

Genetic diversity analysis of mango cultivars using inter simple sequence repeat markers

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With 33 polymorphic Inter Simple Sequence Repeat (ISSR) markers, genetic diversity among 70 mango cultivars and a distant outgroup was analysed. Clustering was done using Dice and Jaccard coefficients with bootstrapping. Multivariate analysis was conducted using the Euclidean distances. Among the total 420 bands, 408 were polymorphic. Probability that any ISSR fragment is shared by two different cultivars was calculated to be 2.54×10^{-1} . Non-Indian mango cultivars were found genetically diverged from Indian mango germ pool. No separation was observed between North Indian and South Indian cultivars. Twelve different cultivar-specific bands were detected for six cultivars, which approved the effectiveness of ISSR markers in mango genetic diversity analysis.

Keywords: DNA finger printing, inter simple sequence repeat, *Mangifera indica*, principle coordinate analysis.

MANGO (*Mangifera indica* L.) (Anacardiaceae) originated in the Indo-Burma region during the earlier period of the Cretaceous era¹ and gradually spread to the tropical and subtropical regions of the world. India is thought to be the primary centre of diversity along with its status as the centre of origin for mango. Presently, India harbours more than 1000 mango cultivars and represents the biggest mango germ pool in the world. Australia, China, USA, Israel and Thailand are the other regions that maintain such healthy mango germ pool.

In the various mango-growing regions, breeding attempts are always in progress for creating better cultivars. Precise information on the genetic relationships within such germplasm diversity is needed for carrying out efficient breeding programmes. In order to assess the genetic diversity in mango, PCR-based DNA markers are among the best tools. A range of DNA markers, viz. AFLP, DAMD, ISSR, ITS and RAPD have already been used for exploring the diversity of the global mango germ pool²⁻¹². Among these, Inter Simple Sequence Repeat (ISSR)¹³ is a repro-

ducible semi-arbitrary primed PCR method that uses simple sequence repeats as primers, combining most of the advantages of microsatellites and Amplified Fragment Length Polymorphism (AFLP), to the universality of Randomly Amplified Polymorphic DNA (RAPD)¹⁴. ISSRs offer greater probability than any other PCR marker system in the repeat regions of the genome, which are the most potent regions for producing cultivar-specific markers. This is also the attribute of ISSRs, which renders them useful as a supplementary system to any of the random, dominant marker systems. Automated PCR base makes ISSRs the markers of choice for screening genotypes. Consequently, we choose the ISSR marker system to assess the diversity among 70 mango cultivars.

For the present experiment, 60 elite Indian cultivars were selected on the basis of their consistency in behaviour for the last 30 years at their growing region, their promising features for breeding and their plausibility to race in the global market. Among these 38 were South Indian cultivars, including 'Villai Kolumban', as the only Indian polyembryonic cultivar in the set and 16 were North Indian cultivars. Six promising Indian cultivars with undecided South Indian or North Indian origin (designated as Indian throughout the analysis) were also included in order to reveal their parentage or at least their alliance in the mango cultivar cladogram. Among the 60 cultivars Alphonso, Badami, Baramahi Hapus, Banarasi Hapus and Kala Hapus real were suspected to be synonymous with each other and were selected especially for confirming their status. Ratna is the F1 progeny cultivar of the Neelum \times Alphonso cross, while Sindhu is the outcome of the Ratna-Alphonso backcross. Ten non-Indian cultivars were included in the analysis to test the assumption that over the centuries, the selection criteria for the world market have been significantly different than those for the Indian market and under such differential selection pressure, the non-Indian cultivars may show isolation from the diversity pool of Indian mango. These include five Floridian cultivars, three Israeli and one Australian ('Kensington' as non-Indian polyembryonic cultivar) and Taiwanese cultivar each. The two above-mentioned polyembryonic cultivars were included as the 'close outgroup' taxa in the study. *Nothopegia colebrookiana* Blume. (Anacardiaceae), a distant relative of the genus *Mangifera* L., which is available in the local forests, was selected as the 'distant outgroup'. The list of cultivars along with their origins is given in Table 1.

