on genetic distances using UPGMA/NJ method using NTSYS software version 1.1. The efficiency of the primers used in the three marker systems were evaluated using the band informativeness and polymorphism information content generated by the primers. Primer banding characteristics such as number of scorable bands (NSB), number of monomorphic bands (NMB), number of polymorphic band (NPB), and percentage of polymorphic bands (PPB) was calculated using the formula: PPB = (NPBx100/NSB). Band informativeness (I_b) and resolving power (Rp) were calculated as given by Prevost and Wilkinson (1999). Band informativeness of a given band is calculated using the formula; $I_b = 1 – (2 X |0.5 – p|)$, where *p* is the proportion of the total genotypes containing the band. Average band informativeness (Av. I_b) is a measure of closeness of a band to be present in 50% of the cultivars under study. Resolving power (Rp) is the sum of Ib values of all the bands amplified by a primer using the formula; $Rp = \sum I_b$. To evaluate the discriminatory power of molecular markers, polymorphism information content (PIC), marker index (MI) and effective multiple ratio (EMR) were calculated. The PIC value was determined by applying the formula $PIC_i = 2f_i (1- f_i)$, where f_i is the percentage of the amplified alleles (bands present) and $(1- f_i)$ is the frequency of the null allele (band absent) for i^{th} allele. The MI was calculated as the product of DI and EMR as described by Prevost and Wilkinson (1999). The DI of the primer is given by $1- \sum(pi)^2$ where pi is the frequency of i_{th} allele, while EMR of a primer represents product of β and the number of polymorphic bands for an individual assay, where β is the fraction of the polymorphic bands (Milbourne *et al*. 1997). Microsatellite based barcode was constructed using Microsoft Excel (2003) by transformation of allele molecular sizes into binary data and converting it into bars.

RESULTS AND DISCUSSION

The litchi, *Litchi chinensis*, is the sole member of the genus in the soapberry family Sapindaceae, native to southern China, It is a diploid with *x* = 15 and 2 *n* = 30, with flow cytometric estimations of genome size being 554 Mbp (VanBuren *et al*. 2011). Comparative evaluation of genetic diversity among 20 Indian litchi cultivars, conserved and sourced from NRC (Litchi), Mushahari, India, using dominant and multiallelic codominant systems, was aimed to reflect overall effectiveness in detecting polymorphism in species with limited germplasm. The study was also designed to testify the most effective marker system and whether the marker systems are compatible for cumulative analysis.

Marker efficiency and diversity analysis

Twenty RAPD primers that amplified reproducible bands were used and out of them 10 were short listed based on polymorphism. These yielded a total of 194 fragments, of which 143 fragments were polymorphic. The number of polymorphic bands per primer ranged from 1 (OPD-18) to 16 (OPA-05), OPB-13 and OPA-03 being 100% polymorphic. Primer characteristics defined by average band informativeness, resolving power and polymorphism information content are shown in Table 1 and 2, wherein OPA-5 and OPA-3 scored best amongst RAPD markers. Amongst ISSR and SSR markers tested, ISSR 01 and 13 had high values for these parameters, while both resolving power and band informativeness of *Lit* 30 was low.

Total number of polymorphic loci (np) for the three classes of primers was 70, 35, and 3, respectively, RAPD being most polymorphic and abundantly available among all classes. Comparison of the three marker classes revealed that average percentage polymorphism of 69.6, 64.07 and 16.67 and average PIC of 0.297, 0.314 and 0.099 was recorded for RAPD, ISSR and SSR marker systems, respectively. For RAPD analysis, effective multiplex ratios (EMR) were estimated at 50.54, while marker index (MI) was 18.649 and cumulatively ISSR and SSR markers estimated 48.847 EMR and 12.896 MI (Table 2). Among SSR markers *Lit* 30 locus containing tri- and di-nucleotide repeats $(AAC)_4…(AG)_{12}$, high heterozygosity estimates of 0.667 (expected 0.59) and PIC value 0.593 were recorded, thus making the locus suitable for genotyping and barcoding. Overall high potential for cumulative microsatellites (SSR and ISSR) was evinced based on β (0.691), DI (0.264), EMR (48.8470) and MI (12.896), which was used for microsatellite barcodes and relationship inferences. Based on the discrimination and resolving power, it could be proved conclusively that besides RAPD, ISSR markers could be deployed with SSR for characterization and clustering analyses.

