African Journal of Biotechnology Vol. 10(75), pp. 17081-17087, 28 November, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1035
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Genetic diversity in green gram accessions as revealed by STMS markers

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Accepted 17 June, 2011

Molecular characterization of green gram (*Vigna radiata* (L.) Wilczek) germplasm is essential for scientific assessment of variability and diversity for its better utilization in breeding programs. In this study, 120 accessions of green gram were analysed for 27 Sequence Tagged Microsatellites (STMS) loci. For data analysis, accessions were grouped into Indian and exotic. DNA profiling of the 120 accessions yielded 42 STMS products. The proportion of polymorphic products was 55.56% in Indian accessions and 51.85% in exotic accessions. Average Jaccard's similarity coefficient among all pairs of comparisons was 0.695. In unweighted pair group method of arithmetic averages (UPGMA) dendrogram, 5 major clusters were identified. High similarities among the Indian and exotic accessions were found and this could be due to free movement of Indian germplasm to other countries through international institutes. Analysis of molecular variance (AMOVA) analysis indicated that sampling should be practiced more extensively within populations to capture maximum variation. The study highlighted the need to make more elaborate collections from India to capture the diversity existing in green gram and to go for genetic enhancement for widening of the genetic base of cultivated green gram using wild relatives.

Key words: Genetic diversity, green gram, *Vigna radiata,* STMS.

INTRODUCTION

Among *Vigna* group, in economic terms, green gram is the most important pulse crop. It has strategic position in Southeast Asian countries for nutritional security and as a sustainable crop. Improved green gram cultivars have a narrow genetic base that limits yield potential and they are poorly adapted to varying growth conditions in different agro-ecological conditions. The genetic potential of landrace germplasm accessions in gene banks therefore needs to be better exploited (Bisht et al., 2004).

Molecular markers employed for the analysis of genetic diversity in green gram include random amplified polymorphic DNA (Santalla et al., 1998; Lakhanpaul et al., 2000; Karuppanapandian et al., 2006); random amplified hybridizing microsatellites (Prasad et al., 1999); amplified fragment length polymorphism (Bhat et al., 2005) and inter simple sequence repeats (Chattopadhyay et al., 2005). However, microsatellite markers are rapidly becoming a preferred type of DNA marker used for germplasm analysis because they are locus specific, widely dispersed throughout the genome, highly polymorphic due to variation in the repeat units and highly informative because of co-dominant nature (Weber, 1994).

Considering the importance of genetic diversity in green gram, this study was undertaken with a view to study the structure of genetic diversity contained in green gram accessions using microsatellite loci.

MATERIALS AND METHODS

Sample preparation

One hundred and twenty (120) accessions of green gram were used for the analysis (Table 1). Young actively growing leaves of 30-day-old plants were collected and used for DNA extraction.

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Table 1. Accession numbers of designated green gram accessions and their source.

