RESEARCH ARTICLE

Comparative evaluation of genetic diversity using RAPD, SSR and cytochrome P450 gene based markers with respect to calcium content in finger millet (*Eleusine coracana* L. Gaertn.)

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Abstract

Genetic relationships among 52 *Eleusine coracana* (finger millet) genotypes collected from different districts of Uttarakhand were investigated by using randomly amplified polymorphic DNA (RAPD), simple sequence repeat (SSR) and cytochrome P450 gene based markers. A total of 18 RAPD primers, 10 SSR primers, and 10 pairs of cytochrome P450 gene based markers, respectively, revealed 49.4%, 50.2% and 58.7% polymorphism in 52 genotypes of *E. coracana*. Mean polymorphic information content (PIC) for each of these marker systems (0.351 for RAPD, 0.505 for SSR and 0.406 for cyt P450 gene based markers) suggested that all the marker systems were effective in determining polymorphisms. Pair-wise similarity index values ranged from 0.011 to 0.999 (RAPD), 0.010 to 0.999 (SSR) and 0.001 to 0.998 (cyt P450 gene based markers) and mean similarity index value of 0.505, 0.504 and 0.499, respectively. The dendrogram developed by RAPD, SSR and cytochrome P450 gene based primers analyses revealed that the genotypes are grouped in different clusters according to high calcium (300–450 mg/100 g), medium calcium (200–300 mg/100 g) and low calcium (100–200 mg/100 g). Mantel test employed for detection of goodness of fit established cophenetic correlation values above 0.95 for all the three marker systems. The dendrograms and principal coordinate analysis (PCA) plots derived from the binary data matrices of the three marker systems are highly concordant. High bootstrap values were obtained at major nodes of phenograms through WINBOOT software. Comparison of RAPD, SSR and cytochrome P450 gene based markers, in terms of the quality of data output, indicated that SSRs and cyt P450 gene based markers are particularly promising for the analysis of plant genome diversity. The genotypes of finger millet collected from different districts of Uttarakhand constitute a wide genetic base and clustered according to calcium contents. The identified genotypes could be used in breeding programmes and a major input into conservation biology of cereal crops.

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Introduction

Eleusine coracana, commonly called finger millet or ragi, is an allotetraploid ($2n = 4x = 36$ chromosomes) whose genomes are designated as AABB. It is an important food crop cultivated widely in India and other Asian countries, and also many parts of Africa. It is rich in protein (~6–13%) and calcium (~0.3–0.4%) (Venkannababu *et al*. 1987). There have been initiatives for finger millet improvement using classical plant breeding approach for different traits. The prerequisite for attaining this goal involves screening of different germplasm for desired trait by using morphological, biochemical or molecular markers. Molecular genetic techniques using DNA polymorphism have been increasingly used to characterize and identify a novel germplasm for uses in the crop breeding process (O'Neill *et al*. 2003). Several DNA marker systems

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are now commonly used in diversity studies of plants. The most commonly used marker systems are random amplified polymorphic DNA (RAPD) (Williams *et al*. 1990; Jacobson and Hedrén 2007), restriction fragment length polymorphism (RFLP) (Soller and Beckmann 1983), amplified fragment length polymorphism (AFLP) (Vos *et al*. 1995), inter simple sequence repeats (ISSRs) (Zietkiewicz *et al*. 1994) and microsatellites or simple sequence repeats (SSRs) (Becker and Heun 1994). Among them, RAPD analysis is quick (Colombo *et al*. 1998), but has problems with reproducibility in amplification of RAPD markers and with data scoring. SSRs are multi-allelic and have high potential for use in evolutionary studies and studies regarding genetic diversity and relationships analysis in a variety of plants. Comparisons of different DNA markers for diversity studies in *Zea mays* (Garcia *et al*. 2004), barley (Russell *et al*. 1997), sorghum (Agrama and Tuinstra 2003), rice (Davierwala *et al*. 2000), wheat (Chao *et al*. 2007) etc. have been tried to evaluate the relative efficiencies of the different techniques available. For research involving finger millet (*Eleusine coracana* L.), the most widely used molecular method has been RAPD (Salimath *et al*. 1995; Fakrudin *et al*. 2007; Babu *et al*. 2007), although isozymes (Hilu 1995), RFLP (Parani *et al*. 2001) and SSRs (Dida *et al*. 2007, 2008) have also been used successfully in genetic diversity analyses. Despite the success of these methods, the level of polymorphism detectable is low. Cyt P450 mono-oxygenases are widely found in animals, plants and microorganisms (Shalk *et al*. 1999). Sequence diversity of P450 gene-analogues in different plant species have been studied and it has been reported that P450 gene-analogues can be used as new genetic markers for diversity studies in plants reflecting both functional and genome-wide regions (Tanksley and McCouch 1997; Watanabe and Iwanaga 1999; Somerville and Somerville 1999). So far there are no reports of using markers based on cyt P450 genes for characterization of accessions of finger millet. Considering the potentials of the DNA-based marker based genetic diversity analysis, the present study aimed to evaluate the relative usefulness of RAPD, SSRs and cytochrome P450 gene based markers, in assessing and analysing the nature and the extent of genetic diversity among the genotypes of finger millet collected from a narrow geographical region of Uttarakhand, India.

Materials and methods

Germplasm collection

A total of 52 genotypes were used in the present study. Seed of these 52 genotypes of *E. coracana* were collected from different districts of Uttarakhand (figure 1) were obtained from Ranichauri Hill Campus, G. B. Pant University of Agriculture and Technology, India. Calcium content of each sample was estimated by atomic absorption spectrophotometer (AAS) method in triplicate (Barbeau and Hilu 1993). The passport data of 52 genotypes are given in table 1.

Figure 1. The geographical map of Uttarakhand showing sites of sample collection.

DNA extraction and PCR amplification

The genomic DNA of different accessions of finger millets were isolated by standard methods (Murray and Thompson 1980), and subsequently quantified and analysed via agarose gel electrophoresis (Maniatis *et al*. 1989).

RAPD, SSR and cytochrome P450 gene based markers analyses

A total of 18 random primers, 10 SSR primers, and 10 pairs of cytochrome P450 gene based primers were used for the polymorphism survey. PCR amplification was performed as per the standard protocol using 50–100 ng of template DNA, 30 ng of primer (Life Tech, Delhi, India), 0.1 mM dNTPS , 1.5 U *Taq* DNA polymerase (Bangalore Genei, Bangalore, India), 1× PCR buffer (10 mM Tris pH 8.0, 50 mM KCl and 1.8 mM $MgCl_2$) in a volume of 25 μL. Amplification was performed with thermal cycler (Eppendorf, Hamburg, Germany). The standardized amplification was: initial denaturation 95°C for 5 min followed by 40 cycles of denaturation 94°C for 1 min; primer annealing based on melting temperature (Tm) value for 1 min; primer extension at 72°C for 2 min; and final primer extension at 72°C for 7 min. The annealing temperatures of the cycling parameter were readjusted for each microsatellite primers according to their calculated Tm based on the sequence composition:

$$Tm = 4^{\circ}(G + C) + 2^{\circ}(A + T) - 3^{\circ}C.$$

Table 1. List of accessions used in the genetic diversity studies.

