Chapter 4
RESULTS AND DISCUSSION

4.1 North Bengal in brief

North Bengal is the general term used to describe the Northern part (from northern bank of river Ganga to end of Darjeeling district) of the Indian State, West Bengal. It l ies in the physiographic zone of Eastern Himalayas and Eastern Plains. It comprises of six Districts viz. Coochbehar, Darjeeling, Jalpaiguri, Malda, North Dinajpur and South Dinajpur covering a total geographic area of 21,855 Km^2 (24.62% of the total geographic area of West Bengal). North Bengal is bordered by Nepal, Bhutan and Sikkim (India) in the North, Bangladesh and Assam (India) in the East and Bihar in the West.

The Darjeeling District constitutes a mountainous topography and is famous for its aggregation of resplendent flora and fauna with a total geographic area of 3149 Km^2 and forest cover of 2289 Km^2. The other districts mainly constitute the terrain dooars plains. Total forest area under North Bengal is 5242 Km^2 (i.e. 40.34% of entire West Bengal). The climate is diverse and varies from subtropical to temperate. Average rainfall is about 5,000 mm (FSI, 2011).

4.2 Collection of plant material

Exploration of different places of North Bengal like Kalimpong, Lataguri, Siliguri, Pundibari, Madarihat, Lava, Sukna, Kalijora, Pankhabari,Gorubathan, Darjeeling and Malda resulted in documentation of thirty five species and varieties of bamboos after proper authentication by the bamboo taxonomist (fig 4.1 and 4.2). The germplasm thus collected were planted in "Bambusetum" at Kurseong Research Range, Sukna,

Darjeeling between 26º47‟26.94‟‟ N Latitude and 88º21‟47.41‟‟ E Longitude with an elevation of 532 feet. List of different species of bamboo encountered in North Bengal is given in table 4.1.

Table 4.1: List of different species and varieties of bamboo found in North Bengal

Sample ID	Name
B1	*Bambusa vulgaris* Schrad „Vittata‟
B2	*B. multiplex* (Lour.) Raeusch. Ex Schult. f „Alphanso-Karr‟
B3	*B. bamboos* (L.) Voss
B4	*B. multiplex* (Lour.) Raeusch. Ex Schult. f „Rivierorum‟
B5	*B. balcooac* Roxb.
B6	*B. vulgaris* Schrad. „Wamin‟
B7	*B. longispiculata* Gamble
B8	*B. atra* Lindl.
B9	*B. oliveriana* Gamble
B10	*B. sinospinosa* McClure
B11	*B. tulda* Roxb.
B12	*B. pallida* Munro
B13	*Cephalostachyum latifolium* Munro
B14	*Dendrocalamus hamiltonii* Munro
B15	*D. sikkimensis* Oliv.
B16	*D. asper* (Schult.) Backer
B17	*D. strictus* (Roxb.) Nees
B18	*Drepanostachyum khasianum* (Munro) Keng f.
B19	*D. intermedium* (Munro) Keng f.
B20	*Gigantochloa* Kurz ex Munro
B21	*Himalayacalamus hookerianus* (Munro) Stapleton
B22	*Melocanna baccifera* (Roxb.) Kurz
B23	*Phyllostachys nigra* (Lodd.) Munro
B24	*Pleioblastus argenteostriatus* (Regel) Nakai
B25	*Pseudosasa japonica* (Steud.) Makino
B26	*Sasaella ramosa* (Makino) Makino
B27	*Shibateae kumasaca* Nakai
B28	*Yushania maling* (Gamble) R.B. Majumdar
B29	Chinese bamboo
B30	*Bambusa nutans* Wallich ex Munro
B31	*Bambusa tuldoides* Munro „Ventricosa‟
B32	*Dendrocalamus giganteus* Munro
B33	*Phyllostachys aurea* Rivière & C.Rivière
B34	*Pleioblastus fortunei* (Van Houtte) Nakai
B35	*Phyllostachys edulis* (Carriere) J.Houz

(Sample ID B1-B29 were used for further downstream processing)

Figure 4.1: Newly emerged bamboo shoots of some species of bamboos encountered in North Bengal during growing season

Figure 4.2: Some of the bamboos encountered in North Bengal during growing season

4.3 Diversity studies of bamboos of North Bengal

4.3.1 DNA extraction, purification and quantification

4.3.1.1 DNA extraction

Bamboo DNA was isolated using the standard protocol of Doyle and Doyle (1987) with minor modifications. The DNA-CTAB complex gave a very good network of whitish precipitate of nucleic acid which was used for further downstream processing. The agarose gel analysis of the DNA thus obtained showed distinct and clear bands.

4.3.1.2 DNA purification

Purification of DNA is very essential since the crude DNA obtained is accompanied by many contaminants which includes the RNA, protein, polysaccharides etc. which severely hampers the downstream process, thus needs to be eliminated. Inclusion of CTAB in the DNA extraction buffer helped in elimination of polysaccharides from DNA precipitations to a great extent. Extraction with phenol:chloroform:isoamyl alcohol eliminated proteins from the sample. The RNAase enzyme was found to be effective in removing RNA.

4.3.1.3 DNA quantification

Two different methods were used to quantify bamboo DNA, one was spectophotometric and the other was agarose gel analysis. Spectrophotometrically the DNA showing A_{260}/A_{280} ratio around 1.8 (table 4.2) was chosen for PCR based molecular documentation methods after quantification by the formula mentioned in the materials and methods section. While as per gel

Table 4.2: List of samples showing their purity

Sample ID	A_{260}/A_{280} ratio **(purity)**
B1	1.84
B2	1.83
B3	1.84
B4	1.83
B5	1.89
B6	1.83
B7	1.84
B8	1.86
B9	1.78
B10	1.87
B11	1.83
B12	1.82
B13	1.76
B14	1.82
B15	1.80
B16	1.85
B17	1.80
B18	1.82
B19	1.84
B20	1.79
B21	1.89
B22	1.77
B23	1.80
B24	1.76
B25	1.82
B26	1.79
B27	1.88
B28	1.82
B29	1.78

analysis the quality of DNA was judged by the presence of single compact and clear band at the corresponding position of the molecular marker λ DNA/*Eco*RI/ *Hin*dIII double digest indicating high molecular weight of the bamboo DNA. The quantity of DNA was estimated by comparing the sample DNA with the control by eye adjustment.

The combination of the above three steps (extraction, purification and quantification) allowed the extraction of sufficient amount of pure DNA from bamboo leaves for PCR amplification.

4.3.2 Random Amplified Polymorphic DNA (RAPD) analysis

RAPD analysis using 30 different primers each of 10 mers in length has been used for the 29 accessions of bamboo. Of the 30 primers screened 16 (sixteen) resulted in producing distinct and scorable bands (table 4.3). The amplification profiles of the total genomic DNA from the 29 accessions of bamboo using 16 primers resulted in production of 326 fragments ranging in between 187-1875 bp of which only two were monomorphic, while rest were polymorphic (table 4.3). The percentage of polymorphism was found to be 99.39%. The number of bands generated by each decamers ranged in between 12 (OPA17) and 34 (OPA07). A representative of RAPD profile of the 29 accessions of bamboo generated using OPA 07 where all the bands generated are polymorphic and OPG 19 which also depicts one monomorphic band is presented in figure 4.3. The similarity matrix obtained using the Dice coefficient of similarity (Nei and li, 1979) depicted in table 4.4. Similarity coefficient among the 29 accessions ranged from 0.583-0.911. The lowest similarity was observed between *Dendrocalamus sikkimensis* and *Phyllostachys nigra*, while the highest value was recorded between *Bambusa multiplex* „Alphonse -Karr" and *Bambusa multiplex* „Rivierorum". The dendrogram constructed on the basis of the data obtained from RAPD analysis is depicted in figure 4.4. In the present study two aspects were taken into consideration to evaluate the genetic diversity and relationships in the 29 different accessions of bamboo. Firstly the comparison was made among generas of bamboo and secondly among bamboo species and varieties. The dendrogram showed that based on the similarity indices, two varieties of the same species i.e. *Bambusa vulgaris* „Vittata" and *Bambusa vulgaris*

Table 4.3: Total number and size of amplified bands, number of monomorphic and polymorphic bands generated and percentage of polymorphism generated by the RAPD primers.