Group	Source	Accession number
Indian	Andhra Pradesh	IC-247897
	Bihar	IC-12434, IC 73430, IC 73465, PLM 3, PLM 16, PLM 32-A, PLM 37, PLM 37-A, PLM 88-A, PLM 100, PLM 119 PLM 224, PLM 243, PLM 256
	Gujarat	IC 10457, IC 10489-2, IC 10492, IC 10497, IC 11379, 1C 11379-A , IC 11303-3, IC 11312, IC 11444,IC 13077-2, IC 39468, IC 39468-A, IC 39480, PLM 651, PLM 695, PLM 688, PLM 694-A, PLM 707, PLM 726, PLM 748-A, PLM 924, PLM 952, PLM 953, PLM 962, PLM 975
	Haryana	PLM 427, PLM 487
	Himachal Pradesh	IC 11488, PLM 346
	India Miscellaneous	ET 52186 (ML 267), ET 52189 (PUSA 9072), ET 52190 (BASANTI)
	J & K	PLM 373, PLM 380
	Madhya Pradesh	IC 22427, IC 22463, IC 22477, PLM 111, PLM 111-A, PLM 303-2, PLM 538
	Maharashtra	AKM 9243, IC 99418, PLM 93, PLM 191, PLM 236, PUSA 103, STV 2635, STV 2643, STV 2665, STV 2669, STV 2685, STV 2761, STV 2762, STV 2763, STV 2766, STV 2768, TAP 7
	Punjab	IC 114, IC-73536, PLM 391-A, PLM 410, PLM 416, PLM 468-A, PLM 573
	Rajasthan	IC 8961-5, IC 8961-5-A, IC 39342, IC 39345, PLM 777-A , PLM 782, PLM 782-A, PLM 806, PLM 818-A, PLM 841, PLM 884-A
	Uttar Pradesh	PLM 250, PLM 334-1, PLM 334-2, PLM 1037, PLM 1060
Exotic	Bangladesh	ET 52185 (BARI MUNG)
	Europe	EC-5478, EC 8837-2, EC-13077-2, EC 25539, EC-25971-4-1, EC-25997-1
	Pacific Islands	EC 260608, EC 261790, EC-27185
	Pakistan	EC 27245, EC 27514, ET 52191 (VC6153B-20G), ET 52192 (CN 9-5), ET 52193 (VC6153B-20P), ET 52194 (VC6173B -6), ET 52195 (VC6372-45-8-1), ET 52196 (NM 54)
	Taiwan	EC 251557-A, EC-251557-A-1, EC 251557-B, EC 314286
	USA	EC-2513-3, EC-10732-A-3

Leaves were harvested and immediately stored at -80°C. Total genomic DNA was extracted using a protocol modified from Saghai-Maroof et al. (1984). The DNA concentration was estimated with a Hoefer DYNA Quant 200 Fluorimeter (Hoefer Scientific San Francisco, USA) using Hoechst 33258 (Bisbenzimide) as the fluorescent dye and calf thymus DNA as the standard (Brunk et al., 1979).

Selection of primers

Twenty seven (27) primer pairs were chosen for STMS analysis (Table 2). The STMS primer sequence as detailed in Li et al. (2001), Wang et al. (2004) and Gwag et al. (2006) was followed, for analysis. Primer pairs were chosen on the basis of amplification and reproducibility.

PCR and gel electrophoresis

PCR reactions were carried out in a Perkin Elmer Thermocycler GenAmp 9600. Each 25 µl reaction mixture contained 1 "x" reaction buffer, 2.5 mM $MgCl_2$, 1 U Taq DNA polymerase; 200 µM each of dATP, dTTP, dCTP and dGTP 0.25 µM of primer and approximately 40 ng of template DNA. The PCR amplification conditions were as follows: initial extended step of denaturation at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at respective annealing temperature for 1 min and primer extension at 72°C for 2 min, and primer elongation at 72°C for 10 min. Reaction products were mixed with 2.5 µl of 6x loading dye and spun briefly in a microfuge before loading. The amplified products were electrophoresed on 6% polyacrylamide gel at 100 V. Gels were stained with ethidium bromide and photographed using a Nighthawk™ gel documentation system (PDI Inc., USA).

Scoring data and analysis

Molecular weights of bands were estimated by using 1 kb ladder and the homology of bands was based on distance

Table 2. Forward and backward primer sequence for polymorphic STMS primers.