Initial studies on litchi germplasm characterization have attended to the use of isozyme and dominant PCR-based markers for germplasm diversity analysis and clonal fingerprinting. RAPD and AFLP markers have generally, been the chosen markers for estimating genetic diversity (Tongpamanak *et al*. 2002 working with Thai cultivars; Jones *et al*. 2006, USDA-ARS germplasm collection at Hawaii) in litchi as in number of other subtropical fruit species, such as mango (Bajpai *et al*. 2008), guava (Bajpai *et al*. 2008a), longan (Yonemoto *et al*. 2006) etc., and the present study further strengthens the capacity of the markers to this effect. Microsatellite or simple sequence repeat (SSR) markers having wide applicability for genetic analysis in crop plant improvement strategies, was used with limited success in litchi due to poor amplification and low

Table 1 Polymorphism, band informativeness and resolving power of primers in Indian litchi cultivars

Marker system	Loci	Sequence (5'-3')	Pattern of scorable bands			PP	Av. Ib	Rp	H	PIC
			NSB	NMB	NPB					
RAPD	OPA-01	CAGGCCCTTC	12	6	6	50	1.561	18.727	0.288	0.246
	OPA-02	TGCCGAGCTG	12	3	9	75	1.682	20.181	0.334	0.278
	OPA-03	AGTCAGCCAC	11	0	11	100	1.421	15.636	0.411	0.327
	OPA-04	AATCGGGCTG	8	3	5	62.5	1.295	10.364	0.442	0.344
	OPA-05	AGGGGTCTTG	17	1	16	94.12	1.433	24.363	0.406	0.324
	OPB-11	GTAGACCCGT	11	3	8	72.73	0.182	19.636	0.191	0.173
	OPB-13	TTCCCCCGCT	5	0	5	100	1.527	7.636	0.361	0.296
	OPB-16	TTTGCCCGGA	3	1	2	66.67	1.394	4.181	0.422	0.333
	OPD-18	GAGAGCCAAC	6	5	1	16.67	1.545	9.273	0.351	0.289
	OPD-19	CTGGGGACTT	12	5	7	58.33	1.212	14.545	0.478	0.363
ISSR	ISSR-08	$G(GATG)_3GAT$	10	1	9	90.0	0.58	5.8	0.412	0.327
	ISSR-13	$(GACA)_4$	8	3	5	62.5	1.513	12.1	0.369	0.301
	ISSR-01	$(AG)_8YC$	11	5	6	54.54	1.127	12.4	0.491	0.370
	ISSR-06	$(GACA)_4$	15	1	14	93.33	0.687	10.3	0.451	0.349
	ISSR-02	$(GA)_8YC$	5	4	1	20.00	1.7	8.5	0.255	0.222
SSR	*Lit*23	$(TC)_6$	1	1	0	0	0.000	0.0	0.0	0.0
	*Lit*24	$(GA)_9 \ldots (AG)_6$	2	2	0	0	0.000	0.0	0.0	0.0
	*Lit*25	$(CT)_{18}$	1	1	0	0	0.000	0.0	0.0	0.0
	*Lit*26/27	$(TC)_6$	1	1	0	0	0.000	0.0	0.0	0.0
	*Lit*30	$(AAC)_4 \ldots (AG)_{12}$	3	0	3	100	0.833	2.5	0.667	0.593
	*Lit*38	$(GT)_4GC(TG)4$	1	1	0	0	0.000	0.0	0.0	0.0

NSB= Total number of scorable bands, NMB= number of monomorphic bands, NPB= number of polymorphic bands, PP= percentage polymorphism, Av. Ib= Average band informativeness, Rp= Resolving power, H = Heterozygousity value, PIC= Polymorphism information content.