Primer ID	Sequence (5'-3')	Total bands amplified	Monomorphic bands	Polymorphic bands	Percentage of polymorphism	Band size (bp)
OPA01	CAGGCCCTTC	21	0	21	100%	367-1633
OPA03	AGTCAGCCAC	17	0	17	100%	240-1875
OPA04	AATCGGGCTG	21	0	21	100%	387-1127
OPA05	ATTTTGCTTG	19	0	19	100%	360-1732
OPA07	GAAACGGGTG	34	0	34	100%	187-1833
OPA08	GTGACGTAGG	17	0	17	100%	230-1640
OPA11	CAATCGCCGT	22	0	22	100%	526-1655
OPA17	GACCGCTTGT	12	0	12	100%	409-1522
OPA20	GTTGCGATCC	21	0	21	100%	344-1567
OPB01	GTTTCGCTCC	20	0	20	100%	299-1387
OPF09	CCAAGCTTCC	14	0	14	100%	419-1557
OPG19	GTCAGGGCAA	22	1	21	95.45%	249-1356
OPH04	GGAAGTCGCC	18	0	18	100%	303-1520
OPN04	GACCGACCCA	18	1	17	94.44%	300-1355
OPN13	AGCGTCACTC	31	0	31	100%	358-1627
OPN19	GTCCGTACTG	19	0	19	100%	294-1444
	Total	**326**	**2**	**324**	**99.39%**	

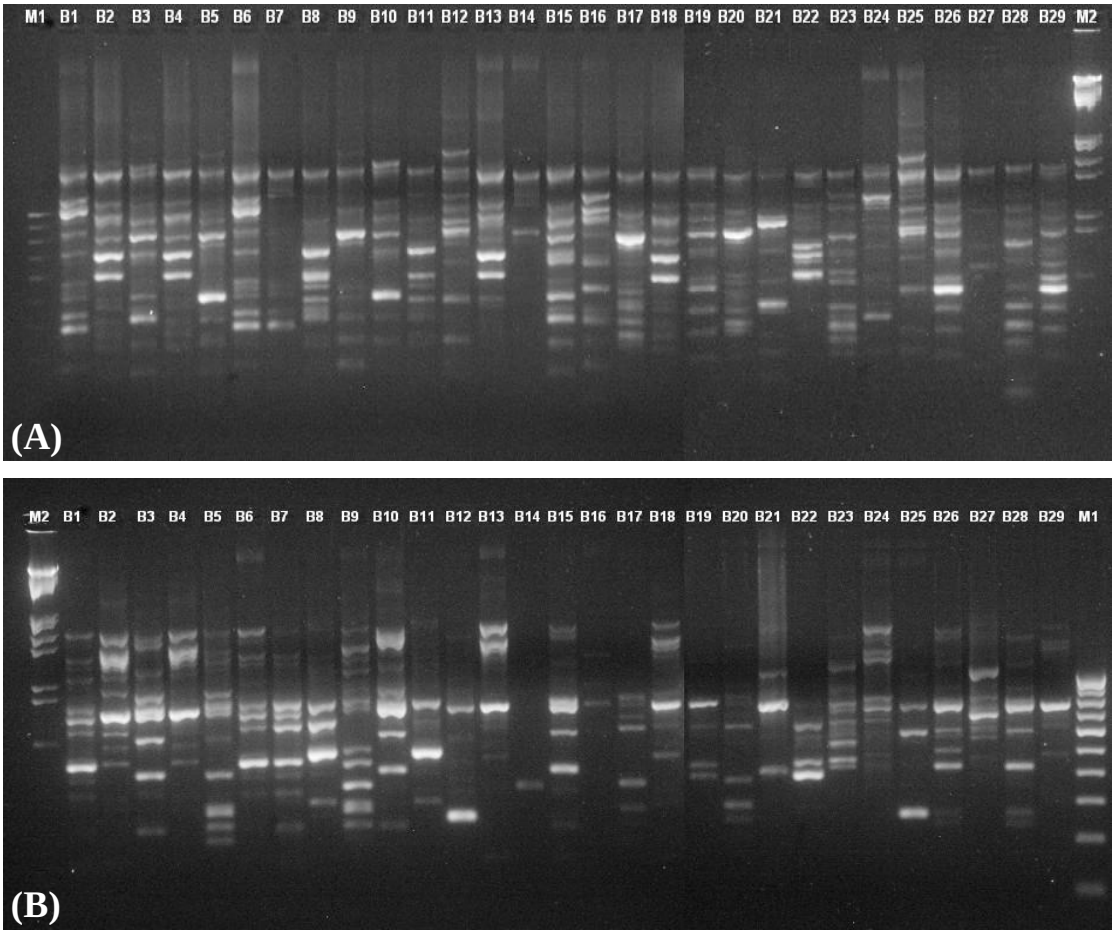


Figure 4.3: A representative RAPD profile of 29 accessions of bamboo amplified with (A) OPG19 primer and (B) OPA07. Lane M1: 100 bp molecular marker; Lane B1-B29 different accessions of bamboo under study (please refer table 4.1 for the species and varieties name); Lane M2: λ DNA/*Eco*RI/*Hin*dIII double digest DNA ladder

„Wamin" on one hand and *Bambusa multiplex* „Alphonse-Karr" and *Bambusa multiplex* „Rivierorum" on another hand formed a cluster sharing a node at 88.3% and 91.1% respectively. Similar results were also documented by Nayak and his coworkers (2003) using RAPD analysis where *Bambusa multiplex* and *Bambusa multiplex* var. Silver stripe clustered together sharing a node, Das *et al*. (2007) also using RAPD analysis found that *Bambusa striata* and *Bambusa wamin* shared a node while as per Mukherjee *et al*. (2010) *Bambusa vulgaris* and *Bambusa vulgaris* var. Vittata showed highest proximity and grouped together using ISSR and EST based primers. The cluster analysis showed that most of the *Bambusa* were close to each other except for *Bambusa balcooa* and *Bambusa oliveriana* which clustered in different clade and *Bambusa atra* and *Bambusa tulda* which clustered in a minor clade showing coefficient similarity of 82.2%. Of all the species

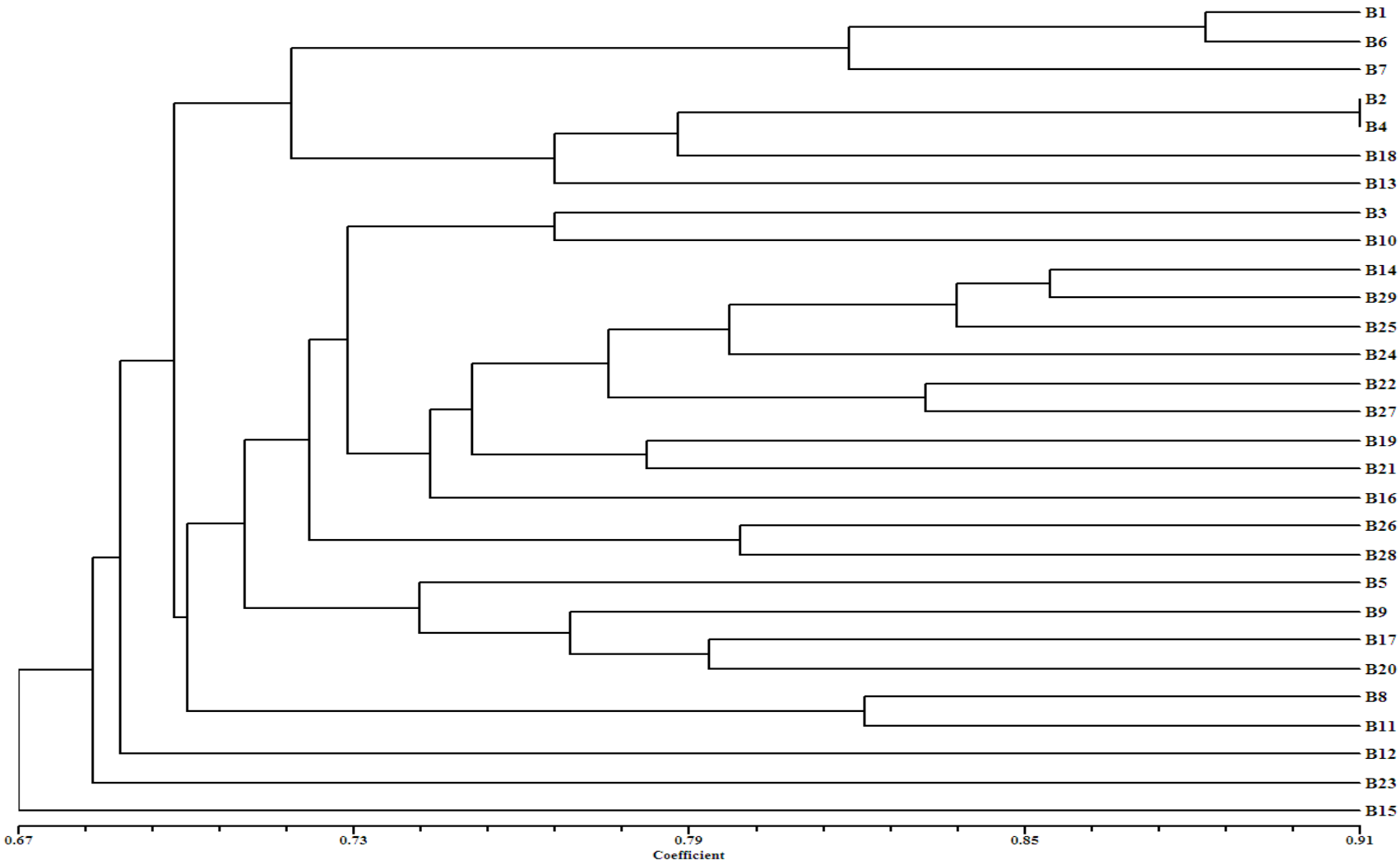


Figure 4.4: Dendrogram derived from UPGMA cluster analysis of RAPD markers illustrating the genetic relationships among the 29 accessions of bamboo