Sl. no.	Genotype	Calcium (mg/100 g)	Sl. no.	Genotype	Calcium (mg/100 g)
1	GPHCPB-1	117.56	27	GPHCPB-27	150.50
2	GPHCPB-2	136.11	28	GPHCPB-28	165.45
3	GPHCPB-3	183.90	29	GPHCPB-29	201.93
4	GPHCPB-4	184.13	30	GPHCPB-30	278.35
5	GPHCPB-5	248.19	31	GPHCPB-31	417.46
6	GPHCPB-6	237.31	32	GPHCPB-32	227.92
7	GPHCPB-7	174.13	33	GPHCPB-33	366.75
8	GPHCPB-8	224.18	34	GPHCPB-34	274.73
9	GPHCPB-9	163.94	35	GPHCPB-35	392.60
10	GPHCPB-10	177.77	36	GPHCPB-36	352.73
11	GPHCPB-11	226.89	37	GPHCPB-37	326.05
12	GPHCPB-12	201.23	38	GPHCPB-38	269.10
13	GPHCPB-13	146.16	39	GPHCPB-39	289.14
14	GPHCPB-14	238.26	40	GPHCPB-40	415.43
15	GPHCPB-15	242.40	41	GPHCPB-41	286.27
16	GPHCPB-16	175.22	42	GPHCPB-42	325.94
17	GPHCPB-17	357.31	43	GPHCPB-43	170.55
18	GPHCPB-18	168.24	44	GPHCPB-44	439.76
19	GPHCPB-19	288.18	45	GPHCPB-45	452.80
20	GPHCPB-20	309.45	46	GPHCPB-46	270.59
21	GPHCPB-21	328.69	47	GPHCPB-47	197.57
22	GPHCPB-22	219.55	48	GPHCPB-48	294.07
23	GPHCPB-23	248.97	49	GPHCPB-49	257.33
24	GPHCPB-24	294.84	50	GPHCPB-50	192.33
25	GPHCPB-25	248.21	51	GPHCPB-51	249.25
26	GPHCPB-26	384.88	52	GPHCPB-52	330.70

PCR amplified products of all the primers were subjected to gel electrophoresis using 1.8% agarose gel in 1× TAE buffer at 100 V. The fragment sizes, ranging from 0.3 to 4.0 kb, were detected by comparing the amplicons with a 100-bp DNA ladder and *Eco*RI/*Hind*III double digest marker (Bangalore Genei, Bangalore, India) and ethidium bromide stained gels were documented using Alpha Imager -1200^{TM} (Alpha Innotech, San Leandro, USA). Duplicated independent DNA preparations for each sample were done and only major bands consistently amplified were scored.

Statistical analysis

DNA fingerprints were scored for the presence (1) or absence (0) of bands of various molecular weight sizes in the form of binary matrix. Data were analysed to obtain Jaccard (1908) coefficients among the isolates by using NTSYS-pc version 2.11W (Rohlf 1997). The SIMQUAL program was used to calculate the Jaccard's coefficients. A common estimator of genetic identity and was calculated as follows:

$$\text{Jaccard's coefficient} = N_{AB}/(N_{AB} + N_A + N_B),$$

where N_{AB} is the number of bands shared by samples, N_A represents amplified fragments in sample A, and N_B represents fragments in sample B. Similarity matrices based on these indices were calculated. Correlation between the matrices obtained with three marker types (RAPD, SSR and cytochrome P450 gene based primers) was estimated by means of Mantel matrix correspondence test (Mantel 1967). Product-moment correlation (r) obtained from this test provides one measure of relatedness among the three matrices. In this instance, the matrix correlation corresponds to three independently derived dendrograms. Similarity matrices were utilized to construct the UPGMA (unweighted pair-group method with arithmetic average) dendrograms. In order to estimate the congruence among dendrograms, cophenetic matrices for each marker and index type were computed and compared using Mantel test. Polymorphic information content (PIC) for SSR markers was calculated as per the formula: $\text{PIC} = 1 - \sum_{i=1}^{n}$, where n is the total number of alleles detected for a locus of a marker and f_i the frequency of the ith allele. PIC or average heterozygosity was calculated as per the formula: $\text{PIC} = 2f_i(1 - f_i)$, where f_i is the frequency of the amplified allele and $1 - f_i$ is the frequency of null allele. Principal coordinate analysis was performed in order to highlight the resolving power of the ordination. To determine robustness of the dendrogram, the data were bootstrapped with 2000 replications along with Jaccard's coefficient by the computer programe WINBOOT (Yap and Nelson 1996).

Results

RAPD analysis

Initial screening of 18 RAPD primers on 52 genotypes showed that seven of these were monomorphic. The total number of fragments observed among the finger millet genotypes based on RAPD analysis with 11 polymorphic primers were 90. The number of scorable fragments produced per primer ranged from 3 to 15, and size of the products ranged from 300 bp to 3034 bp. The total number of polymorphic fragments and the percentage of polymorphism was 48 and 49.43 respectively (table 2). A representative RAPD profile obtained by primer RAPD-15 is shown in figure 2. Of a total of nine bands (0.15–3.5 kb), five were polymorphic (55.5%). The 0.4-kb band marked 'A', and the 1.2-kb band marked 'B', were unique to genotypes containing high calcium (300–450 mg/100 g). The similarity coefficients based on RAPD markers ranged from 0.011 to 0.999 with an average value of 0.505. The PIC values, a reflection of allele diversity and frequency among the varieties, were not uniformly higher for all the RAPD loci tested. The PIC value ranged from 0.141 (RAPD-09) to 0.5 (RAPD-030) with a mean of 0.351.

SSR analysis

A total of 70 scorable markers were yielded by five polymorphic primers with an average of 14 bands per primer. Thirty-four markers (50.2%) with an average of 6.8 per primer were polymorphic (table 3). A representative fingerprint pattern generated by primer SSR-06 is shown in (figure3). Out of 10 alleles generated by this primer (size range 0.3–2.3 kb), six were monomorphic. A 1.0-kb allele 'A' is present only in genotypes containing high calcium (300–450 mg/100 g). The PIC value ranged from 0.274 (SSR-10) to 0.758 (SSR-02) with a mean of 0.557. The similarity coefficients based on SSR markers ranged from 0.010 to 0.999 with an average value of 0.504. Jaccard's pair-wise similarity coefficient values ranged from 0.255 to 0.950 with an average value of 0.602.

Cytochrome P450 gene based markers analysis

A total of 111 scorable markers were yielded by the 10 pairs of polymorphic primers with an average of 11.1 bands per primer (table 4). Sixty-seven markers (58.7%) with an average of 6.7 per primer were polymorphic. A representative cytochrome P450 gene based marker profile obtained by primer cyt09(F) and cyt010(R) is shown in figure 4. Of a total of 15 bands (0.15–2.8 kb), 10 are polymorphic (66.6%). The 0.4-kb band marked 'A' is unique to genotypes containing high calcium (300–450 mg/100 g) and genotypes containing medium calcium (200–300 mg/100 g) but absent in genotypes containing low calcium. The PIC value ranged from 0.311 cyt03(F) and cyt04(R) to 0.5 cyt015(F) and cyt016(R) with a mean of 0.406. The similarity coefficients based on cytochrome P450 gene based markers ranged from 0.001 to 0.998 with an average value of 0.499.