S/N	Primer*	Forward primer sequence (5'- 3')	Backward primer sequence (5'- 3')	Repeat motif
1.	AB128093	CCCGATGAACGCTAATGCTG	CGCCAAAGGAAACGCAGAAC	(AG)
2.	VM27	GTCCAAAGCAAATGABGTCAA	TGAATGACAATGAGGGTG C	(AAT) (TC) (AC)
3.	MB7/2	CTTGCTTGCGAGGATGAG	TCCAGTGCAGCAGATTGA	(CT)
4.	MB14	TGGAATTTGGAAGGAAGGA	GATGCAGGTGTTTGGGAG	(AAGA)
5.	MB77	GGAGAGGAAGGAACAGGG	GGCAGAGCATAACATGGC	(GTT) (GA) A (AG)
6.	MB91	GAGGCCAATCCCATAACTTT	AGCACCACATCAGAGATTCC	(AG) (GA)
7.	MB122A	TGGTTGGTTGGTTCACAAGA	CACGGGTTCTGTCTCCAATA	(TGGT)
8.	MB733B	GAGAGCAACGATTGAAAAATG	GTTCGTAGTTACATTGTCCC	(GA)
9.	Gi1150683A	GGTAATGAAATTATCCGTGACG	CCTTCTTTTAGGAAAAGATTGTGCC	(AG)
10.	Gi32454119	CTCTTTTAATATCACCACCACACC	GCCACAGAAAAAGACACAACACG	(AG)
11.	Gi32454132	CTCTTTTAATATCACCACCACACC	ACATTTCTCCTACATTACCACCC	(AG)
12.	VJ31122A	TGGTTGGTTGGTTCACAAGA	CACGGGTTCTGTCTCCAATA	(TGGT)
13.	DQ094299	TCTCTCTCTCTCTGCATGCACG	CACTTGATTCTCAAGTTCTTCTCC	(AG)
14.	AY297425	CATTATCATTCAATTCCTCCACC	ACACATGGGTTAAAACACCTCC	(AG)
15.	AY301991	TACGATTCAAGACGTCCTCG	AAACATGGGTTAAAACACCTCC	(AG)

*The 12 monomorphic STMS primers used in the analysis were AB128079, AB128113, AB128143, VM14, VM15, VM21, VM22, VM23, VM24, VM32, VM69 and MB319B.

of migration in the gel. STMS amplicon obtained from each entry were resolved as a single band on the non-denaturing PAGE system and the data set were used to calculate pair wise similarity coefficients following Jaccard (1908). The similarity matrices constructed were subjected to cluster analysis by unweighted pair group method of arithmetic average (UPGMA) analysis to generate dendrogram. These computations were performed using NTSYS-PC (ver. 2.02j; Exeter Software, N.Y., Rohlf, 1993). Mantel's correlation test (Mantel, 1967) was performed by calculating correlations between Jaccard's similarity coefficients and cophenetic values for each pair of comparisons. The green gram accessions were grouped into two groups according to source, as belonging to Indian and exotic classes. They were further sub-grouped into 18 categories depending on their source of collection. The exotic accessions were classified as European (including Russia), Pakistan, Bangladesh, USA, Taiwan and Pacific (includes Philippines and Surinam). POPGENE 32 software was used (Yeh et al., 2000) to estimate the genetic diversity parameters. The Ewens–Watterson test of neutrality was also performed, using the earlier mentioned software to detect deviations from a neutral equilibrium model.

The data were subjected to analysis of molecular variation (AMOVA) using Arlequin 3.1 software (Excoffier et al., 2005). Fixation indices and population pairwise F_{ST} values were also computed.

RESULTS

Properties of STMS markers

Out of 27 primer pairs used for the genetic diversity analysis, 15 were found to be polymorphic. The selected primers generated a total of 42 alleles and the overall size of amplified products ranged from 120 (AB128093) to 470 bp (VM15). The number of alleles ranged from one (in 12 primers) to two (in 15 primers), with an average of 1.55 per locus. Maximum number of STMS fragments (42) was observed in accessions from Bihar, while it was the least (31) in accessions from USA. The proportion of polymorphic products varied from 51.85% in exotic accessions to 55.56% in Indian accessions. The number of polymorphic loci was 15 in the case of Indian accessions, whereas in exotic accessions, it was 14.