heterozygosity (Viruel and Hormaza 2004, Li *et al*. 2006, Madhou *et al*. 2010). Although the molecular diversity of litchi cultivars from Mauritius, Hawaii and China was investigated using other dominant marker systems, SRAP and AFLP detected high polymorphism, they could not corroborate morphological or phenotypic classification (Tongpamnak *et al.* 2002; Ganjun *et al.* 2003). An interesting situation of high polymorphism but low gene diversity being evinced by RAPD (DI=0.369) and ISSR (DI=0.395) markers was demonstrated in this study (Table 2). Similarly recent studies by Pathak *et al.* (2014), on Indian litchi cultivars estimated gene diversity for AFLP markers at 0.083 to 0.499 with a mean of 0.25 ± 0.13 per assay units, highlighting low diversity in Indian litchi germplasm, echoing findings of the Chinese group using molecular markers (Chen *et al.* 2004). Though the climatic and geological conditions influence the morphological traits, clear cut discrimination could be established through molecular markers, even for cultivars like Dehrarose and Dehradun from Northern Indian plains, often mistaken as synonyms.

Microsatellite based barcodes

In this study, microsatellite allele sizes, determined at six loci in 20 litchi genotypes were used to construct a DNA fingerprint or barcode system, thereby facilitating documentation by presenting banding pattern at multiple loci in one diagram. Pairwise comparison of the microsatellite data led to the identification of nonredundancy in nomenclature of the cultivars under study (sample size being 20), and established differences between two litchi cultivars, Dehradun and Dehrarose, previously described as identical. The results also pointed to several unique alleles in cultivar Kasba, Madarji, Late Large Red, Yogda Selection and Seedless late. Here, alleles at *Lit* 30 and five polymorphic ISSR loci arranged as per their molecular weight (allele size) clearly establish the uniqueness of the twenty litchi cultivars (Fig 1, discriminating alleles are marked with *). Similar work has previously been described for grapes (Galbacs *et al*. 2009) and mango (unpublished data). Besides SSR based barcodes could also arrive at cultivar specific fingerprinting pattern as described for AFLP fingerprints (Pathak *et al*. 2014).

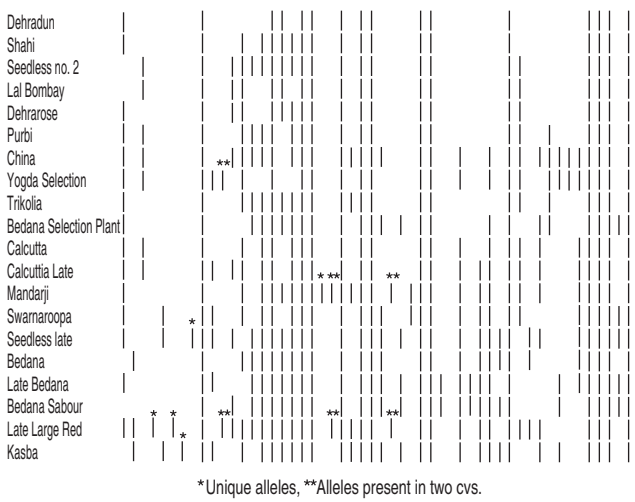
Phylogenetic analysis

Genetic diversity was assessed using RAPD ISSR and new SSR markers reported for assessment of Chinese litchi cultivars (Li *et al.* 2006). Heterozygosity estimates were very low for individual SSR markers, being moderately higher for RAPD markers(0.191-0.478) (Table 1), similar to Chinese cultivars studied by Li *et al*. (2006), wherein 2-3 alleles per locus were reported with heterozygosity being 0.021-0.9. Comparative analysis of three marker systems

Table 2 Comparative analysis of three marker systems (RAPD, ISSR and SSR)

Parameter	RAPD	ISSR	SSR	Combined micro-satellite markers* (ISSR & SSR)
Number of primers used	40	13	8	21
Number of primers which produced reproducible amplicons	20	6	6	12
Number of polymorphic primers	10	5	1	6
Scorable band classes per primer	2-8	1-5	1-3	1-5
Average no. of bands per primer	9.7	4.9	1.5	5.8
Scorable band size range (bp)	300-3000	500-3500	150-280	150-3000
Total number of loci scored (n)	97	49	9	58
Total number of polymorphic loci (np)	70	35	3	38
Beta (Fraction of polymorphism)	0.722	0.714	0.333	0.691
Average percentage polymorphism	69.6	64.07	16.67	70.061
Average PIC	0.297	0.314	0.099	0.213
Similarity coefficients	0.63-0.90	0.58-0.95	0.70-0.90	0.63-0.90
Diversity index or Average Heterozygosity (DI)	0.369	0.395	0.111	0.264
Effective multiplex ratio (EMR)	50.54	24.99	0.999	48.847
Marker Index	18.649	9.871	0.112	12.896

* Includes only *Lit* 30 SSR locus + polymorphic ISSR loci.