Cluster analysis was performed based on Jaccard (1908) similarity coefficient matrices, calculated from RAPD, SSR and cytochrome P450 gene based markers to generate dendrogram of finger millet genotypes. The dendrogram generated from RAPD (figure 5a) and SSR (figure 5b) data separated finger millet genotypes into four clusters. First cluster included genotypes containing high calcium (300–450 mg/100 g). Second cluster included genotypes containing medium calcium (200–300 mg/100 g) GPHCPB-19, -48, -23, -29 had 100 per cent similarity with medium calcium range. Third cluster included genotypes containing medium calcium (200–250 mg/100 g). Fourth cluster included genotypes containing low calcium (100–200 mg/100 g). Several reasons could be attributed to the low intrazonal diversity detected in the present investigation. On the other hand, cluster analysis of cytochrome P450 gene based markers separated the finger millet genotypes into four

Table 2. *Eleusine coracana*: summary of genetic diversity study using RAPD primers.

	Name of primer	Primer sequence (5′ to 3′)	Tm value of primer (°C)	Range of markers (kb)	Polymorphic bands/total no. of bands	% Polymorphism	PIC
1	RAPD-010	CCA CAC TAC C	38.6	2.0–0.40	4/7	57.11	0.269
2	RAPD-012	CGG CCA CTG T	38.6	3.0–0.30	7/11	63.63	0.484
3	RAPD-015	CGG CCC CGG C	38.6	2.7–0.40	5/9	55.55	0.467
4	RAPD-04	CGG AGA GCG A	42.7	1.3–0.35	1/3	33.33	0.449
5	RAPD-09	GAC GGA GCA G	42.7	3.0–0.600	3/6	50.0	0.141
6	RAPD-036	GAA GAA CCG C	38.6	1.5–0.550	5/8	62.5	0.281
7	RAPD-021	GAC GGA TCA G	48.6	1.8–0.300	4/7	57.14	0.267
8	RAPD-011	CGG AGA GCC C	46.8	1.6–0.400	6/11	54.54	0.427
9	RAPD-031	GGG TAA CGC C	42.7	3.0–0.400	4/8	50.0	0.364
10	RAPD-030	GGA CTG GAG T	38.6	2.7–0.350	9/15	60.0	0.500
11	RAPD-14	CGG CCC CGG T	42.7	1.5–0.700	0/5	0	0.209
Total					48/90	543.8	
Average					4.36/8.18	49.43%	0.351

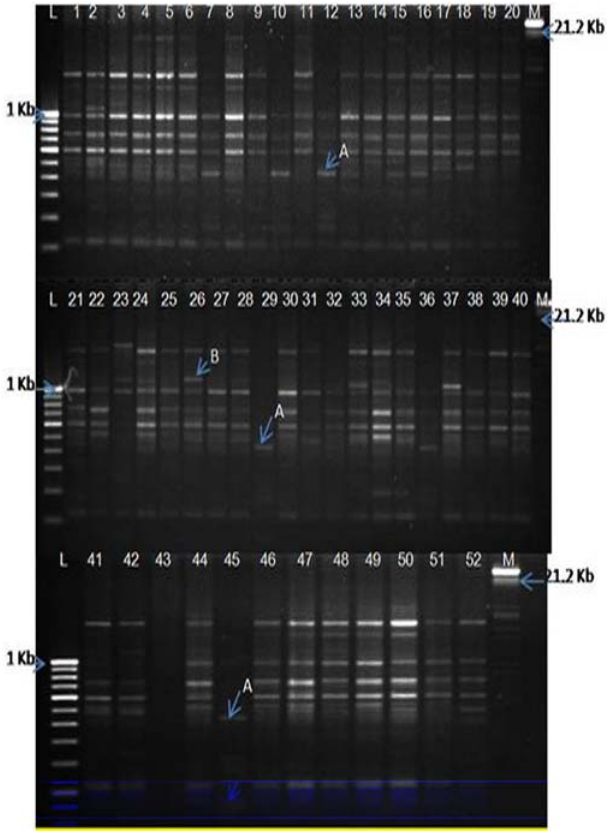

Figure 2. RAPD profile of 52 finger millet genotypes generated by random primer (RAPD-15). The lanes represent, lane L, 100-bp ladder; lanes 1-52 (finger millet genotypes), lane M, marker (*Eco*RI/*Hind*III).

distinct clusters. The first cluster had genotypes containing low calcium (100–200 mg/100 g). Second cluster included genotypes containing high calcium (300–450 mg/100 g). In this subcluster, GPHCPB-17, -35, -40, -52, -20, -45 had 100 per cent similarity. Third cluster included genotypes containing medium calcium (200–300 mg/100 g). Fourth cluster formed different subclusters with genotypes containing medium calcium (200–250 mg/100 g) and low calcium (100–200 mg/100 g) (figure 5c). In *E. coracana* combined analysis of all the three markers (RAPD, SSR and cytochrome P450

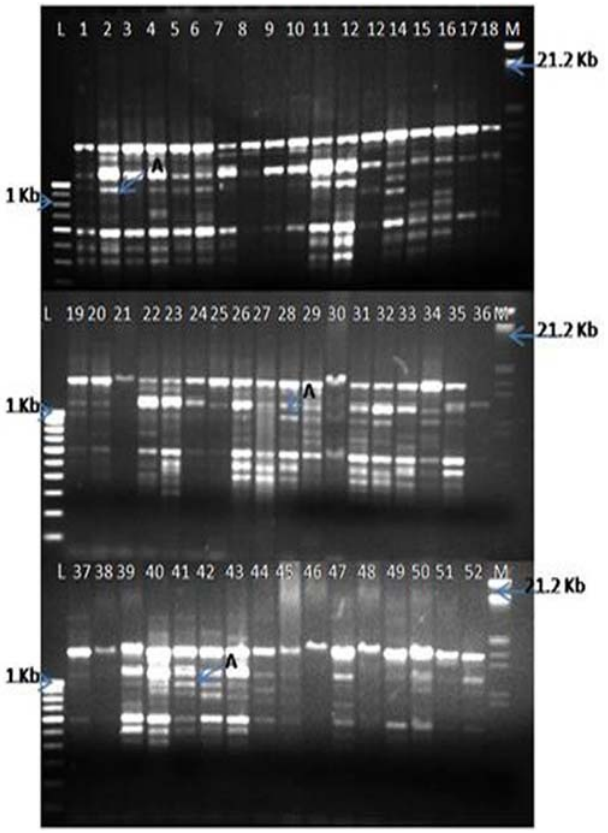

Figure 3. SSR profile of 52 finger millet genotypes generated by primer (SSR-6). The lanes represent, lane L, 100-bp ladder; lanes 1-52 (finger millet genotypes), lane M, marker (*Eco*RI/*Hind*III).

Table 3. *Eleusine coracana*: summary of genetic diversity study using SSR primers.