Flushing leaves from 70 mango cultivars (Table 1) were collected from the experimental orchards at the Regional Fruit Research Station (RFRS) of Dr Balasaheb Savant Konkan Krishi Vidyapeeth, Vengurle (Maharashtra, India) and those of *N. colebrookiana* were collected from the forest of Amboli, Maharashtra. All leaf samples were frozen in liquid nitrogen for transportation to the laboratory and subsequently stored at -80°C until processed.

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Table 1. Mango cultivars used in the ISSR analysis along with their region of cultivation

Serial no.	Cultivar	Region of cultivation	Serial no.	Cultivar	Region of cultivation
1	13-3	Israel	36	Keitt	Florida
2	Alphonso	South India	37	Kent	Florida
3	Badaigol	India	38	Kensington	Australia
4	Badami	South India	39	Kesar	North India
5	Banarasi Batli	North India	40	Kingphone	Taiwan
6	Banarasi Hapus	South India	41	Ladaio	South India
7	Bangalore Goa	South India	42	Langra	North India
8	Baramahi Hapus	India	43	Lili	Israel
9	Baramasia	South India	44	Mahalanjeo	South India
10	Bengali pairi	North India	45	Maharaja of Mysore	South India
11	Bekurad	India	46	Makaram	India
12	Bombay green	South India	47	Maya	Israel
13	Borsha Kalamshar	North India	48	Mulgoba	South India
14	Chandrama	South India	49	Musharad	South India
15	Chinna Suvarnaksha	South India	50	Naliaro	South India
16	Chittur Badami	South India	51	Neelum	South India
17	Creeping	South India	52	Osteen	Florida
18	Dadamio	North India	53	Pairi	South India
19	Dasher	North India	54	Palmar	Florida
20	Devrukhio	South India	55	Pau	India
21	Dilpasand	South India	56	Police	India
22	Dudh peda	South India	57	Rajapuri	North India
23	Fakira	North India	58	Rangar	India
24	Fernandin	South India	59	Ratna	South India
25	Gadhemar	South India	60	Roos	South India
26	Goamankur	South India	61	Rumani	South India
27	Gopta of Navasari	North India	62	Sabja	South India
28	Hamlet	South India	63	Saleem	South India
29	Hathizool	North India	64	SB Chausa	North India
30	Jamadar	North India	65	Sindhu	South India
31	Kajalio	South India	66	Tankij- amadi	India
32	Kala Pahad	North India	67	Tomy-Atkins	Florida
33	Kalahapus real	South India	68	Totapuri	South India
34	Karanjio	South India	69	Vanraj	North India
35	Karelia	North India	70	Villai Kolumban	South India
			71	<i>Nothopegia colebrookiana</i>	South India

DNA was extracted as described by Doyle and Doyle¹⁵. DNA concentrations were determined by comparison with the intensity of standard DNA (λ DNA, Bangalore Genei, India) after electrophoresis in ethidium bromide-stained 0.8% agarose gel.

Initially 100 UBC primers were screened with ten mango cultivars, wherein at least one cultivar, represented each geographic region along with one outgroup. Primers that generated polymorphism were used for the final experiment with all the 70 cultivars along with one outgroup.

A set of 100 ISSR primers, procured from University of British Columbia (UBC, Vancouver, USA), was used for amplification of plant DNA. Polymerase chain reaction (PCR) was carried out in 25 μ l volume, as detailed by Deshpande *et al.*¹⁶. The amplified products were separated on 2.0% agarose gel in 0.5 \times TAE buffer and bands were detected by ethidium bromide staining, as suggested by Deshpande *et al.*¹⁶. The size of each fragment was estimated with reference to a DNA size marker ϕ X 174/*Hae*III digest (Bangalore Genei).

The band pattern obtained by each ISSR primer was scored by visual inspection and the bands were recorded as present (1) or absent (0). From the band patterns obtained with each primer, the cultivar-specific bands (if any) along with their sizes were recorded.