*Unique alleles, **Alleles present in two cvs.

Fig 1 Microsatellites based barcodes for 20 Indian litchi cultivars {Each row represents barcode for specific cultivar based on 6 polymorphic microsatellite markers (SSR and ISSR) and each bar indicates presence of allele at that locus}.

revealed low genetic distances (0.63 - 0.90 RAPD,0.58 - 0.95 ISSR,0.70 - 0.90 SSR) and diversity indices (0.369 0.395, 0.111, respectively). Other SSR markers developed for litchi (Viruel and Hormaza 2004) could not be used for the study due to poor amplification products and difficulty in allele sizing. Based on the presence or absence of the amplification of alleles, the pair-wise genetic similarity according to Jaccard's coefficient was analyzed which ranged from 0.63-0.90 for RAPD and cumulative polymorphic microsatellites. The similarity matrix was used to construct a dendrogram with UPGMA method displaying clustering pattern which could be arranged into four clusters (Fig.2) within low rage of dissimilarity (0.28 to 0.04). The first one comprised of cultivars like Shahi, Dehrarose and Dehradun etc. in which Purbi and Tirkoila were also placed. China, a commercial variety with very high yield potential, tolerant to extreme weathers; similar to Calcuttia, Bombaiya etc. for few external characters grouped along with Mandarji and Yogda Selection, sharing more than 86% similarity with the earlier group. Interestingly Late Large Red and Mandarji grouped together forming a distinct cluster corresponding to their morphological distinctions and favorable response in Bihar and eastern parts of the country. While Bedana selections and Swarnaroopa grouped into another cluster, Kasba was isolated and formed an outgroup, even though sharing 87% genomic information with other accessions in the cluster (IV). Clearly the grouping and similarity values indicated low diversity as differentiation was depicted within a very narrow range (0.28 to 0.04). Li *et al*. (2006) also found limited genetic diversity when evaluating 58 litchi cultivars from Hainan Island using SSR markers thus corroborating the findings of our study on Indian cultivars. Interestingly, phylogenetic analysis of molecular data of Indian litchi cultivars revealed that the clustering followed 4 basic groups, viz. Bedana (i.e. seedless;Grp IV), Mandarji (Grp III), Shahi(Grp I) and China groups(China II)(Table 3 molecular grouping combined with morphological attributes). This clustering is in confirmation with the identification of litchi cultivars based on morphological attributes, viz. flush colour, leaf shape/colour, panicle size, fruits shape etc (Singh and Babita 2002). Previous classification by Dwivedi and Mitra (1995) into 3 complexes based on TSS and acidity ratios, suggested hybridization among complex I and II for improving cultivars. The grouping based on reproducible markers like microsatellites along with RAPD markers displayed low genetic distances (0.28 to 0.04) and gene diversity estimates, thus exposing narrow genetic background of Indian litchi germplasm, and recommends cross breeding and widening genetic base for future breeding programs.

Thus, molecular characterization of Indian litchi genetic resources accomplished the documentation of the native germplasm diversity based on microsatellite barcodes. Cumulative marker analysis precisely arranged the cultivars into groups concurring with known morphological classifications. The narrow genetic base revealed by low gene diversity, warrant immediate introduction of diverse

Table 3 Morphological attributes of important Indian litchi cultivars* and their grouping based on molecular phylogeny(Singh, Gorakh, Nath, Vishal, Pandey S D, Ray P K and Singh H S (2012). The Litchi: FAO, New Delhi: 1-219)