	Name of primer	Primer sequence (5′ to 3′)	Tm value of primer (°C)	Range of markers (kb)	Polymorphic bands/total no. of bands	% Polymorphism	PIC
1	SSR-01	(F) GCGAAAACACAATGCAAAAA (R) GCGTTGGTTGGACCTGAC	55	4.0–0.7	7/12	58%	0.721
2	SSR-02	(F) TCCTCCCTCCCTTCGCCCACTG (R)CGATGTTCGCCATGGCAGCGACC	60	3.0–0.6	9/15	60%	0.758
3	SSR-06	(F) GCCTCGAGCATCATCATCAG (R) ATCAACCTGCACTTGCCTGG	55	4.2–0.7	6/10	60%	0.523
4	SSR-08	(F) TTCCCTGTTAAGAGAGAAATC (R) GTGTATTTGGTGAAAGCAAC	55	1.2–0.4	6/15	40%	0.511
5	SSR-10	(F) CTTTGTCTATCTCAAGACAC (R) TTGCAGATGTTCTTCCTGATG	55	1.9–0.35	6/18	33%	0.274
Total					34/70	251	
Average					6.8/14	50.2%	0.557

Table 4. *Eleusine coracana*: summary of genetic diversity study using cytochrome P450 gene based primers.

	Name of primer	Primer sequence (5′ to 3′)	Tm	Reference	Range of markers (kb)	Polymorphic bands/total no. of bands	% Polymorphism	PIC
1	CYP2B6(F)	GACTCTTGCTACTCCTGGTT	66.0	Inui *et al*. (2000)	2.0–0.40	9/13	69.23	0.480
	CYP2B6(R)	CGA ATACAG AGCTGATGAGT	66.0					
2	CYP2C19(F)	TCCTTGTGCTCTGTCTCTCA	55.0	Inui *et al*. (2000)	3.0–0.35	3/5	60.0	0.499
	Hem2C19(R)	TCCCACACAAATCCGTTTTCC	55.0					
3	Cyt01(F)	GATGGTCTTCCGCGGTA	62.3	Kumar *et al*. (1997)	3.5–0.10	9/15	60.0	0.355
	Cyt02(R)	CACTGGAAGGCGTGCA	63.6					
4	Cyt03(F)	CGG CTTGCTCATGGA	60.0	Kumar *et al*. (1997)	3.5–0.40	10/16	62.5	0.311
	Cyt04(R)	GAGAAATAGGTGGGTGGA	59.0					
5	Cyt05(F)	GACCCA AGC AACGTCA	59.0	Kumar *et al*. (1997)	3.0–0.40	3/7	42.85	0.499
	Cyt06(R)	GTGGGTTATGGCCCACA	59.0					
6	Cyt07(F)	GACGTGCCACTCTGCA	60.0	Kumar *et al*. (1997)	3.5–0.15	3/8	37.50	0.493
	Cyt08(R)	ACCCTAGGCTAAGGTGGA	60.0					
7	Cyt09(F)	CCACCT TGACGACCCAA	63.0	Kumar *et al*. (1997)	2.0–0.15	10/15	66.66	0.470
	Cyt010(R)	TGGCCCACATATTCACCA	63.0					
8	Cyt011(F)	ACGTGCCACTCTGCAA	60.0	Kumar *et al*. (1997)	3.0–0.35	7/10	70.0	0.368
	Cyt012(R)	ACCCTAGGCTAAGGTGGA	59.6					
9	Cyt013(F)	GGGCCATAACCCACGA	64.0	Kumar *et al*. (1997)	3.5–0.10	7/12	58.33	0.491
	Cyt014(R)	ATTGGAGCGCCGGTGA	64.0					
10	Cyt015(F)	CCTGTACGACCCAAGCA	61.0	Kumar *et al*. (1997)	2.5–0.30	6/10	60.0	0.500
	Cyt016(R)	TGG CCCACA TATTCACCA	61.0					
Total	10 primers					67/111	587	
Average						6.7/11.1	58.7%	0.406

gene based markers) grouped the finger millet genotypes into three distinct clusters. The first cluster had genotypes containing low calcium (100–200 mg/100 g). Second cluster included genotypes containing high calcium (300–450 mg/100 g). Third cluster included genotypes containing medium calcium (200–300 mg/100 g).

All the phenograms confirm the consistency of data. The three molecular marker systems were compared on the basis of different criteria (table 5). In case of polymorphism detection, cytochrome P450 gene based primers as a tool scored higher than SSR and RAPD. The coefficient of correlation between RAPD and SSR, RAPD and cytochrome P450 gene based primers and SSR and cytochrome P450 gene based primers was 0.719, 0.575 and 0.628, respectively.

Mantel test (Mantel 1967) was employed to determine the coefficient of correlation between the similarity matrices generated by these markers. Mantel test was also employed to analyse the 'goodness of fit' for the UPGMA dendrograms generated by each marker system (RAPD, SSR and cytochrome P450 gene based markers). This was done by making the cophenetic similarity matrices from each UPGMA phenogram and then comparing these cophenetic similarity matrices with the original similarity matrices (generated from binary data) for each marker technique. It revealed values higher than 0.95 for all the markers used (RAPD ($r = 0.999, P = 0.01$), SSR ($r = 0.999, P = 0.01$), cytochrome P450 gene based markers ($r = 0.998, P = 0.01$)), thus confirming their authenticity. Bootstrap analysis of this data was performed to determine the confidence values as percentage at each node. Very high bootstrap values were obtained from the major nodes.

Discussion

The finger millet genotypes constitute a rich source of biodiversity and their conservation and utilization requires that their genetic structure is well characterized and understood. DNA fingerprinting is a routine method employed to study the extent of genetic diversity across a set of germplasm or cultivars and group them into specific categories. DNA markers have been used to evaluate genetic diversity in different crop species (Cooke 1995). Therefore, random amplified polymorphic DNA (RAPD) is increasingly being employed in genetic research owing to its speedy process and simplicity (Williams *et al*. 1990). Development of co-dominant markers, such as SSRs, which are currently available in finger millet will greatly aid in diversity and genome analysis efforts. Comparative studies in finger millet species involving RAPD, RFLP, isozymes and SSR marker systems were successfully used by very limited researchers (Dida *et al*. 2007, 2008; Hilu 1995; Salimath *et al*. 1995), although it has been carried out in many other crops. The discriminatory power of DNA markers used as tool to characterize the finger millet genotypes is very important because they can be used to assess the genetic diversity among the finger millet genotypes.

Bulk analyses are useful to obtain information on genetic variability among different populations (Loarce *et al*.

1996). During the characterization of finger millet genotypes, from Uttarakhand, we evaluated the discriminative power of primers for RAPD, SSR and cytochrome P450 gene based marker systems.

Among the three marker systems employed, 18 RAPD primer pairs produced a total of 90 markers; 10 SSR primers produced only 70 markers whereas 10 pairs of cytochrome P450 gene based marker produced 111 markers. The level of polymorphism revealed by RAPD (49.43%) is lower than SSR (50.2%) and cytochrome P450 gene based marker (58.75%). (Salimath *et al*. 1995) detected 10% and 26% of polymorphism respectively in 17 accessions of *E. coracana* from Africa and Asia with RAPDs. On the other hand, Babu *et al*. (2007) detected 91% of polymorphism in 32 accessions of *E. coracana*.