Similarity estimate, D was calculated as $2Nab/Na + Nb$ for each primer and also collectively for all 33 primers¹⁷. The probability that a fragment in one cultivar is also found in another for all pairwise comparisons (fingerprinting potential of a marker) was then calculated for each primer as $[(X_D)^n]$, where X_D represents the average similarity index for all pairwise comparisons and n is the average number of bands amplified by the primer¹⁸. $[(X_D)^n]$ was also calculated considering data from all 33 primers together.

The binary score data from ISSR amplifications were directly fed to the multivariate statistical package (MVSP)¹⁹ for Principal Coordinate (PCO) analysis.

The genetic distance matrices were generated using the Windist software option of the Winboot package²⁰, with

Table 2. Performance of various ISSR primers in the genetic diversity analysis of mango

UBC primer no.	Primer sequence	Loci scored	No. of polymorphic loci	Percentage polymorphism	Average no. of loci produced in 71 genotypes (<i>n</i>)	Average similarity [(X) _D] ± SD	Probability of identical match by chance [(X) _D] ⁿ
807	(AG) ₈ T	16	16	100	6.01 ± 1.43	0.70 ± 0.13	1.25 × 10 ⁻¹
808	(AG) ₈ C	18	18	100	6.21 ± 1.43	0.75 ± 0.11	1.68 × 10 ⁻¹
809	(AG) ₈ G	08	08	100	5.23 ± 1.12	0.81 ± 0.15	3.39 × 10 ⁻¹
810	(AG) ₈ T	11	08	72.72	6.15 ± 1.09	0.83 ± 0.11	3.24 × 10 ⁻¹
811	(GA) ₈ C	13	12	92.30	4.69 ± 1.17	0.76 ± 0.13	2.90 × 10 ⁻¹
812	(GA) ₈ A	15	14	93.33	8.33 ± 1.58	0.76 ± 0.11	1.09 × 10 ⁻¹
813	(CT) ₈ T	09	09	100	3.88 ± 0.49	0.92 ± 0.13	7.27 × 10 ⁻¹
815	(CT) ₈ G	10	10	100	3.38 ± 1.38	0.64 ± 0.17	2.30 × 10 ⁻¹
830	(TG) ₈ G	11	11	100	2.35 ± 1.26	0.75 ± 0.18	5.15 × 10 ⁻¹
834	(AG) ₈ YT	12	12	100	4.94 ± 1.47	0.71 ± 0.17	1.96 × 10 ⁻¹
835	(AG) ₈ YC	16	15	93.75	3.91 ± 1.18	0.82 ± 0.10	4.67 × 10 ⁻¹
836	(AG) ₈ YA	15	13	86.66	8.18 ± 1.22	0.83 ± 0.08	2.18 × 10 ⁻¹
840	(GA) ₈ YT	16	16	100	6.18 ± 1.27	0.77 ± 0.11	2.06 × 10 ⁻¹
844	(CT) ₈ RC	13	13	100	3.88 ± 1.59	0.64 ± 0.15	1.87 × 10 ⁻¹
845	(CT) ₈ RG	13	13	100	6.42 ± 2.04	0.62 ± 0.15	5.10 × 10 ⁻¹
848	(CA) ₈ RG	10	09	90.00	3.71 ± 0.81	0.84 ± 0.12	5.33 × 10 ⁻¹
852	(TC) ₈ RA	18	18	100	3.76 ± 1.30	0.86 ± 0.10	5.74 × 10 ⁻¹
855	(AC) ₈ YT	17	17	100	5.47 ± 1.28	0.66 ± 0.13	1.09 × 10 ⁻¹
856	(AC) ₈ YA	16	15	93.75	6.49 ± 1.26	0.79 ± 0.10	2.30 × 10 ⁻¹
857	(AC) ₈ YG	14	14	100	3.70 ± 1.91	0.72 ± 0.14	3.01 × 10 ⁻¹
859	(TG) ₈ RC	08	08	100	3.97 ± 1.24	0.70 ± 0.16	2.44 × 10 ⁻¹
862	(AGC) ₆	08	08	100	5.83 ± 0.56	0.95 ± 0.15	7.58 × 10 ⁻¹
864	(ATG) ₆	07	06	85.71	3.32 ± 0.95	0.77 ± 0.14	4.19 × 10 ⁻¹
866	(CTC) ₆	11	11	100	4.59 ± 1.34	0.79 ± 0.13	3.52 × 10 ⁻¹
876	(GATA) ₂ (GACA) ₂	14	14	100	4.39 ± 1.52	0.65 ± 0.13	1.54 × 10 ⁻¹
878	(GGAT) ₄	11	10	90.90	5.49 ± 1.30	0.77 ± 0.12	2.40 × 10 ⁻¹
881	GGG(TGGGG) ₂ TG	09	09	100	4.73 ± 1.06	0.73 ± 0.16	2.27 × 10 ⁻¹
884	HBH(AG) ₇	14	14	100	7.78 ± 1.75	0.68 ± 0.13	5.00 × 10 ⁻¹
886	VDV(CT) ₇	16	16	100	7.28 ± 1.59	0.78 ± 0.12	1.79 × 10 ⁻¹
887	DVD(TC) ₇	16	16	100	5.47 ± 1.1	0.80 ± 0.11	3.14 × 10 ⁻¹
889	DBD(AC) ₇	08	08	100	2.28 ± 1.32	0.70 ± 0.18	4.54 × 10 ⁻¹
890	VHV(GT) ₇	15	15	100	6.46 ± 1.31	0.79 ± 0.12	2.20 × 10 ⁻¹
891	HVH(TG) ₇	12	12	100	3.22 ± 1.64	0.79 ± 0.12	4.70 × 10 ⁻¹
Total no. of primers: 33		Total loci: 420	Total polymorphic loci: 408	Average per cent polymorphism: 97.14	Mean average no. of loci produced by 33 ISSR primers: 5.08 ± 1.58	0.76 ± 0.06	2.54 × 10 ⁻¹