In Indian accessions, the average number of alleles was 1.56 per locus. The effective number of alleles detected ranged from one to two with the mean value of 1.4 per locus. Shannon's information index was found to be highest for Gi32454119 locus which indicates that this locus was the most informative and useful, for the diversity analysis of Indian accessions. In exotic accessions, the average number of alleles was 1.52 per locus. The effective number of alleles detected varied from one to two (MB122A) with the mean value of 1.3 per locus. Shannon's information index was highest for MB122A, making it the most informative primer for the diversity analysis of exotic accessions. The mean of observed heterozygosity and expected heterozygosity was 0.08 and 0.23, respectively, for Indian accessions, while it was 0.06 and 0.19, respectively, for exotic accessions. The lower than expected level of observed heterozygosity value suggested a predominantly self pollinated nature of green gram.

Gene flow in population

The rate of gene flow is inferred from the amount of genetic differentiation among population. The population

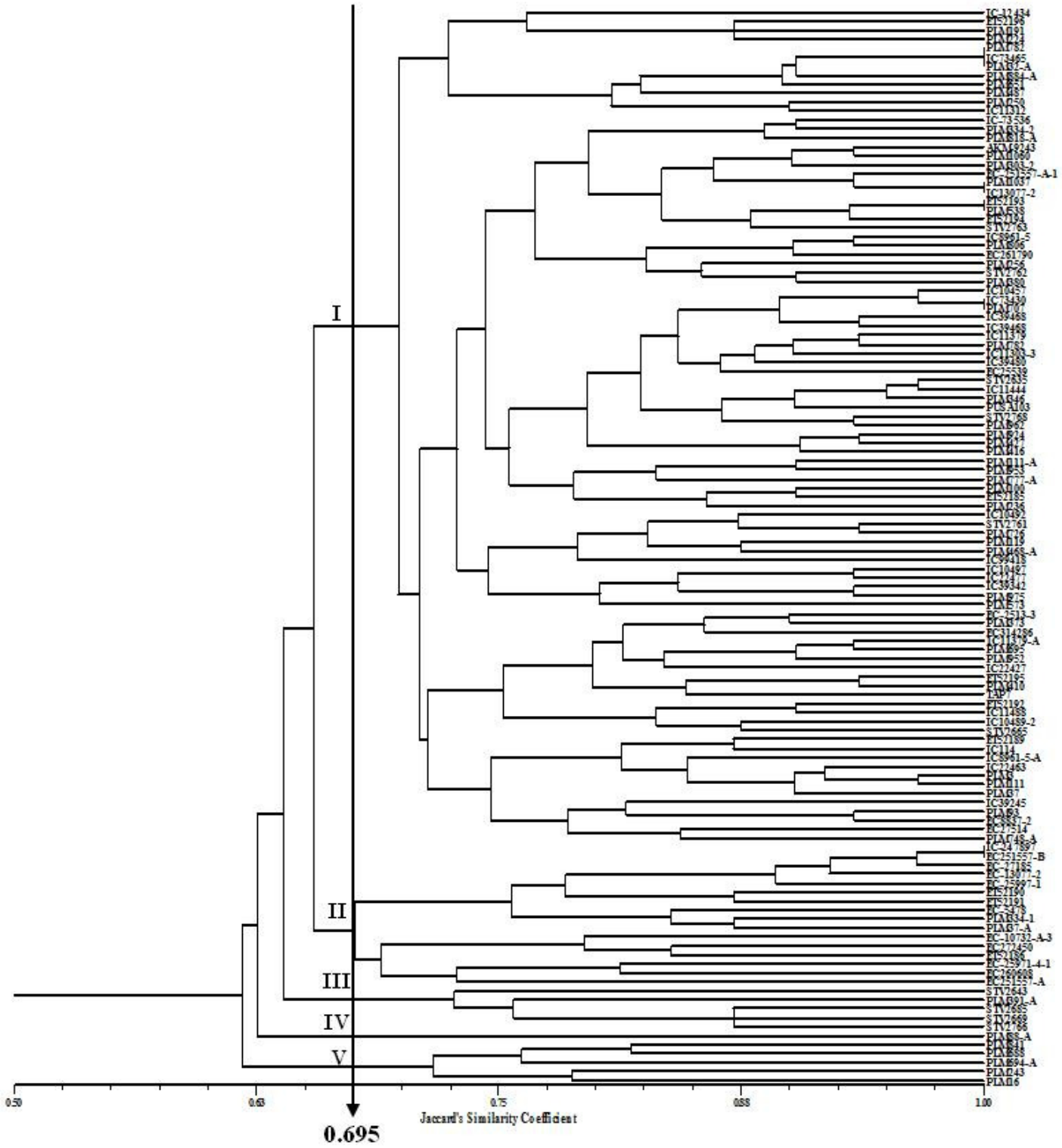

Figure 1 UPGMA dendrogram based on Jaccard's similarity coefficients constructed using STMS data. The cut off value of 0.695, the average Jaccard's similarity coefficients between all pairs of comparison, was used to identify the clusters.

differentiation (F_{ST}) value calculated from F-statistics was used to determine the amount of gene flow (Nm). There is an inverse relationship between F_{ST} and Nm. The mean F_{ST} value for Indian accessions was 0.28 and 0.14

for exotic accessions. The mean Nm value was higher in the exotic accessions (1.49) than that in the Indian accessions (0.63). Nm value was found to be highest for MB122A (14.08) locus in group 1, whereas in group 2, MB72 and Gi32454119 had the highest values (5.537 each).

Cluster analysis

The STMS data was used to calculate pair wise Jaccard's similarity coefficients between the accessions analysed. Average Jaccard's similarity coefficient among all pairs of comparisons was 0.695. Accordingly, in the UPGMA dendrogram based on JAC, 5 major clusters were identified (Figure 1). Cluster III comprised of only the accessions of exotic origin, cluster IV and V included only indigenous accessions while I and II included both exotic and Indian origin. The validity of the grouping observed in the dendrogram was tested by calculating Mantel's correlation between cophenetic values and similarity coefficients. The Mantel's correlation coefficient between Jaccard's similarity coefficient and cophenetic value was 0.62.