Cultivar	Grouping (Molecular phylogeny)	Dominant variety from different Indian states	Availability	Descriptors	Fruit Wt (g)	Pulp (%)	Seed (%)	Leaf shape	Fruit shape/Apex
Dehradun	I	Uttar Pradesh, Uttarakhand, Himachal Pradesh, Punjab, Haryana	Early season	Fruit oblique heart to conical shape, bright pink color, cracking 32.8%, precocious in bearing, average yield 85 kg	15.2	74.34	16.18	Boat-shaped	Oblong with round apex
Shahi	I	Bihar, Jharkhand, Uttar Pradesh	Early season	Color of fruit deep pink, cracking 31.5%, fruit haveexcellent aroma and quality, average yield 100.30 kg	20.98	58.77	18.49	Boat-shaped	Oblong with round apex
Purbi	I	Bihar, Jharkhand, West Bengal	Mid season	Fruit egg round to lopsided, heart-shaped, deep pink, cracking 7.6%, second most important cultivar in Australia, average yield 80.65 kg	20.77	64.51	19.06	Curved upward from the midrib and down along their length	Heart-shaped with uneven shoulder distinctly pointed
Trikolia	I	Bihar, Jharkhand	Early season	Color of fruit deep pink, fruit weight 18.25 g, cracking 32.3%, resemble Shahi, average yield 42.37 kg	18.25	72.77	11.95	Boat-shaped	
China	II	Bihar, Jharkhand, West Bengal, Assam	Mid season	Fruit oblong in shape tyrant rose in color with dark tubercles at maturity, fruit weight 22 g, cracking 0.24%, aril is creamy white, soft, juicy and sweet, matures when all other cultivars have been harvested, average yield 95.33 kg	20.30	60.59	18.84	Curved upward from the midrib and down along their length	Oblong with pointed apex
Yogda Selection	II	Bihar (Ranchi, Muzaffarpur)	Late season	Seedling selection from Yogda Ashram, Ranchi, fruit round, fruit weight 15 g, considered as one of the oldest plant in the premises	ND	ND	ND	ND	Round
Calcuttia	II	Haryana, Punjab, Uttar Pradesh, Uttarakhand, Himanchal Pradesh	Mid season	Fruit oblong with tyrian rose color and dark tubercles at maturity, fruit weight 22 g, cracking 0.5%, pulp is very sweet with agreeable aroma, average yield is 90 kg	22.00	61.50	15.86	Curved upward from the midrib and down along their length	Oblong with pointed apex
Mandarji	III	Bihar, Jharkhand	ND	Fruit oblong with shoulders, deep pink, fruit weight 22 to 26 g, cracking not reported, pulp is soft, juicy with pleasant smell	ND	ND	ND	Curved upward from the midrib and down along their length	Oblong with pointed apex
Late Large Red	III	Bihar and Uttar Pradesh	ND	Fruits are large in size, oval or oblong conical with crimson red tubercles. Pulp is grayish white, soft, moderately juicy with a TSS of 20.3° B.	ND	62.5	ND	ND	ND

Contd.

Table 3 (*Concluded*)

Cultivar	Grouping (Molecular phylogeny)	Dominant variety from different Indian states	Availability	Descriptors	Fruit Wt (g)	Pulp (%)	Seed (%)	Leaf shape	Fruit shape/Apex
Swarnaroopa	IV	Bihar, Jharkhand	Mid season	Fruit deep pink, midseason maturity, fruit weight 19 g, clonal selection of seedless group released by CHES, Ranchi, recommended for commercial production	18.95	76.62	16.36	Oval shaped leaves	Round
Seedless late	IV	ND	ND	ND	21.39	72.79	4.2	ND	ND
Bedana	IV	Uttar Pradesh, Uttarakhand, Himanchal Pradesh	Mid season	Fruit oval and deep red, cracking 0.29%, fruit with high percentage of chicken tongued seeds, overall fruit quality is good, average yield 32.75 kg	18	83.39	8.83	ND	ND
Late Bedana (*Syn.* Seedless late)	IV	Bihar, Uttar Pradesh	Late Season	Fruits are medium to large in size, conical in shape, colour at maturity vermilion to carmine with dark-blackish brown tubercles; pulp is creamy-white,soft, juicy.	16.70	75.08	ND	ND	Conical
Bedana Sabour	IV	Bihar	Mid season	An excellent variety recently released from Sabour in Bihar; average yield is 80-90 kg/ tree; fruits are large in size (24-30 g); fruit colour attractive carmine red with uranium green skin background; sweet, soft, and juicy; small, chicken tongue seeds 80 to 90 per cent	24-30	75-80	ND	ND	ND
Kasba	IV	West Bengal, Assam	Mid season	Fruit conical, fruit color marigold-orange, fruit weight 23 to 27 g, cultivar performs better in marginal soil because it has better capacity to absorb nutrients, average yield 37 kg	20.30	69.95	13.79	Boat-shaped	Largest size fruit