Dice and Jaccard coefficients. Bootstrapping²¹ was done using the Winboot software to construct dendrograms using UPGMA algorithm with 500 replicates.

The entire analysis, from tissue collection to the statistical data analysis, was repeated twice.

Out of 100 ISSR primers, 40 showed amplification in 11 genotypes that were used for the initial screening. Among these, 33 primers generated reproducible polymorphic DNA amplification patterns in all the 71 genotypes. Twenty-seven of these 33 primers belonged to the anchored dinucleotide repeat class; remarkably, 15 of these 27 belong to either 'AG repeat' class or its complementary 'CT repeat' class (Table 2).

Table 2 explains the performance of each of the 33 ISSR primers with 71 genotypes. These primers yielded a total of 420 scorable bands on amplification and their sizes ranged between 200 and 2000 bp. The number of

scorable loci generated by the individual primers ranged between seven (UBC 864) and 18 (UBC 809 and UBC 852). Out of 420 bands 408 (97.14%) were polymorphic and only 12 bands (2.86%) were monomorphic. Most of the primers (24 of 33 = 67%) exhibited 100% polymorphism, while least polymorphism (8 of 11 loci = 72.72%) was shown by UBC 810. The average number of bands amplified from the pool of 71 genotypes by UBC 889 was 2.28 and that by UBC 812 was 8.33; other primers produced average number of scorable bands within the range of these two values.