Neutrality test for STMS loci

Ewens-Watterson test for neutrality was done using 1000 simulated samples. The loci will be neutral to selection pressure if the observed F-value is in lower and upper 95% limit. Three loci, Gi32454119, MB122A and AY297425, were found to be non-neutral to selection pressure as their observed F (Obs. F) values were below the lower 95% limit.

Analysis of molecular variance (AMOVA)

The percentage of variation was much higher within population than both among groups and among population within groups (Table 3). Variation was least in the case of among population within groups. For comparison of pairs of population samples, pairwise F_{ST} were calculated by using AMOVA. Indian accessions showed high population differentiation as compared to exotic ones, particularly Taiwan and USA samples. However, F_{ST} value between European accessions and accessions from Bihar was lesser.

DISCUSSION

In spite of the large collections of diverse germplasm made over the years, very few have found their way into green gram improvement programs. So, it is imperative to further explore the genetic diversity available in this crop for better utilization of genetic resources in yield improvement. Information on the levels and distribution of genetic diversity of any plant species may contribute to the knowledge of their evolutionary history and potential, and is critical to their conservation and management (Hamrick and Godt, 1996).

In this study, a total of 42 alleles were detected in 120 green gram accessions by using 27 STMS markers. The mean value for effective number of loci in each group was low which indicates overall high genetic similarity among the green gram accessions. High genetic similarity in green gram was earlier reported by Lakhanpaul et al. (2000), Afzal et al. (2004) and Karuppanapandian et al. (2006) in their RAPD analysis of green gram cultivars. Bhat et al. (2005) also reported narrow genetic base in their AFLP analysis of Indian green gram cultivars.

The clustering pattern did not show any relationship between geographic distribution and genotypic diversity as the genotype of different geographic origin were grouped in the same clusters as earlier observed by Manivannan et al. (1998). This was ascribed to the fact that genetic drift and selection in different environment could cause greater diversity than geographic distances. Bisht et al. (1998) also reported no correlation between geographic diversity and genotypic diversity in green gram.