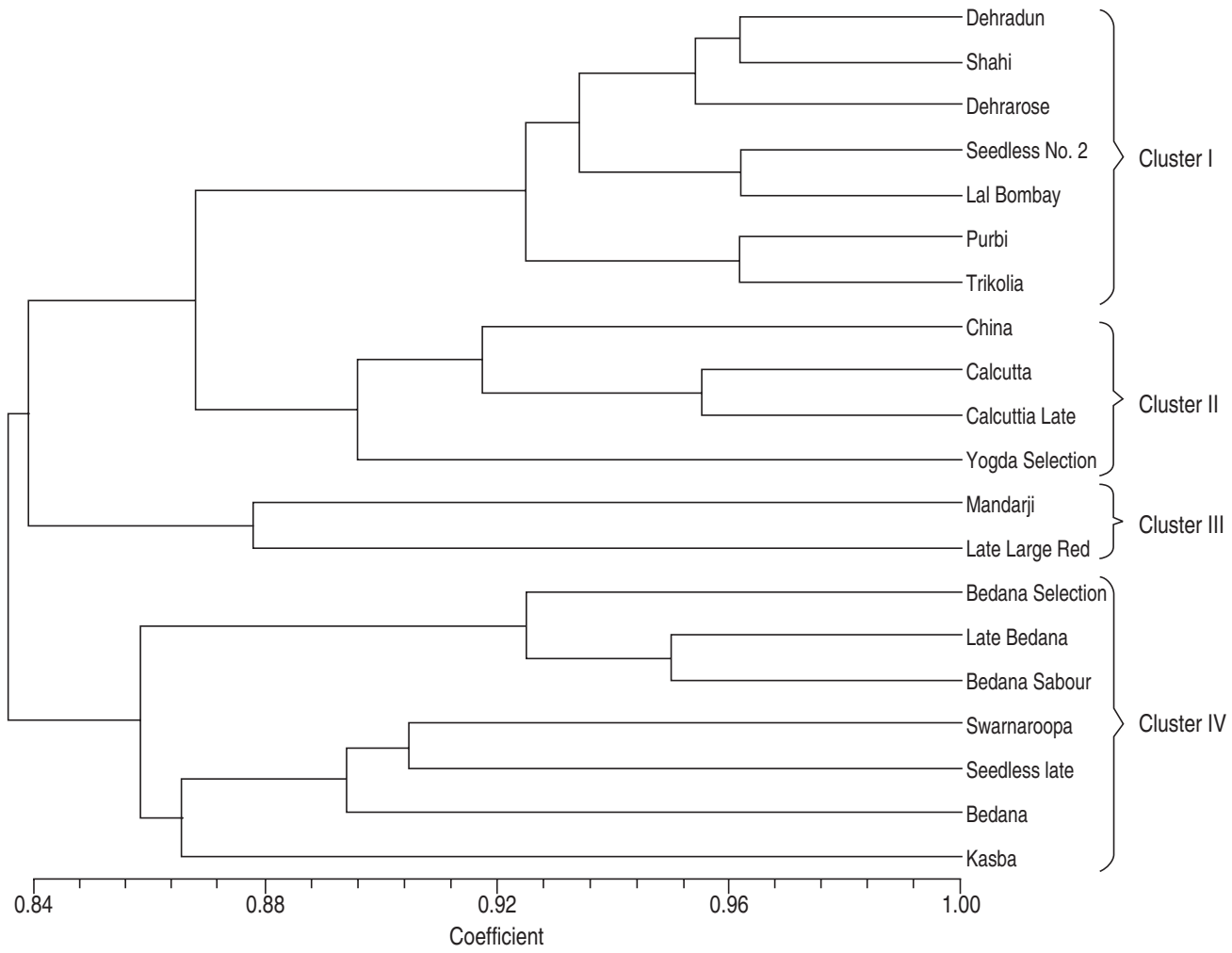


Fig 2 Dendrogram depicting genetic relationship among Indian litchi cultivars using molecular markers

gene pool, germplasm exchange and cross breeding for realizing genetic gains.

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