We obtained 12 different cultivar-specific bands from the amplification profiles with eight ISSR primers (Table 3). Tomy-Atkins was a unique cultivar with maximum of five specific bands produced by various primers. Primer UBC 813 produced three specific bands for Vanraj thus making three as the highest number of specific bands that

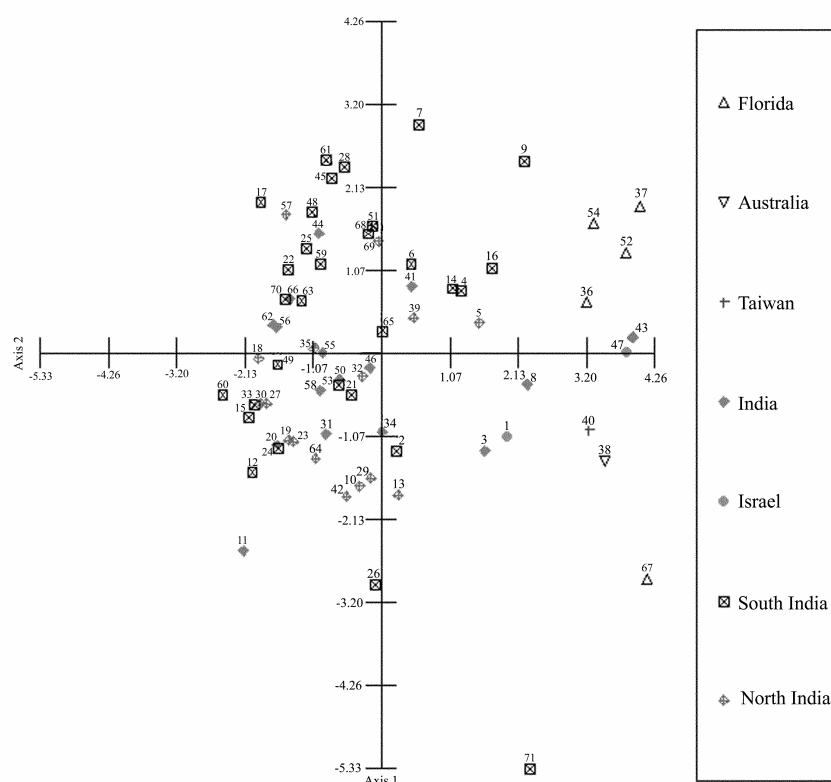


Figure 1. Principle coordinate analysis based on ISSR marker data for 70 mango cultivars (Indian as well as foreign) along with *Nothopegia colebrookiana*, the outgroup. Numbers denoting the plotted data-points represent the respective mango cultivars as listed in Table 1.

Table 3. Cultivar-specific bands obtained with various ISSR primers (bands specific to the distant outgroup are not mentioned here)

Cultivar	Primer producing specific band(s)	Size of the specific band (bp)
Fakira	UBC 878	500
Gadhemar	UBC 836	2000
Neelum	UBC 884	500
S B Chausa	UBC 852	400
Tomy-Atkins	UBC 811	300
	UBC 834	700
	UBC 852	1000
	UBC 866	1400
	UBC 866	1500
Vanraj	UBC 813	1000
	UBC 813	400
	UBC 813	300
Total no. of cultivars: 6	No. of primers generating cultivar-specific bands: 8	Total no. of cultivar-specific bands: 12

any primer has produced. UBC 852 produced one specific band each in S B Chausa and Tomy-Atkins and was the only primer that could produce specific bands in two different cultivars.

The probability of matching DNA fingerprints of any two mango cultivars $[(X_D)^n]$ was 2.54×10^{-1} when calculated for the entire 33 primer sets of ISSR (420 loci; Table 2). When calculated for each primer, it ranged between 1.09×10^{-1} (UBC 812 and UBC 855) and 5.1×10^{-2} (UBC 845; Table 2).

Separation of non-Indian cultivars from Indian cultivars was revealed in the PCO analysis (Figure 1). Foreign cultivars were placed in the first and the fourth quadrants with the close cluster of Floridian cultivars, including the Israeli cultivar Maya (cultivar no. 47, Table 1). However, Tomy-Atkins (cultivar no. 67, Table 1), the Floridian cultivar was placed distantly in the fourth quadrant from the pool of mango cultivars. Cultivar 13-3 (cultivar no. 1, Table 1) was placed centrally to the clusters of Indian and foreign cultivars. *N. colebrookiana* (outgroup no. 71, Table 1) was placed completely aloof in the fourth quadrant. All the Indian cultivars were observed to be grouped together closely. However, seven Indian cultivars: Badai-gol (cultivar no. 3, Table 1), Badami (cultivar no. 4, Table 1), Banarasi Batli (cultivar no. 5, Table 1), Banarasi Hapus (cultivar no. 6, Table 1), Baramahi Hapus (cultivar no. 8, Table 1), Baramasia (cultivar no. 9, Table 1), Chandrama (cultivar no. 14, Table 1) and Chittur Badami