and varieties of *Bambusa* under study, *Bambusa pallida* was found to be segregated in a distinct clade showing a strong indication of the polyphyletic origin of the genus *Bambusa*. These findings are in close correspondence with the previous phylogenetic analysis on bamboo using AFLP markers (Loh *et al*., 2000), RAPD primers (Nayak *et al*., 2003; Das *et al*., 2007), ISSR and EST based primers (Mukherjee *et al*., 2010). The four accessions of *Dendrocalamus* experimented here showed considerable divergence among themselves in contrast to traditional taxonomy. Similar pattern of clustering were noted by Loh *et al*. (2000) between *Dendrocalamus giganteus* and *D. brandissi* using AFLP analysis and by Nayak *et al*. (2003) between *Dendrocalamus strictus* and *D. giganteus*. The two species of *Drepanostachyum* i.e. *Drepanostachyum khasianum* and *Drepanostachyum intermedium* were distantly placed in the dendrogram sowing the similarity coefficient of as low as 72.8%. The appearance of this divergence might be due to their growth habit together with their morphological characteristics. The cluster analysis showed genetic proximity of *Melocanna baccifera* and *Shibateae kumasaca*, *Sasaella ramose* and *Yushania maling*, *Dendrocalamus strictus* and *Gigantochloa* sp. with similarity coefficients of 83.3%, 80% and 79.4% respectively. Species of all other genera were intermingled in the dendrogarm irrespective of their generic connectivity. The principal coordinate analysis which is based on the similarity coefficients or variance-covariance among the traits validated the dendrogram (Akond *et al*., 2007) (fig 4.5). Both the dendrogram and the principal coordinate analysis showed the similar cluster. Thus it can be concluded from the RAPD marker study that the bamboo taxonomy should be reconstituted taking into account both the data obtained from classical approaches and molecular evidences, since individual approaches will not be able to solve the taxonomic disputes which is already severely hampered.

4.3.3 ISSR (Inter Simple Sequence repeat) analysis

We accessed phylogenetics among the 29 accessions of bamboo documented from North Bengal, India using DNA based technique complemented with 9 ISSR primers. Fifteen ISSR primers were initially screened to generate polymorphic bands, out of which only

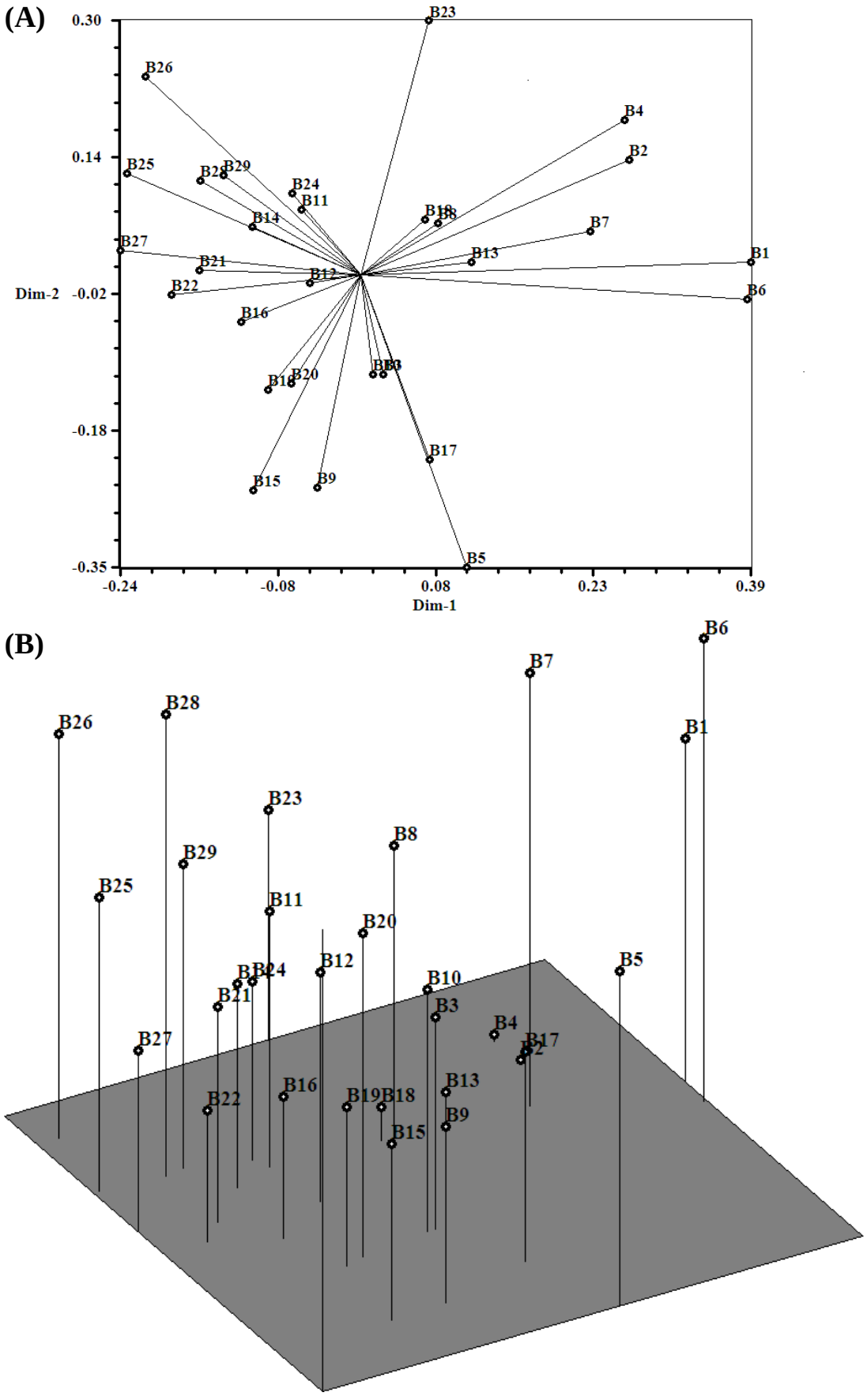


Figure 4.5: Principal coordinate analysis of 29 species and varieties of bamboo based on RAPD analysis data. (A) 2-dimensional plot and (B) 3-dimensional plot.

Table 4.4: The similarity matrix obtained using Dice coefficient of similarity among the 29 accessions of bamboo based on RAPD profiling