In the present investigation, the average number of fragments amplified by RAPD primers among the genotypes were 8.18 with a range of 1 to 15. Fakrudin *et al*. (2007) reported an average of six markers per primer in *E. coracana* and similar results were reported by Salimath *et al*. (1995) and Babu *et al*. (2007). Such a high variation in the number of fragments produced by these arbitrary primers may be attributed to the differences in the binding sites throughout genome of the genotypes included. SSR primers generated 3 to 18 markers with an average of 14.0 per genotype and generation of EST-SSR markers were also reported in finger millet (Dida *et al*. 2008). The distribution of different microsatellite sequences in different plant genomes determines the possibility of using this method for DNA fingerprinting. Cytochrome

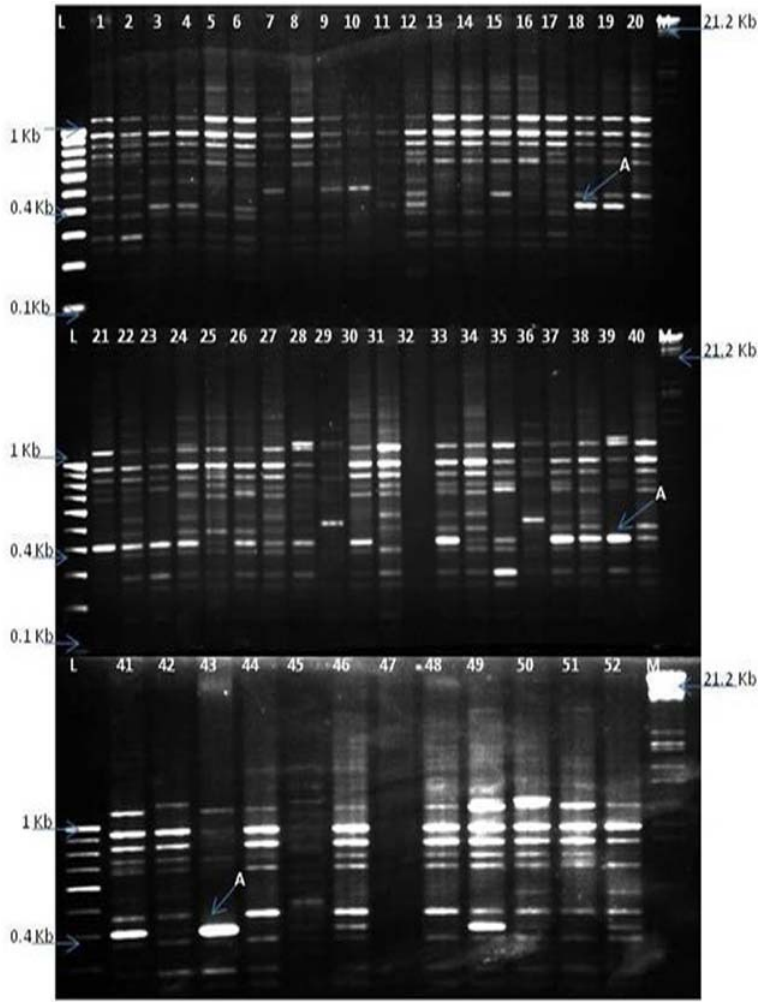

Figure 4. Cytochrome P450 gene based primer profile of 52 finger millet genotypes generated by primer combination cyt09(F) and cyt010(R). The lanes represent lane L, 100-bp ladder; lanes 1-52 (finger millet genotypes), lane M, marker (*Eco*RI/*Hind*III).

P450 gene based primers generated 2 to 13 markers with an average of 10.3 per genotype. Earlier, generation of seven markers in *Cymbopogon* species by cytochrome P450 gene based primers were reported (Kumar *et al*. 2007).

Comparison of PIC values for three marker systems (a parameter associated with the discriminating power of markers) indicated that the range of PIC values for RAPD primers was from 0.0141 (RAPD-09) to 0.5 (RAPD-030). Among the 10 SSR markers surveyed across the finger millet genotypes, only five markers showed better resolving power. The PIC value ranged from 0.274 (SSR-10) to 0.758 (SSR-02). The comparison of the average PIC values of cytochrome P450 gene based primers revealed that the lowest was with 0.311 cyt03(F) and cyt04(R) and the highest was with 0.5 cyt015 (F) and cyt016(R).

A close correspondence between the similarity matrices of RAPD, SSR and cytochrome P450 gene based markers was established by means of high value of matrix correlation value of 0.999, 0.999 and 0.998, respectively. Hence, the three marker systems RAPD, SSR and cytochrome P450 gene-based markers either individually or combined can be effectively used in determination of genetic relationships among finger millet genotypes. However, the results concluded that SSR and cytochrome P450 gene based markers would be a better tool than RAPD for phylogenetic studies. The marker index values of cytochrome P450 gene based primers also added strength to the above results.

Cluster analysis was carried out on three sets of marker profiling data based on i) RAPD; ii) SSR;

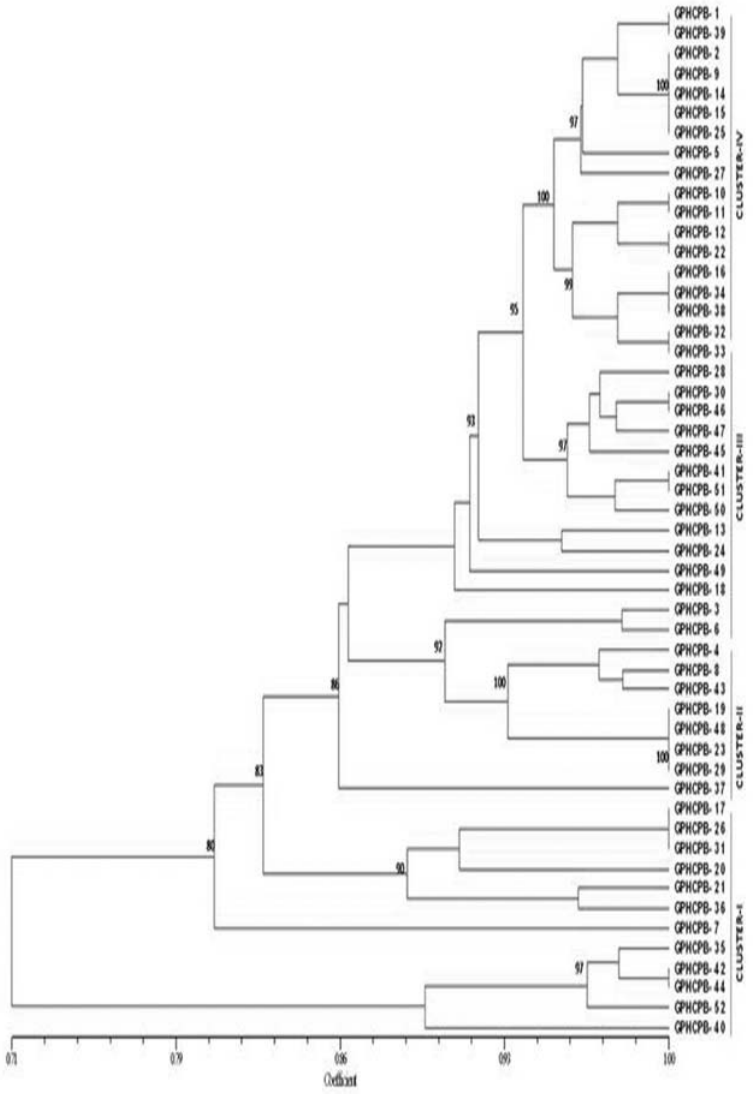

Figure 5a. UPGMA cluster analysis showing the relationship and diversity among 52 finger millet genotypes produced by random primers.