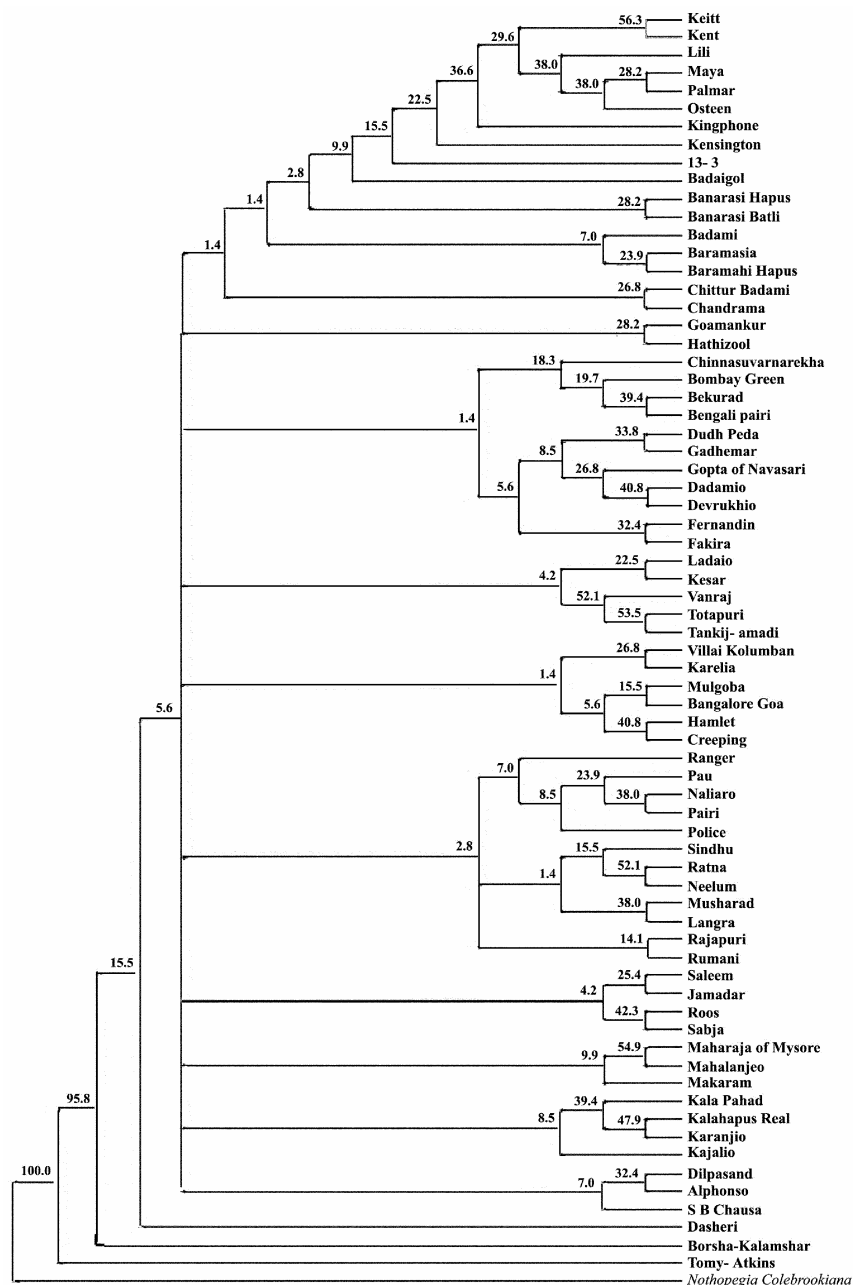


Figure 2. UPGMA dendrogram for 70 mango cultivars and the distant outgroup, drawn using Dice coefficient and Winboot software.