	B1	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**	**B9**	**B10**	**B11**	**B12**	**B13**	**B14**	**B15**	**B16**	**B17**	**B18**	**B19**	**B20**	**B21**	**B22**	**B23**	**B24**	**B25**	**B26**	**B27**	**B28**	**B29**
B1	1.000																												
B2	0.783	1.000																											
B3	0.739	0.767	1.000																										
B4	0.772	**0.911**	0.711	1.000																									
B5	0.711	0.683	0.750	0.661	1.000																								
B6	0.883	0.722	0.689	0.722	0.728	1.000																							
B7	0.778	0.717	0.672	0.694	0.711	0.861	1.000																						
B8	0.728	0.700	0.711	0.722	0.650	0.722	0.739	1.000																					
B9	0.656	0.672	0.717	0.661	0.711	0.650	0.678	0.706	1.000																				
B10	0.706	0.722	0.767	0.711	0.706	0.722	0.694	0.711	0.683	1.000																			
B11	0.650	0.678	0.644	0.689	0.617	0.656	0.694	0.822	0.683	0.722	1.000																		
B12	0.683	0.678	0.700	0.667	0.639	0.678	0.672	0.656	0.661	0.678	0.644	1.000																	
B13	0.711	0.750	0.694	0.772	0.644	0.706	0.700	0.728	0.711	0.717	0.683	0.706	1.000																
B14	0.733	0.772	0.772	0.761	0.711	0.728	0.756	0.772	0.756	0.783	0.750	0.761	0.778	1.000															
B15	0.633	0.661	0.706	0.628	0.678	0.628	0.622	0.661	0.678	0.728	0.672	0.672	0.667	0.700	1.000														
B16	0.656	0.683	0.672	0.694	0.711	0.661	0.678	0.683	0.689	0.694	0.717	0.717	0.722	0.778	0.700	1.000													
B17	0.706	0.733	0.711	0.711	0.761	0.722	0.750	0.722	0.783	0.756	0.711	0.711	0.750	0.794	0.683	0.728	1.000												
B18	0.700	0.794	0.672	0.783	0.689	0.672	0.733	0.672	0.700	0.683	0.683	0.717	0.778	0.767	0.656	0.744	0.739	1.000											
B19	0.694	0.722	0.767	0.744	0.750	0.667	0.694	0.700	0.694	0.756	0.722	0.644	0.706	0.794	0.750	0.761	0.744	0.728	1.000										
B20	0.689	0.661	0.706	0.683	0.756	0.694	0.722	0.717	0.756	0.706	0.694	0.672	0.733	0.800	0.644	0.689	0.794	0.711	0.794	1.000									
B21	0.656	0.683	0.706	0.672	0.678	0.628	0.678	0.683	0.689	0.694	0.683	0.694	0.711	0.789	0.656	0.689	0.694	0.744	0.783	0.767	1.000								
B22	0.650	0.689	0.733	0.700	0.672	0.644	0.694	0.722	0.728	0.711	0.722	0.711	0.717	0.828	0.694	0.761	0.711	0.717	0.789	0.717	0.717	1.000							
B23	0.683	0.733	0.689	0.733	0.606	0.656	0.728	0.711	0.617	0.656	0.689	0.611	0.672	0.739	**0.583**	0.661	0.667	0.672	0.700	0.694	0.672	0.689	1.000						
B24	0.694	0.756	0.733	0.722	0.672	0.711	0.728	0.700	0.728	0.711	0.689	0.700	0.750	0.828	0.728	0.739	0.722	0.772	0.733	0.728	0.739	0.744	0.744	1.000					
B25	0.644	0.694	0.739	0.672	0.678	0.650	0.711	0.683	0.678	0.717	0.694	0.750	0.689	0.844	0.667	0.744	0.706	0.711	0.717	0.733	0.722	0.750	0.694	0.783	1.000				
B26	0.639	0.656	0.689	0.678	0.639	0.622	0.672	0.678	0.650	0.689	0.689	0.667	0.639	0.761	0.628	0.694	0.644	0.661	0.689	0.706	0.706	0.689	0.700	0.722	0.761	1.000			
B27	0.650	0.700	0.733	0.667	0.628	0.633	0.694	0.722	0.717	0.733	0.700	0.689	0.672	0.817	0.694	0.739	0.722	0.717	0.756	0.728	0.739	0.833	0.678	0.767	0.794	0.722	1.000		
B28	0.672	0.644	0.700	0.656	0.672	0.700	0.694	0.700	0.639	0.689	0.700	0.700	0.661	0.750	0.694	0.683	0.667	0.683	0.711	0.728	0.739	0.700	0.667	0.744	0.761	0.800	0.711	1.000	
B29	0.711	0.717	0.717	0.728	0.689	0.706	0.744	0.706	0.711	0.761	0.728	0.717	0.744	0.856	0.667	0.744	0.761	0.767	0.772	0.789	0.756	0.750	0.706	0.783	0.833	0.817	0.761	0.772	1.000

For details on sample ID (B1– B29) please refer table 4.1 in the results and discussion section

9 primers were able to produce distinct, scorable bands (table 4.5) and were selected for further study. Among the primers used, the primer (TC)8A produced only 22 bands while (TC)8G amplified the highest number of bands i.e. 33. On an average each primer produced about 27.11 scorable, distinct bands. A total of 244 amplified bands were produced by the 9 primers of which all the 244 were polymorphic. The frequency of polymorphism was found to be 100%. The band size ranged between 137bp to 2017bp. A representative of ISSR profile of the 29 accessions of bamboo generated with UBC815 and UBC824 primer is depicted in figure 4.6. Nei‟s genetic similarity between each pair of species ranged between 0.613 - 0.960 (table 4.6). The lowest was found between *Bambusa vulgaris* „Vittata‟ and *Bambusa pallida*, while the highest value was recorded between *Bambusa multiplex* „Alphonse-Karr‟ and *Bambusa multiplex* „Rivierorum‟.

The dendrogram constructed based on the data from the ISSR based random primers (fig 4.7) showed that most of the *Bambusa* species and varieties clustered together except for *Bambusa atra* and *Bambusa oliveriana* which clustered in a minor clade showing coefficient similarity of 74.7%. *Bambusa pallida* was found to be segregated in a distinct clade from all other genera, species and varieties of bamboo under study indicating the non -monophylyl origin of the genus *Bambusa*. Similar views were held by different workers (Soderstorm and Ellis, 1987; Loh *et al*., 2000; Das *et al*., 2007 and Mukherjee *et al*., 2010). *Bambusa multiplex* „Alphonse-Karr‟

Table 4.5: Total number and size of amplified bands and number of polymorphic bands generated by ISSR primers

Primer ID	Sequence (5'-3')	Total amplified bands	Polymorphic bands	Percentage of polymorphism	Band size (bp)
UBC810	(GA)8T	27	27	100%	148-1800
UBC815	(CT)8G	24	24	100%	190-1600
UBC818	(CA)8G	32	32	100%	137-1843
UBC822	(TC)8A	22	22	100%	179-1843
UBC824	(TC)8G	33	33	100%	205-1888
UBC825	(AC)8T	25	25	100%	204-1711
UBC841	(GA)8YC	25	25	100%	243-1325
UBC856	(AC)8YA	29	29	100%	153-1975
UBC873	(GACA)4	27	27	100%	217-2017
	Total	**244**	**244**	**100%**	

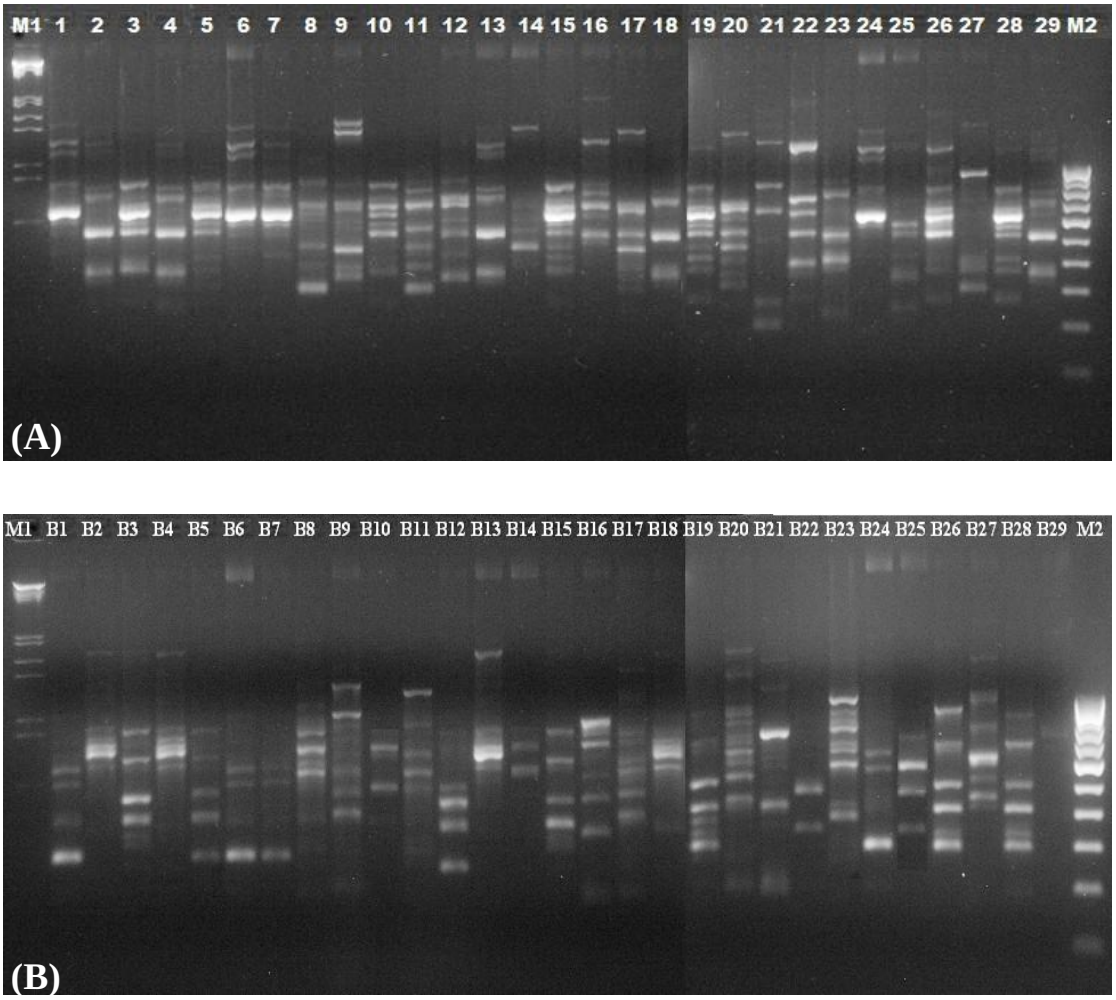


Figure 4.6: ISSR banding patterns of 29 accessions of bamboo generated by (A) UBC824 primer and (B) UBC815. Lane M1: λ DNA/*Eco*RI/*Hin*dIII double digest DNA ladder; Lane B1-B29 different accessions of bamboo under study (please refer table 4.1 for the species and varieties name); Lane M2:100 bp molecular marker