Table 5. Comparison of various molecular markers in evaluating genetic diversity of finger millet.

Molecular marker	RAPD	SSR	Cytochrome P450 gene based primers
No. of genotypes	52	52	52
Total no. of bands	90	70	111
Polymorphic bands	48	34	67
Total no. assays/primer combinations	11	5	10
Percentage polymorphic (%)	49.43%	50.2%	58.7%
Multiplex ratio (n/T)	8.18	14.0	11.1
Average heterozygosity (H_{av})	0.351	0.505	0.406
Marker index (MI) = $H_{av} \times$ MR	2.87	7.07	4.50

MR, multiple ratio (MR was estimated by dividing the total number of bands (m (monomorphic) and p (polymorphic)) amplified by total number of assays (primer combinations employed (n)).

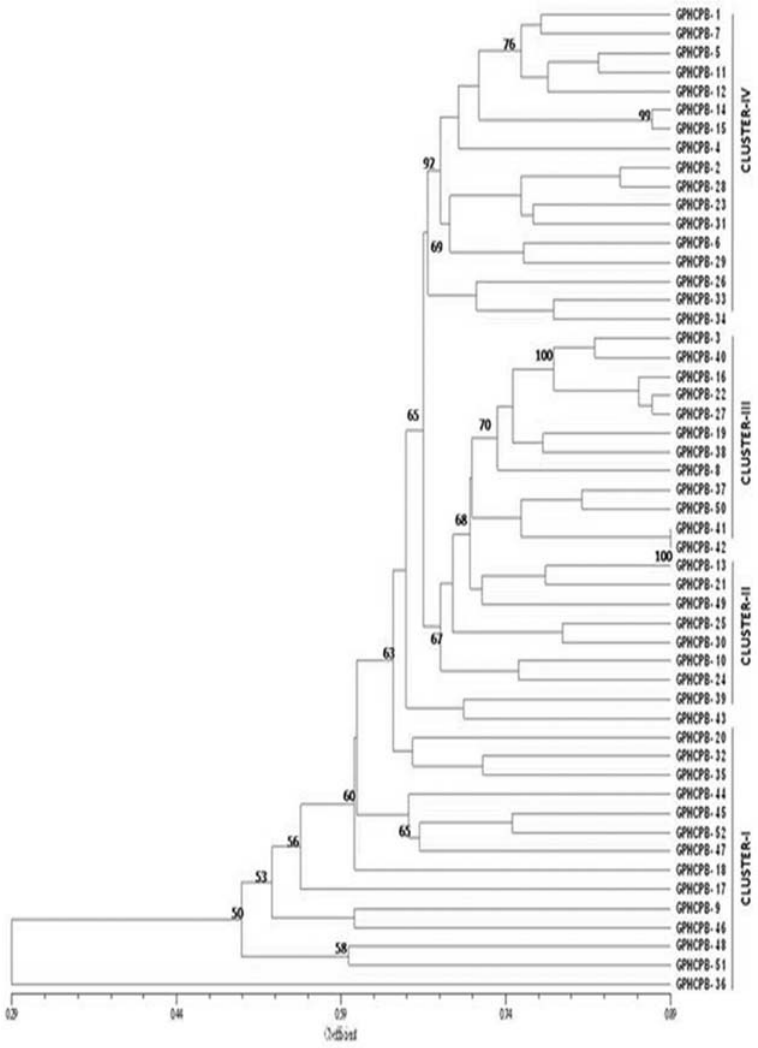

Figure 5b. UPGMA cluster analysis showing the relationship and diversity among 52 finger millet genotypes produced by SSR primers.

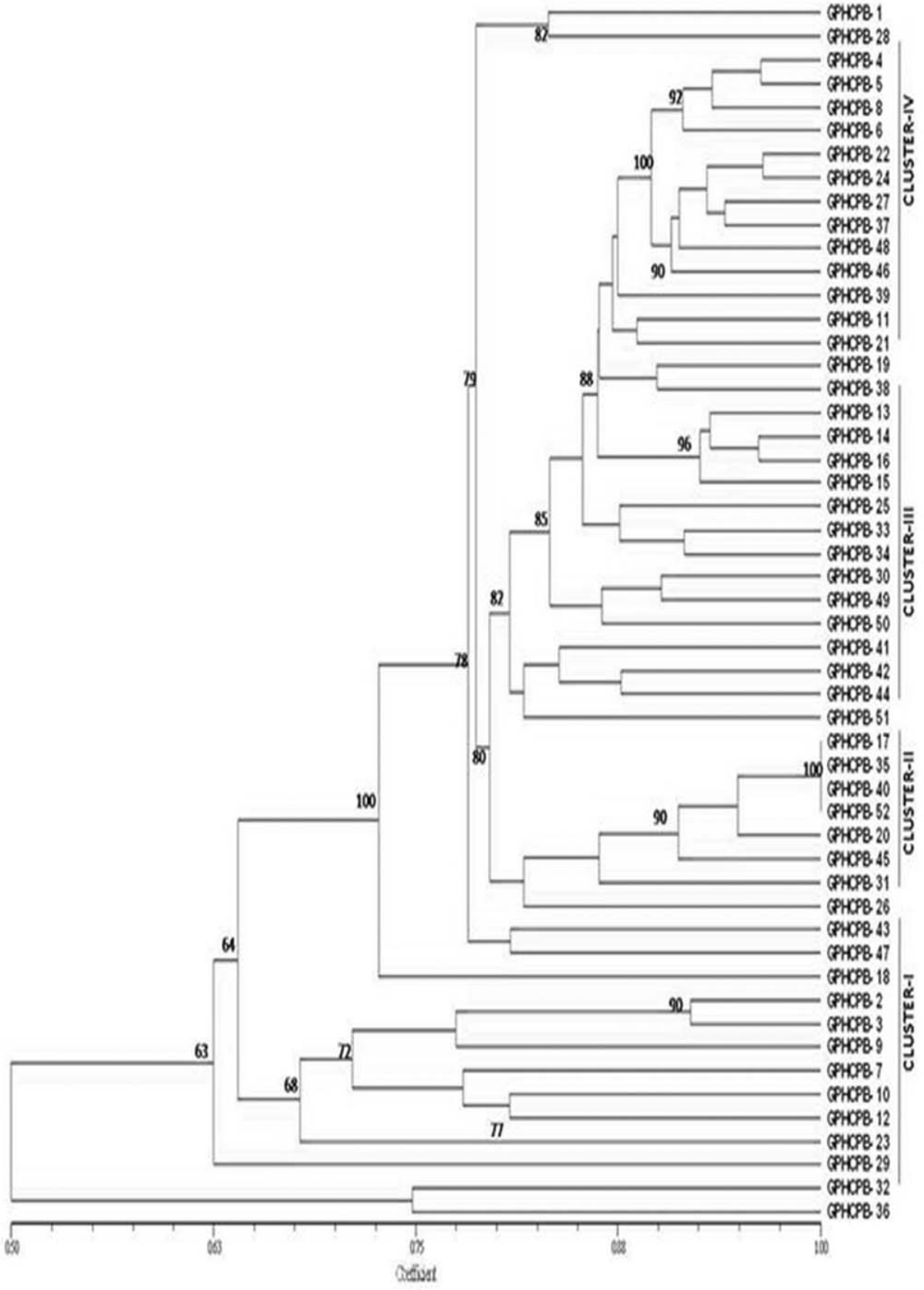

Figure 5c. UPGMA cluster analysis showing the relationship and diversity among 52 finger millet genotypes produced by cytochrome P450 gene based primers.