(cultivar no. 16, Table 1) were not placed close to the cluster of Indian cultivars and 13-3 was placed with these cultivars.

No geographical separation was revealed between the North and South Indian cultivars by any of these statistical combinations.

Both UPGMA dendrograms drawn using Dice (Figure 2; representative of the two dendrograms) and Jaccard

coefficients showed highly similar grouping of cultivars. Both showed high bootstrap separation of outgroup (100%) from the mango clade and that of Tomy-Atkins (average 95 and 94 % respectively) from the big cluster of 69 mango cultivars. Though non-Indian cultivars grouped separately from most of the Indian cultivars, eight Indian cultivars grouped with the foreign cultivars. Congruence in the composition of small clusters (3–5 OTU) or OTU

pairs was remarkable in both the dendrograms. However, for most of the clusters, Dice coefficient produced higher bootstrap values than Jaccard coefficient.

Separation among the Indian cultivars was seen with low bootstrap values. However, cultivars that were suspected to be synonymous, opted different subgroups. Villai Kolumban subgrouped with Karelia within the group of Indian cultivars in both the trees. Similarly, Kensington grouped with the non-Indian cultivars. Six cultivars of unknown origin were found scattered over the dendrograms. Among these, Badaigol remained in the eight Indian cultivars that grouped with the foreign cultivars. The remaining five subgrouped with various South Indian cultivar clusters; Bekurad with Bombay green and Chinnasuvarnarekha; Makaram with Mahalanjeo and Maharaja of Mysore; and Pau, Police and Rangar with Naliaro and Pairi.

To the best of our knowledge there are only two reports^{5,12} where the genetic diversity in mango has been assessed using ISSR markers. The report by Eiadthong *et al.*⁵ mainly dealt with the amplification in Thai cultivars by seven ISSR primers, of which four were anchored dinucleotide repeat primers. Srivastava *et al.*¹² have also used seven ISSR primers which include two anchored dinucleotide repeat primers. Here we have screened 100 ISSR primers available from UBC, Canada and have found that majority of the anchored ISSR primers (>80%) that can produce multilocus amplifications in mango belong to the dinucleotide repeat class. It indicates that the mango genome is likely to be rich in the dinucleotide repeat regions. Eiadthong *et al.*⁵ also reported variability in the band patterns in order to distinguish any two cultivars, which could not be obtained in the current set of cultivars. Indian cultivars showed higher homogeneity in the ISSR-targeted regions; it was also true for the selected foreign cultivars. Based on the present study, that includes the highest number of cultivars, number of primers as well as the number of loci scored, we postulate that the variability observed by previous researchers could be because of small sample size used and such variability might be eliminated with the increase in sample size and rule out the possibility that ISSRs can become a direct and comprehensive marker system in mango cultivar identification. Nonetheless, we report cultivar-specific bands from the present set of cultivars (Table 3). It should be noted that these specific bands are more in the case of two most diverged cultivars, Tomy-Atkins and Vanraj. These two probably define the extremes of the dendrograms in the present set of cultivars, as the similarity between them calculated using all Dice as well as Jaccard similarity coefficients was the lowest among all the pairwise cultivar comparisons (0.504 and 0.337 respectively). Rest of the cultivar pool was considerably homogenous for the ISSR-targeted genomic regions, which is evident from the 'close' pairwise similarity values. Srivastava *et al.*¹² reported RAPD and DAMD bands that were specific to cultivar Neelum and its hybrid descendents; no such

bands could be found in Alphonso, Neelum, Ratna and Sindhu group with the ISSR marker system.