and *B. multiplex* „Rivierorum" formed a cluster sharing a node at 96% similarity being varieties of the same species. This was in accordance with the results obtained by Nayak and his coworkers (2003) where *Bambusa multiplex* and *Bambusa multiplex* var. Silver stripe were found to be very close to each other. Similarly *Bambusa vulgaris* „Vittata" and *Bambusa vulgaris* „Wamin" with 85.3% similarity were found to be closely linked being two varieties of the same species. This close proximity between the two have already been documented (Nayak *et al.*,2003; Das *et al.*,2007 and Mukherjee *et al.*,2010). Among all the 29 accessions least proximity (0.613) was found between *Bambusa vulgaris* „Vittata" and *Bambusa pallida*. Of the 4 accessions of the genus *Dendrocalamus* , *Dendrocalamus strictus* and *Dendrocalamus hamiltonii* were close to each other and formed a

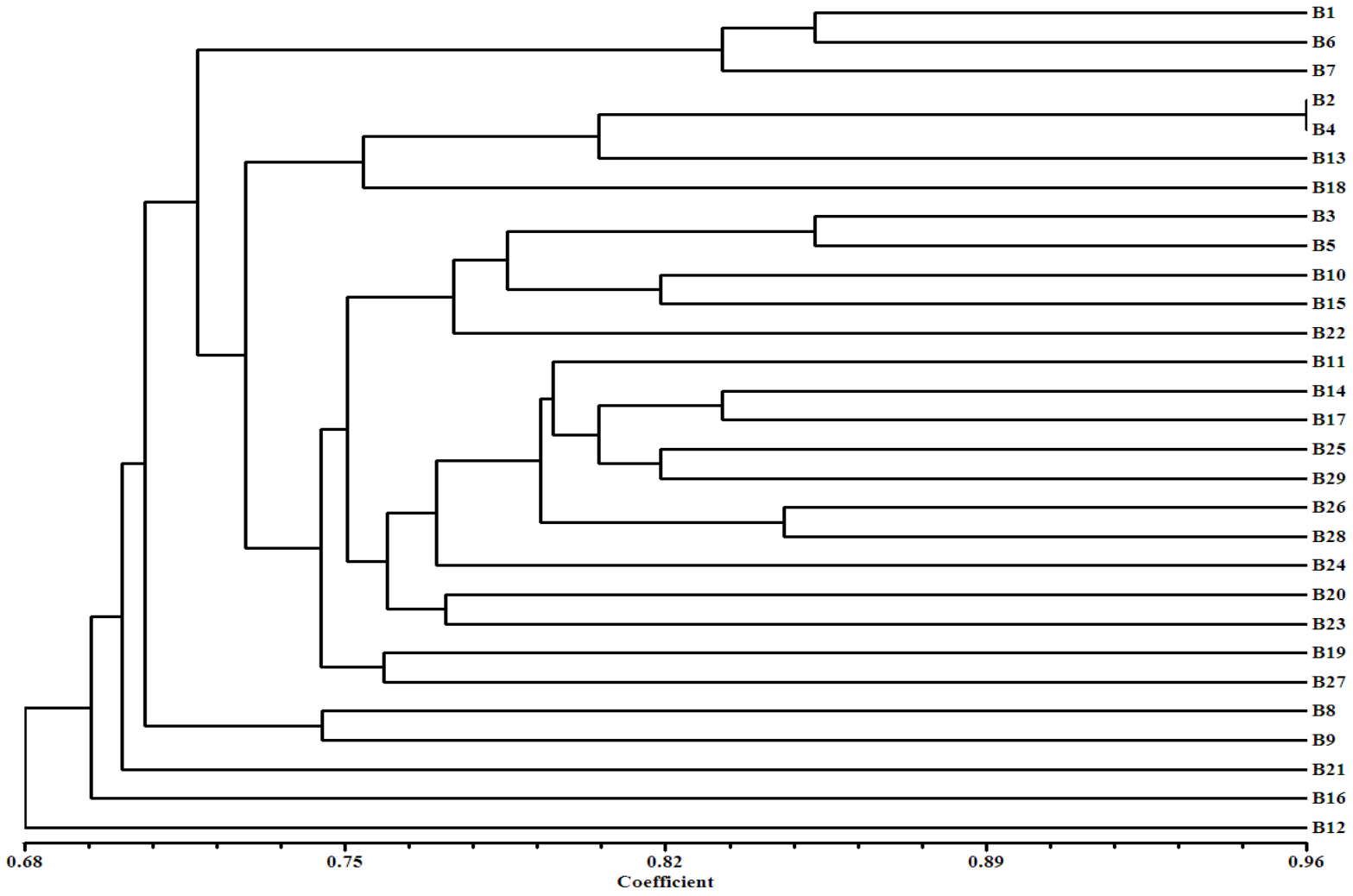


Figure 4.7: Dendrogram generated from the cluster analysis of ISSR markers of 29 bamboo accessions

Table 4.6: The similarity matrix obtained using Dice coefficient of similarity among the 29 accessions of bamboo based on ISSR profiling

	B1	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**	**B9**	**B10**	**B11**	**B12**	**B13**	**B14**	**B15**	**B16**	**B17**	**B18**	**B19**	**B20**	**B21**	**B22**	**B23**	**B24**	**B25**	**B26**	**B27**	**B28**	**B29**
B1	1.000																												
B2	0.687	1.000																											
B3	0.700	0.707	1.000																										
B4	0.673	**0.960**	0.707	1.000																									
B5	0.727	0.733	0.853	0.733	1.000																								
B6	0.853	0.673	0.727	0.673	0.793	1.000																							
B7	0.833	0.733	0.760	0.733	0.813	0.833	1.000																						
B8	0.693	0.740	0.767	0.740	0.753	0.667	0.740	1.000																					
B9	0.680	0.660	0.687	0.660	0.727	0.707	0.740	0.747	1.000																				
B10	0.693	0.700	0.820	0.700	0.793	0.720	0.780	0.733	0.733	1.000																			
B11	0.687	0.720	0.733	0.720	0.773	0.713	0.760	0.780	0.713	0.767	1.000																		
B12	**0.613**	0.633	0.673	0.647	0.673	0.653	0.673	0.680	0.680	0.720	0.713	1.000																	
B13	0.653	0.793	0.700	0.820	0.740	0.707	0.740	0.733	0.680	0.760	0.740	0.707	1.000																
B14	0.713	0.760	0.733	0.760	0.773	0.713	0.773	0.753	0.700	0.767	0.827	0.767	0.780	1.000															
B15	0.687	0.720	0.747	0.707	0.787	0.713	0.773	0.727	0.753	0.820	0.747	0.700	0.753	0.747	1.000														
B16	0.647	0.680	0.693	0.640	0.680	0.633	0.707	0.660	0.673	0.727	0.720	0.647	0.673	0.773	0.733	1.000													
B17	0.733	0.753	0.740	0.740	0.753	0.720	0.807	0.760	0.760	0.747	0.767	0.707	0.760	0.833	0.793	0.767	1.000												
B18	0.667	0.727	0.700	0.767	0.713	0.680	0.740	0.720	0.680	0.760	0.753	0.733	0.773	0.807	0.740	0.727	0.800	1.000											
B19	0.667	0.673	0.740	0.687	0.767	0.693	0.753	0.693	0.667	0.733	0.740	0.653	0.720	0.753	0.727	0.647	0.707	0.667	1.000										
B20	0.680	0.713	0.713	0.700	0.767	0.680	0.740	0.680	0.693	0.720	0.780	0.640	0.693	0.780	0.713	0.700	0.733	0.720	0.760	1.000									
B21	0.660	0.693	0.680	0.680	0.720	0.660	0.720	0.660	0.673	0.713	0.733	0.673	0.660	0.733	0.680	0.640	0.713	0.700	0.687	0.713	1.000								
B22	0.673	0.720	0.747	0.720	0.813	0.700	0.733	0.700	0.673	0.753	0.787	0.740	0.753	0.787	0.787	0.693	0.767	0.740	0.753	0.767	0.747	1.000							
B23	0.667	0.767	0.700	0.767	0.780	0.693	0.740	0.680	0.707	0.693	0.767	0.653	0.720	0.767	0.727	0.687	0.773	0.720	0.747	0.773	0.740	0.740	1.000						
B24	0.680	0.687	0.727	0.673	0.727	0.693	0.753	0.680	0.653	0.747	0.780	0.653	0.720	0.780	0.713	0.700	0.733	0.693	0.693	0.733	0.700	0.713	0.693	1.000					
B25	0.673	0.707	0.733	0.720	0.800	0.740	0.800	0.700	0.687	0.753	0.800	0.740	0.727	0.827	0.760	0.720	0.780	0.767	0.780	0.780	0.720	0.773	0.740	0.780	1.000				
B26	0.680	0.687	0.713	0.700	0.753	0.707	0.753	0.680	0.640	0.747	0.767	0.640	0.707	0.767	0.753	0.700	0.733	0.693	0.787	0.733	0.673	0.727	0.733	0.773	0.793	1.000			
B27	0.693	0.687	0.740	0.687	0.780	0.693	0.767	0.667	0.680	0.707	0.753	0.693	0.693	0.767	0.713	0.687	0.733	0.720	0.760	0.747	0.727	0.727	0.720	0.760	0.780	0.760	1.000		
B28	0.727	0.773	0.720	0.787	0.773	0.713	0.787	0.740	0.727	0.793	0.813	0.727	0.793	0.827	0.787	0.733	0.807	0.767	0.740	0.780	0.760	0.773	0.793	0.780	0.813	0.847	0.753	1.000	
B29	0.693	0.780	0.740	0.767	0.807	0.707	0.793	0.733	0.720	0.787	0.793	0.667	0.787	0.820	0.753	0.767	0.800	0.773	0.760	0.813	0.740	0.793	0.773	0.773	0.820	0.787	0.760	0.833	1.000