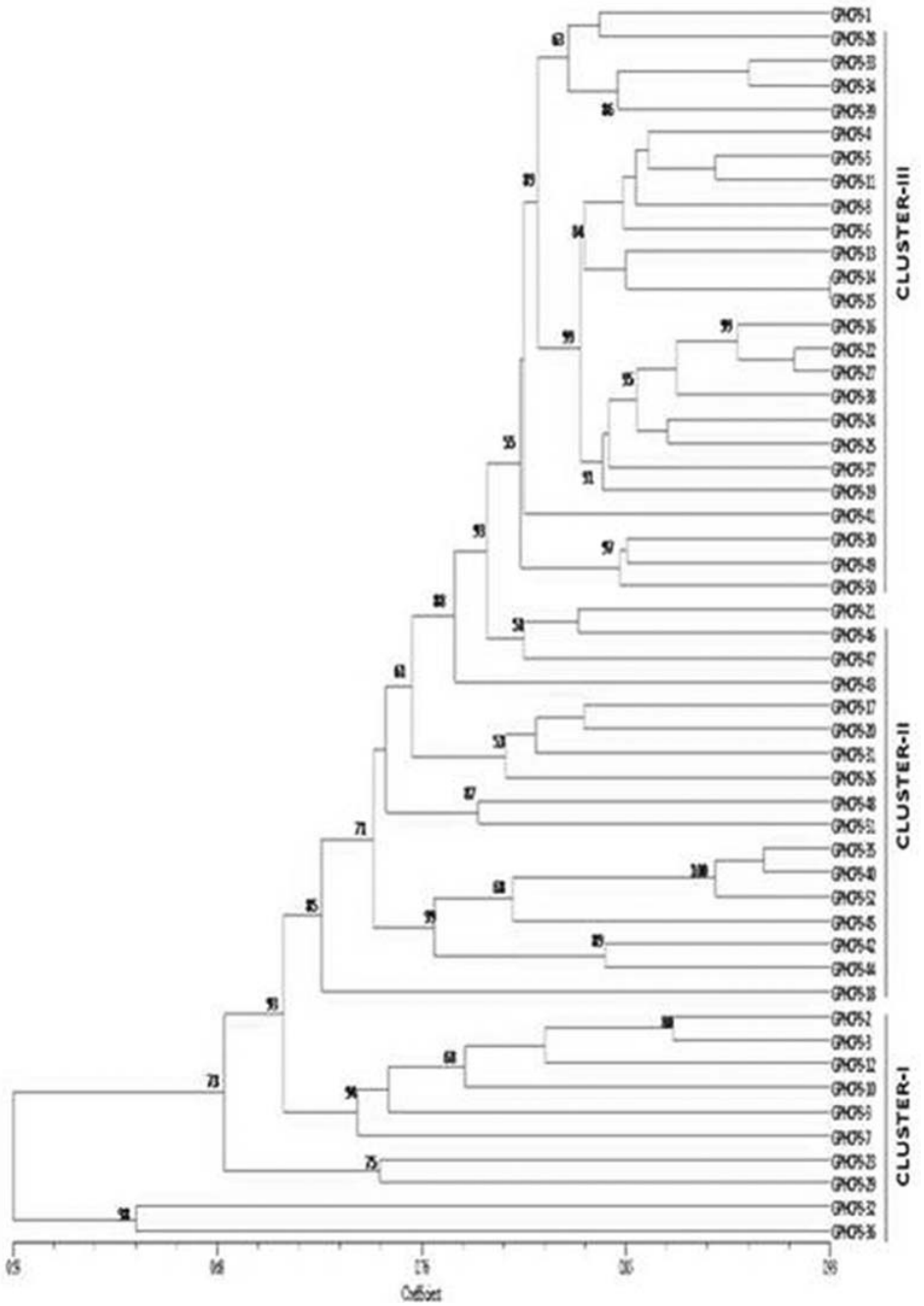

Figure 5d. UPGMA cluster analysis showing relationships among *Eleusine coracana* genotypes as revealed by data from combination of RAPD, SSR and cytochrome P450 gene based marker.

iii) cytochrome P450 gene-based primers; and iv) combination of RAPD, SSR and cytochrome P450 gene based primers. The results based on the three DNA marker profiles and combined analysis broadly grouped the 52 genotypes into distinct clusters showing relation on the basis of calcium content. The genotypes containing high calcium, medium calcium and low calcium grouped in different clusters. Based on the seed colours as phenotypic descriptor, attempt has been made to analyse 36 genotypes of finger millet and correlate protein and calcium contents with grain colour (Vadivoo *et al*. 1998).

In the present investigation, various specific bands were amplified, like 0.4-kb and 1.2-kb band amplified by primer RAPD-15, a 1.0-kb SSR band amplified by primer SSR-06 and 0.4-kb band amplified by primer cyt9(F) and cyt10(R) which are present only in genotypes containing high calcium

but absent in genotypes containing low calcium content. Phenograms generated from RAPD, SSR and cyt P450 gene based primers data showed similarity in relative placement of genotypes. The cophenetic correlation values of Mantel test are above 0.95 for each marker system. This validates the phenograms obtained after UPGMA analysis of binary matrices data. The identified markers (RAPD-$15_{0.4}$ and $_{1.2}$, SSR-$06_{1.0}$,$SSR10_{0.4}$ and cyt9(F) and cyt$10(R)_{0.4}$ which are present only in genotypes containing high calcium but absent in genotypes containing low calcium content) and associated with calcium content with highly positive correlation ($P < 0.001$). These findings can be used in authentication of the finger millet and perhaps in recognizing high calcium content genotypes in a particular area.

Inclusion of genotypes for specific objectives like yield and quality parameters over different geographical location resulting in narrowing of genetic base and the marker system used could be the reason for clustering most of the genotypes in one cluster. The relationships between the genotypes are not necessarily reflecting the agronomic traits. Molecular markers are scattered throughout the genome and their association with various agronomic traits is influenced by the cultivator under selection pressure induced by domestication. Exploration and evaluation of diversity among these genotypes would be of great significance for *in situ* conservation and finger millet breeding programmes.

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References

Agrama H. A. and Tuinstra M. R. 2003 Phylogenetic diversity and relationships among sorghum accessions using SSRs and RAPDs. *Afr. J. Biotech*. **2**, 334–340.

Babu B., Senthil N., Gomez S., Biji K., Rajendraprasad N., Kumar S. and Babu R. 2007 Assessment of genetic diversity among finger millet *Eleusine coracana* L Gaertn accessions using molecular markers. *Genet. Resour. Crop Evol*. **54**, 399–404.