Various markers that have been used to assess the genetic diversity in mango have proved to be successful in different dimensions. RAPD markers in the case of mango could clearly resolve the monoembryonic and polyembryonic cultivars^{7,8}. Such a differentiation has not been reported by AFLP, ISSR or any other system. ISSR marker system could not resolve the two polyembryonic cultivars, Kensington and Villai Kolumban, which were the *priori* assumed close outgroups. The present data are enough to state that no such differentiation has taken place in the SSR regions of the mango genome. Secondly, Lopez-Valenzuela *et al.*⁸ and Schnell *et al.*¹¹ mentioned the geographical clustering of cultivars with RAPDs, which we have also obtained clearly and consistently with ISSRs. Considering the extent of genomic regions spanned by the ISSR and the RAPD, this phenomenon can be an evidence for the initiation of spatial isolation in the global mango germ pool. Isolation within Indian cultivars as North and South Indian, has also been reported by Karihaloo *et al.*⁷ and Ravishankar *et al.*¹⁰ (dendrogram as well as PCO results) using RAPD. This grouping was observed to be dissolved by an increase in the sample size⁹ and by change in the selected cultivar set³. ISSR in the present study completely merges both the groups. This was evident from the high probability of finding shared bands among any two cultivars, from the scatter plot as well as from the low bootstrap values in the dendrograms. This could be because of the cultivar spreading in the recent past and higher rate of new cultivar generation in India. In order to obtain a clearer picture, such studies should be carried with out with a larger sample size that will truly represent the diversity of Indian cultivars and different types of markers, each in a statistically significant number. India being the centre of origin and the primary centre of diversity for mango, such studies are of scientific importance.

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ACKNOWLEDGEMENTS. The Department of Scientific and Industrial Research, Government of India and Council of Scientific and Industrial Research (CSIR), New Delhi supported this work under the network programme on Cell and Tissue Engineering, to National Chemical Laboratory, Pune. We thank the staff members at RFRS, Vengurle, for maintaining the mango orchards. S.S.P thanks CSIR for Senior Research Fellowship.

Economic viability of cultivation of the Himalayan herb *Angelica glauca* Edgew. at two different agro climatic zones

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***Angelica glauca* (Apiaceae), endemic to the Himalaya, is an endangered medicinal herb for which, besides *in situ* conservation, *ex situ* cultivation is also recommended. Observations were carried out for economic yield trails at two different agro-climatic zones in Garhwal Himalaya. Seedlings of different age groups were transplanted at 2200 m asl (Pothivasa, PV) and 3600 m asl (Tungnath, TN) to observe comparative yield as well as climate and land suitability for future cultivation. Yield was minimum in youngest seedlings (60-days-old) transplanted at TN in ordinary alpine soil (control) and maximum under polyhouse beds at PV after two years of growth. Results indicate that plants raised through younger seedlings (60-days-old) had minimum yield, while those raised through 90- or 120-days-old seedlings had better yield with no or very less variation in yield. In addition, economic yield was found maximum at lower altitude (PV, 2200 m) in comparison to higher altitude (TN, 3600 m). Observations on yield under different treatments at the two sites are presented here and on the basis of these observations, suggestions for commercial cultivation of the species are made.**

Keywords: Agroclimatic zone, cultivation, profit, yield.

ANGELICA glauca Edgew. (Choru, Gandhrayan) belonging to, family Apiaceae is a glabrous, aromatic herb, 90–190 cm in height, found in high altitudes of Himalaya (2600–3700 m asl). Roots and rhizomes of this herb are used as spices and condiments by indigenous communities¹. The rhizomes are considered cardio-active, useful in constipation², for curing rheumatism and urinary disorders. The powdered root along with milk is used to treat bronchitis³. All parts of the herb are reported to be useful to cure stomach ailments, bilious complaints, menorrhagia, infantile atrophy and as a stimulant^{2,4}.

In the temperate and alpine zone of the Himalaya, increasing intensity of harvesting of medicinal plants and change in climatic conditions have adversely affected the habitats of many species, leading to a gradual loss in regeneration potential and diversity of many economically

Received 4 January 2007; revised accepted 9 August 2007

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