For details on sample ID (B1– B29) please refer table 4.1 in the results and discussion section

cluster sharing the node at 83.3% similarity while *Dendrocalamus sikkimensis* clustered with *Bambusa sinospinosa* where as *Dendrocalamus asper* formed a totally separate cluster. The divergence between the species of the same genus might be possibly due to their growth habit or morphological features (Nayak *et al*., 2003). This divergence in the genus *Dendrocalamus* has been previously encountered by Loh *et al*. (2000), where they found *Dendrocalamus giganteus* and *Dendrocalamus brandissi* forming different cluster using AFLP technique and both Nayak *et al* (2003) and Mukherjee *et al* (2010) found *Dendrocalamus strictus* and *Dendrocalamus giganteus* in different clade employing RAPD and ISSR marker analysis respectively. The two species of *Drepanostachyum* i.e. *Drepanostachyum khasianum* and *Drepanostachyum intermedium* were distantly placed in the dendrogram sowing the similarity coefficient of as low as 66.7%. This also might be due to their growth habit together with their morphological characteristics as in case of *Dendrocalamus*. Species of all other genera were intermingled in the dendrogarm irrespective of their generic connectivity. The principal coordinate analysis which is based on the similarity coefficients or variance-covariance among the traits validated the dendrogram (Akond *et al*., 2007) (fig 4.8). Both the dendrogram and the principal coordinate analysis showed the similar cluster. Mukherjee *et al*. (2010) also reported similar results while accessing the genetic relationships among 22 taxa of bamboo using ISSR and EST-based random primers. Thus it can be inferred from the ISSR marker study that not only morphological features must be considered for the taxonomy of bamboo but multidisciplinary approach including the molecular techniques must be employed to have correct taxonomic demarcation.

4.3.4 Combined RAPD and ISSR based analysis

The similarity coefficients of the 29 accessions of bamboo based on 326 RAPD and 244 ISSR loci ranged in between 0.629 to 0.934 (table 4.7). The highest similarity was found between *Bambusa multiplex* „Alphonse-Karr‟ and *B. multiplex* „Rivierorum‟ (0.934) like RAPD and ISSR analysis separately, while the lowest was noted between *B. vulgaris* „Vittata‟ and *Himalayacalamus hookerianus* (0.629). Cluster analysis performed from the

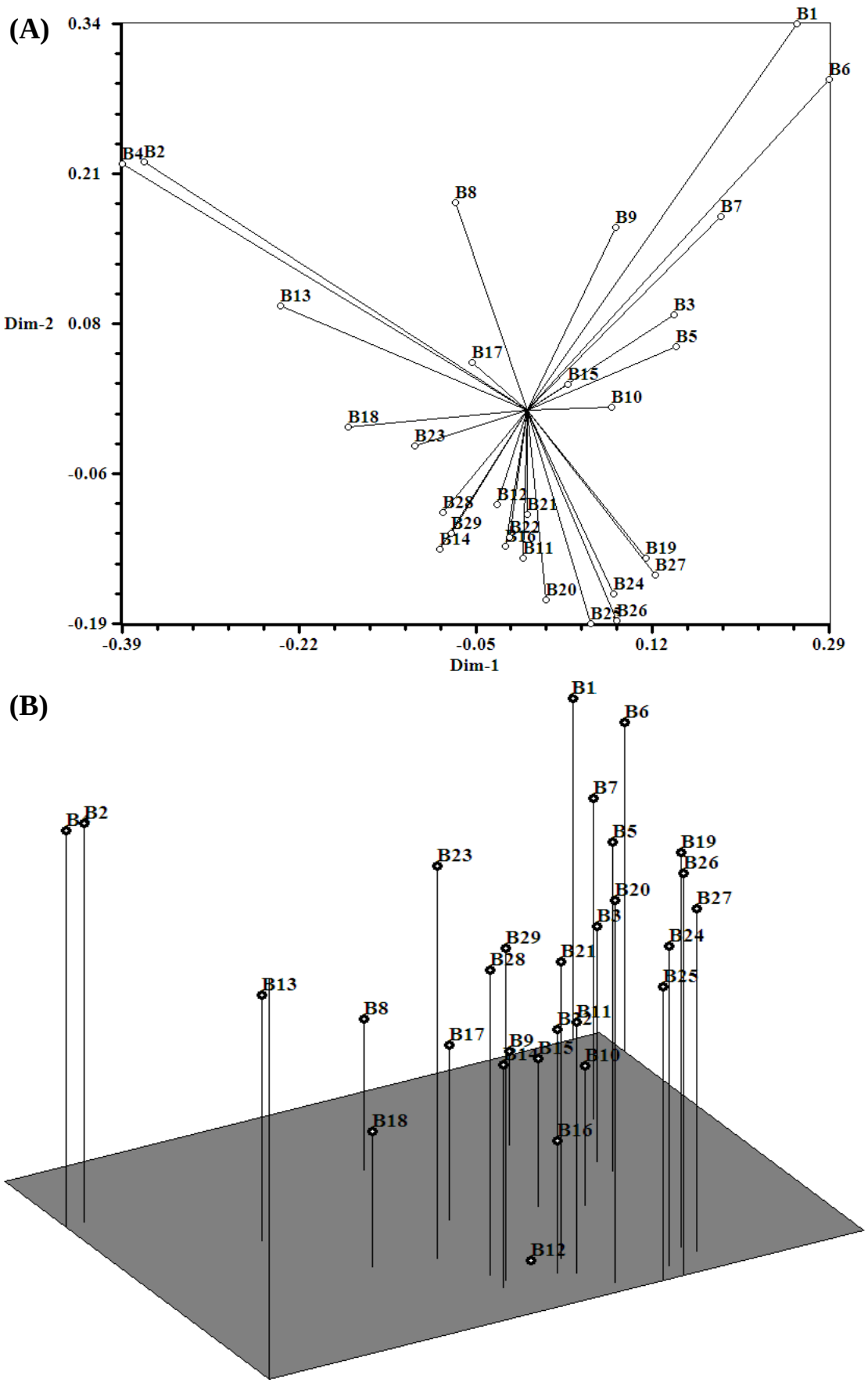


Figure 4.8: Principal coordinate analysis of 29 species and varieties of bamboo based on ISSR analysis data. (A) 2-dimensional plot and (B) 3-dimensional plot

combined data sets of both RAPD and ISSR markers generated a dendrogram as illustrated in fig. 4.9. The dendrogram gave clustering pattern similar to that of ISSR and RAPD analysis separately, except for that in both the individual analysis (ISSR and RAPD), three accessions were segregated from the cluster and evolved as independent distinct clade, *Bambusa pallida* being common. In the combined analysis only *B. pallida* was found to diversify from the main clade. The correspondence analysis both 2D and 3D (fig 4.10) corroborated the cluster analysis results. However, the ISSR based cluster was found to be more similar as compared to RAPD based cluster.