Barbeau W. E. and Hilu K. W. 1993 Protein, calcium, iron and amino acid content of selected wild and domesticated cultivars of finger millet. *Plant Foods Hum. Nutr*. **43**, 97–104.

Becker J. and Heun M. 1994 Barley microsatellites: allele variation and mapping. *Plant Mol. Biol*. **274**, 835–845.

Chao S., Zhang W., Dubcovsky J. and Sorrells M. 2007 Evaluation of genetic diversity and genome wide linkage disequilibrium among US wheat *Triticum aestivum* (L.) germplasm representing different market classes. *Crop Sci*. **47**, 1018–1030.

Colombo C., Second G., Valle T. L. and Charrier A. 1998 Genetic diversity characterization of cassava *Manihot esculenta* Crantz cultivars. I. RAPD markers. *Genet. Mol. Biol*. **21**, 69–84.

Cooke R. J. 1995 Variety identification of crop plants. In *New diagnostics in crop sciences* (ed. J. H. Skerrit and R. Appels), pp. 33–63. CAB International, Wallingford, UK.

Davierwala A. A. P., Chowdari K. V., Shiv K., Reddy A. P. K., Ranjekar P. K. and Gupta V. S. 2000 Use of three different marker systems to estimate genetic diversity of Indian elite rice varieties. *Genetica* **108**, 269–284.

Dida M. M., Srinivasachary), Ramakrishnan S., Bennetzen J. L., Gale M. D. and Devos K. M. 2007 The genetic map of finger millet, *Eleusine coracana. Theor. Appl. Genet*. **114**, 321–332.

Dida M. M., Wanyera N., Dunn M. L. H., Bennetzen J. L. and Devos K. M. 2008 Population structure and diversity in finger millet *Eleusine coracana* germplasm. *Trop. Plant Biol*. **1**, 131–141.

Fakrudin B., Kulkarni R. S., Shashidhar H. E. and Hittalmani S. 2007 Genetic diversity assessment of finger millet, *Eleusine coracana*, germplasm through RAPD analysis. *PGR Newsletter* **138**, 52–54.

Garcia A. A. F., Benchimol L. L., Barbosa A. M. M., Geraldi I. O., Souza C. L. J. and Souza A. P. 2004 Comparison of RAPD, RFLP, AFLP and SSR markers for diversity studies in tropical maize inbred lines. *Genet. Mol. Biol*. **27**, 579–588.

Hilu K. W. 1995 Evolution of finger millet: evidence from random amplified polymorphic DNA. *Genome* **38**, 232–238.

Inui H., Komada T., Ohkawa Y. and Ohkawa H. 2000 Herbicide metabolism and cross-tolerance in transgenic potato plants co-expressing human CYP1A1, CYP2B6 and CYP2C19. *Pest. Biochem. Physiol*. **66**, 116–129.

Jaccard P. 1908 Nouvelles recherches sur la distribuition florale. *Bull. Soc. Vaudoise Sci. Nat*. **44**, 223–270.

Jacobson A. and Hedrén M. 2007 Phylogenetic relationships in *Alisma* (Alismataceae) based on RAPDs, and sequence data from ITS and trnL. *Plant Syst. Evol*. **265**, 27–44.

Kumar J., Verma V., Qazi G. N. and Gupta P. K. 2007 Genetic diversity in *Cymbopogon* species using PCR-based functional markers. *J. Plant Biochem. Biotech*. **16**, 119–122.

Loarce Y., Gallego R. and Ferrer E. A. 1996 Comparative analysis of genetic relationships between rye cultivars using RFLP and RAPD markers. *Euphytica* **88**, 107–115.

Maniatis T., Sambrook J. and Fritsch E. F. 1989 *Molecular cloning: a laboratory manual*, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.

Mantel N. 1967 The detection of disease clustering and generalized regression approach. *Cancer Res*. **27**, 209–220.

Murray M. G. and Thompson W. F. 1980 Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res*. **8**, 4321–4326.

O'Neill R., Snowdon R. J. and Kohler W. 2003 Population genetics aspects of biodiversity. *Prog. Bot*. **64**, 115–137.

Parani M., Rajesh K., Lakshmi M., Parducci L., Szmidt A. E. and Parida A. 2001 Species identification in seven small millet species using polymerase chain reaction -restriction fragment length polymorphism of trnS-psbC gene region. *Genome* **44**, 495–499.

Rohlf F. J. 1997 NTSYS-pc numerical taxonomy and multivariate analysis system, version 20, Exeter Publications, New York, USA.

Russell R. J., Fuller J. D., Macaulay M., Hats B. G., Jahoor A., Powell W. and Waugh R. 1997 Direct comparison of levels of genetic variation among barley accessions detected by RFLPs, AFLPs, SSRs and RAPDs. *Theor. Appl. Genet*. **95**, 714–722.

Salimath S. S., Olivera A. C. D., Godwin I. D. and Bennetzen J. L. 1995 Assesment of genome origins and diversity in the genus *Eleusine* with DNA markers. *Genome* **38**, 757–763.

Shalk M., Nedelkina S., Schoch G., Batard Y. and Werck-Reichhart D. 1999 Role of unusual amino-acid residues in the proximal and distal heme regions of a plant P450, CYP73A1. *Biochemistry* **38**, 6093–6103.

Soller M. and Beckmann J. S. 1983 Genetic polymorphism in varietal identification and genetic improvement. *Theor. Appl. Genet*. **67**, 25–33.

Somerville C. and Somerville S. 1999 Plant functional genomics. *Science* **85**, 380–383.

Tanksley S. D. and McCouch S. R. 1997 Seed banks and molecular maps: unlocking genetic potential from the wild. *Science* **277**, 1063–1066.

Vadivoo A. S., Joseph R. and Ganesan N. M. 1998 Genetic variability and diversity for protein and calcium contents in finger millet *Eleusine coracana* (L. Gaertn.) in relation to grain color. *Plant Foods for Human Nutrition*. **52**, 353–364.

Venkannababu B., Ramana T. and Radhakrisnan T. M. 1987 Chemical composition and protein content in hybrid varieties of finger millet. *Ind. J. Agric. Sci*. **57**, 520–522.

Vos P. R., Hogers R., Bleeker M., Reijans M., Lee T., Hornes M. *et al*. 1995 AFLP: a new technique for fingerprinting. *Nucleic Acids Res*. **21**, 4407–4414.

Watanabe K. N. and Iwanaga M. 1999 Plant genetic resources and its global contribution. *Plant Biotech*. **16**, 7–13.

Williams J. G. K., Kubelik A. R., Livak K. J., Rafalski A. J. A. and Tingey S. V. 1990 DNA polymorphism amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res*. **22**, 6531–6535.

Yap I. V. and Nelson R. J. 1996 *Winboot: a program for performing bootstrap analysis of binary data to determine the confidence of UPGMA-based dendrograms*. IRRI, Manilla, Phillipines.

Zietkiewicz E., Rafalski A. and Labuda D. 1994 Genome fingerprinting by simple sequence repeat SSR-anchored polymerase chain reaction amplification. *Genomics* **202**, 176–183.

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