The percentage of variation was much higher in the case of within population than both among groups and among population within groups (Table 3). Variation was least in the case of among population within groups. This indicated that the sampling should be done more extensively within populations which are identified to be more diverse in order to capture the maximum genetic variation. The pair wise F_{ST} calculated by using AMOVA reveals that European accessions were very close to accessions from Bihar State (Figure 2). This suggested that these materials were of Indian origin. The high value of Jaccard's similarity coefficients also supported the earlier conclusions. The high similarity among Indian sub-continent accessions and exotic accessions suggested that majority of exotic accessions have an Indian parentage in their pedigree. This was also supported by the fact that AVDRC, Taiwan, imported large numbers of green gram germplasm from Indian sub-continent and it is possible that these accessions made their way to different countries in the process of exchange.

This study highlights the importance of molecular markers in identifying the collection gap in existing germplasm accessions. It also shows the need to ascertain the molecular basis of the diversity reported, using morpho-agronomic traits in green gram accessions. The analysis also emphasizes the need to go for genetic enhancement to widen the genetic base of cultivated green gram using wild relatives since the evidence suggests the presence of a bottleneck situation in green gram domestication.

Table 3. Analysis of molecular variance (AMOVA).

Source of variation	d. f.	Sum of square	Variance component	Percentage of variation
Among Indian and exotic	1	24.114	0.690^{Va}	11.77
Among population within Indian and exotic	16	99.216	0.190^{Vb}	3.24
Within population	102	508.669	4.986^{Vc}	84.99
Total	119	632.000	5.867	

Fixation indices: F_{SC}, 0.037; F_{ST}, 0.150; F_{CT}: 0.11.

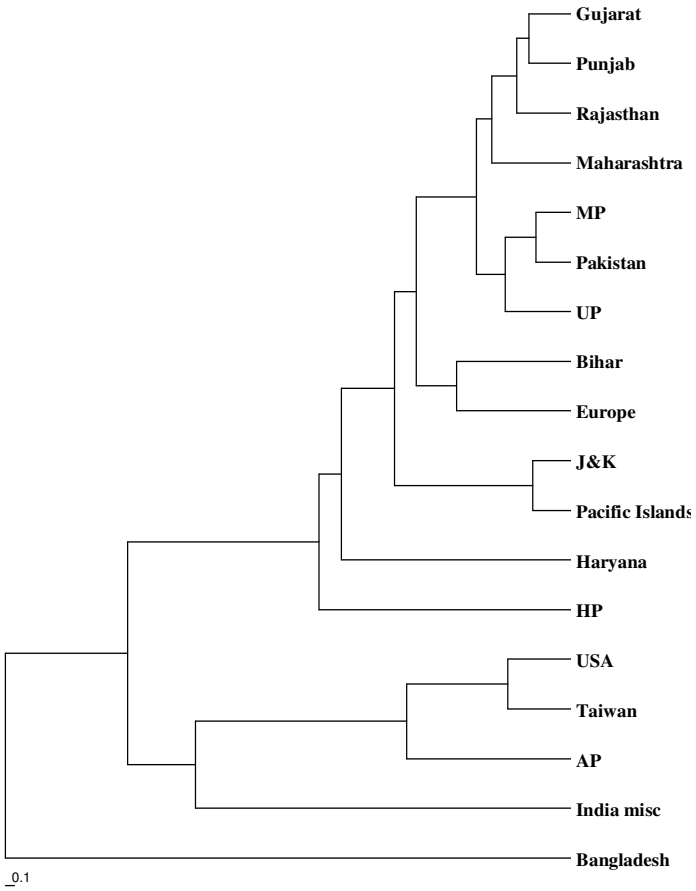

Figure 2. Dendrogram based on pairwise F_{ST} value.

ACKNOWLEDGEMENT

The authors are thankful to Dr. K.V. Bhat, Principal Scientist, National Research Centre on DNA Fingerprinting, NBPGR, New Delhi, India for providing the facilities for the study.

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