4.3.5 *PCR-RFLP analysis of the trnL-trnF region of the bamboo chloroplast genome*

PCR-RFLP being a simple and inexpensive method plays an important role in accessing the genetic diversity of different plant species and was thus applied to study the fingerprinting of different species and varieties of bamboo found in North Bengal.

4.3.5.1 PCR amplification and agarose gel analysis

In the present study 29 species and varieties of bamboo were subjected to PCR amplification with locus specific primer pair

Tab c 5‟-CGAAATCGGTAGACGCTACG-3‟

&

Tab f 5‟-ATTTGAACTGGTGACACGAG-3‟

developed based on the Tab c-f in "Taberlet" (TrnL-TrnF) region of the chloroplast genome of bamboo for which the nucleotide information was available with respect to other plant species in the public domain. The primer pair successfully amplified the Tab c-f in "Taberlet" (TrnL-TrnF) region of the chloroplast genome of bamboo. Using the template DNA, the primer pair generated a single band following PCR of expected length i.e. 1029 bp. The amplified product is shown in fig 4.11.

4.3.5.2 PCR product restriction digestion and agarose gel analysis

The PCR product obtained from the prime pair Tab c-f were subjected to restriction digestion using six different restriction enzymes like *Taq*I, *Alu*I, *Hinf*I, *Hae*III, *Hpa*I and *Msp*I to short out the degree of genetic variation among different species and varieties of bamboo. Three restriction enzymes (*Hae*III, *Hpa*I and *Msp*I) under study failed to digest the PCR products while the summary of the digestion by the other three restriction enzymes are depicted in table 4.8. Digestion with

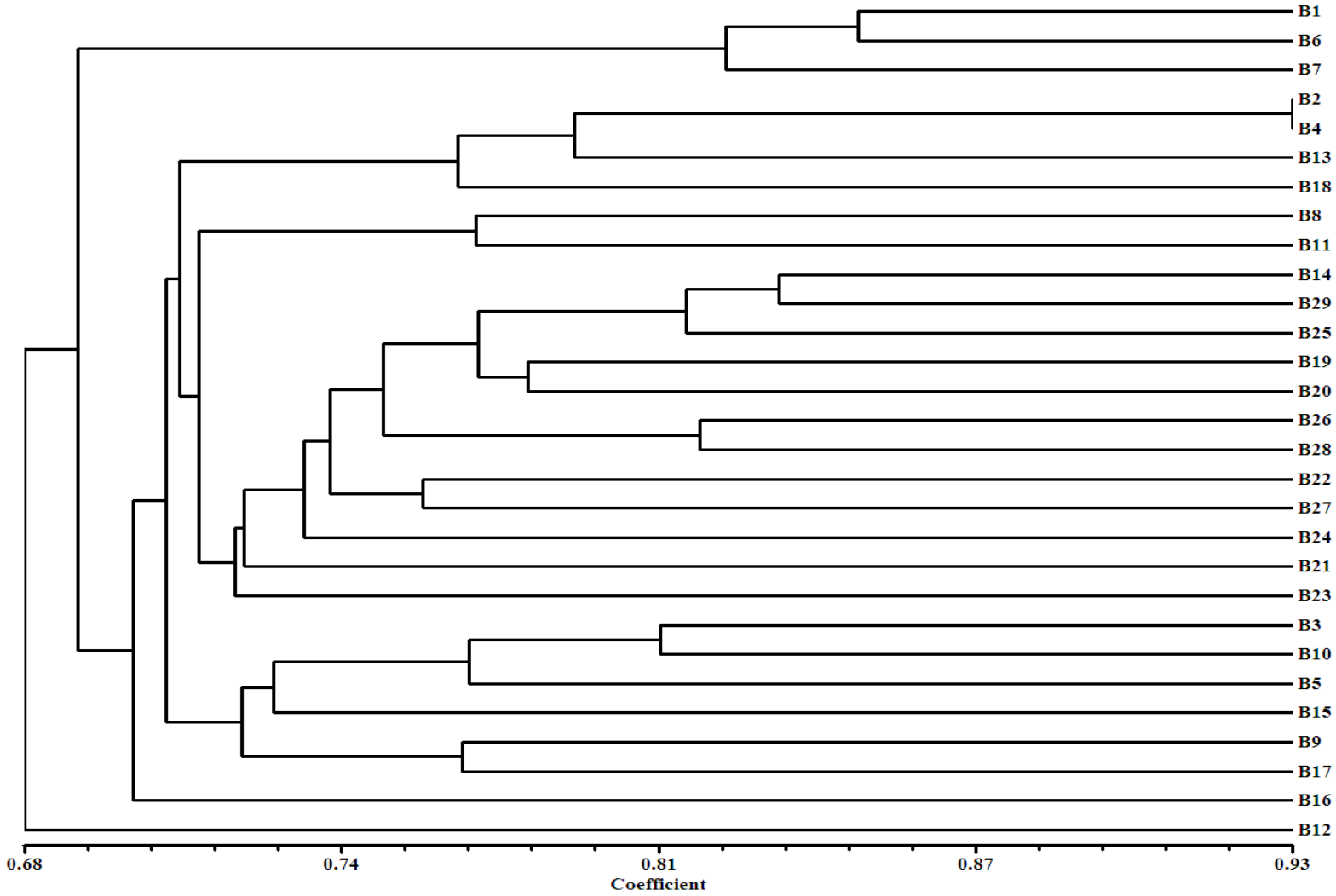


Figure 4.9: Dendrogram constructed on the basis of data obtained from the combined RAPD and ISSR analysis

Table 4.7: The similarity matrix obtained using Dice coefficient of similarity among the 29 accessions of bamboo based on combined RAPD and ISSR profiling

	B1	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**	**B9**	**B10**	**B11**	**B12**	**B13**	**B14**	**B15**	**B16**	**B17**	**B18**	**B19**	**B20**	**B21**	**B22**	**B23**	**B24**	**B25**	**B26**	**B27**	**B28**	**B29**
B1	1.000																												
B2	0.729	1.000																											
B3	0.711	0.743	1.000																										
B4	0.721	**0.934**	0.719	1.000																									
B5	0.695	0.716	0.793	0.703	1.000																								
B6	0.846	0.698	0.695	0.700	0.737	1.000																							
B7	0.793	0.729	0.706	0.721	0.743	0.846	1.000																						
B8	0.698	0.724	0.737	0.737	0.700	0.676	0.735	1.000																					
B9	0.658	0.679	0.703	0.676	0.724	0.674	0.706	0.727	1.000																				
B10	0.687	0.724	0.806	0.716	0.743	0.714	0.729	0.714	0.706	1.000																			
B11	0.653	0.684	0.666	0.687	0.682	0.658	0.706	0.769	0.687	0.721	1.000																		
B12	0.634	0.666	0.684	0.658	0.668	0.639	0.655	0.671	0.663	0.692	0.679	1.000																	
B13	0.676	0.782	0.695	0.796	0.695	0.703	0.714	0.729	0.700	0.735	0.695	0.703	1.000																
B14	0.711	0.764	0.745	0.756	0.745	0.706	0.753	0.764	0.740	0.759	0.777	0.764	0.775	1.000															
B15	0.653	0.679	0.714	0.660	0.719	0.653	0.679	0.679	0.708	0.753	0.698	0.668	0.695	0.708	1.000														
B16	0.650	0.682	0.684	0.674	0.695	0.645	0.692	0.682	0.679	0.703	0.706	0.676	0.687	0.775	0.711	1.000													
B17	0.711	0.737	0.724	0.714	0.751	0.695	0.753	0.732	0.767	0.743	0.719	0.706	0.743	0.809	0.719	0.743	1.000												
B18	0.671	0.767	0.690	0.769	0.695	0.660	0.729	0.698	0.690	0.719	0.700	0.714	0.761	0.769	0.679	0.735	0.764	1.000											
B19	0.660	0.698	0.743	0.711	0.748	0.655	0.698	0.692	0.674	0.729	0.737	0.655	0.714	0.769	0.727	0.692	0.716	0.698	1.000										
B20	0.679	0.690	0.698	0.692	0.761	0.668	0.716	0.700	0.714	0.695	0.729	0.658	0.716	0.782	0.671	0.684	0.751	0.711	0.780	1.000									
B21	0.645	0.687	0.690	0.679	0.690	**0.629**	0.671	0.666	0.674	0.687	0.684	0.660	0.682	0.748	0.647	0.660	0.700	0.719	0.729	0.732	1.000								
B22	0.653	0.695	0.729	0.703	0.724	0.647	0.690	0.700	0.687	0.721	0.745	0.706	0.716	0.793	0.714	0.711	0.729	0.716	0.764	0.735	0.732	1.000							
B23	0.660	0.740	0.684	0.737	0.690	0.645	0.708	0.698	0.653	0.660	0.721	0.634	0.692	0.743	0.637	0.660	0.700	0.687	0.729	0.743	0.703	0.716	1.000						
B24	0.679	0.706	0.714	0.687	0.687	0.668	0.706	0.679	0.676	0.706	0.708	0.668	0.716	0.777	0.703	0.706	0.714	0.706	0.690	0.714	0.706	0.714	0.721	1.000					
B25	0.647	0.690	0.729	0.692	0.719	0.663	0.732	0.700	0.671	0.721	0.729	0.732	0.690	0.809	0.682	0.711	0.714	0.727	0.748	0.751	0.700	0.751	0.711	0.751	1.000				
B26	0.647	0.679	0.692	0.692	0.692	0.642	0.690	0.679	0.639	0.700	0.708	0.668	0.663	0.745	0.660	0.679	0.671	0.663	0.716	0.703	0.684	0.698	0.711	0.735	0.756	1.000			
B27	0.650	0.687	0.721	0.663	0.679	0.655	0.714	0.682	0.695	0.719	0.711	0.682	0.671	0.769	0.684	0.703	0.721	0.719	0.735	0.716	0.714	0.759	0.682	0.743	0.759	0.716	1.000		
B28	0.684	0.721	0.708	0.729	0.719	0.695	0.727	0.716	0.676	0.727	0.735	0.700	0.727	0.772	0.719	0.700	0.719	0.721	0.727	0.751	0.748	0.729	0.732	0.740	0.761	0.814	0.711	1.000	
B29	0.695	0.737	0.719	0.735	0.740	0.684	0.748	0.716	0.714	0.753	0.745	0.690	0.753	0.830	0.698	0.743	0.767	0.764	0.769	0.798	0.732	0.751	0.737	0.751	0.814	0.782	0.732	0.793	1.000

For details on sample ID (B1– B29) please refer table 4.1 in the results and discussion section

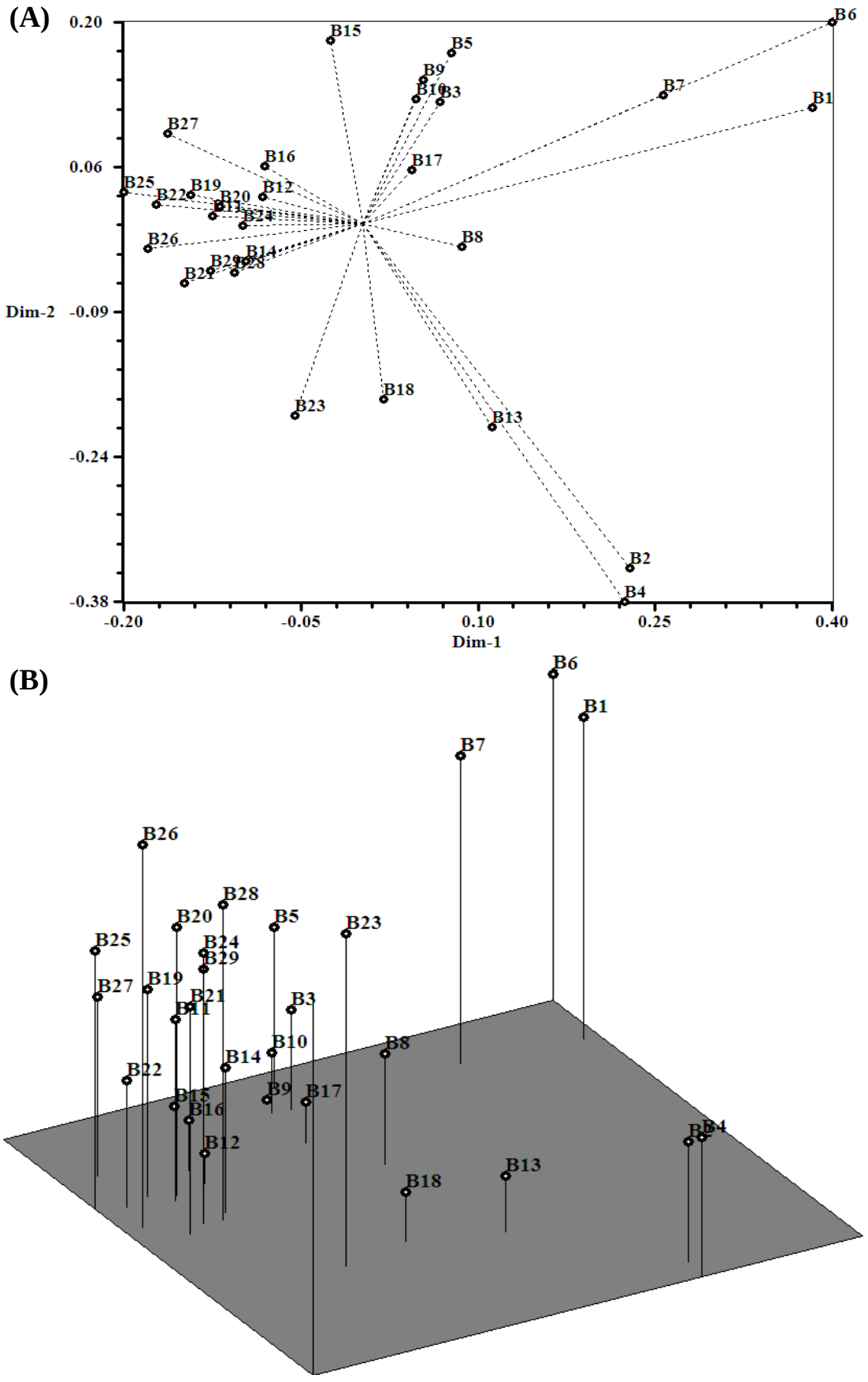


Figure 4.10: Principal coordinate analysis of 29 accessions of bamboo based on combined RAPD and ISSR analysis data. (A) 2-dimensional plot and (B) 3-dimensional plot

*Taq*I resulted in producing four bands in all except for *Bambusa multiplex* „Alphanso-Karr", *B. multiplex* „Rivierorum", *Cephalostachyum latifolium*, *Drepanostachyum khasianum* and Chinese bamboo (Unidentified) where five bands were produced (fig 4.12a). The polymorphism percentage was found to be 20% with band size between 354-740 bp. Restriction enzyme *Alu*I showed maximum polymorphism (70%) generating five bands in most of the accessions of bamboo, six bands were produced in *Bambusa multiplex* „Alphanso-Karr", *B. multiplex* „Rivierorum", *Cephalostachyum latifolium* and four bands in *Bambusa bamboos*, *Drepanostachyum khasianum*, *Himalayacalamus hookerianus* and *Shibateae kumasaca* (fig 4.12b). When the PCR products were subjected to restriction digestion with *Hinf*I it produced four bands in almost all the bamboo species and cultivars under study with the exception of *Himalayacalamus hookerianus*, *Pseudosasa japonica* and *Shibateae kumasaca* where only three bands were visible (fig 4.12c), while

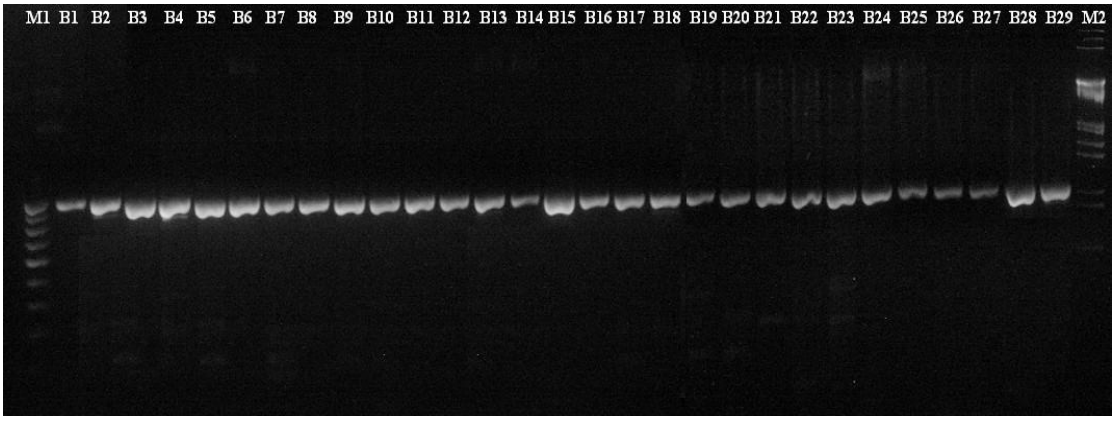


Figure 4.11: Amplification of bamboo accessions with primer Tab c-f (TrnL-TrnF).
Lane M1: 100bp DNA ladder; Lane B1-B29: Different species of bamboo as listed in table 4.1 and M2: λ DNA/*Eco*RI/*Hin*dIII double digest DNA ladder

Table 4.8: Total fragments, number of monomorphic and polymorphic bands generated by using different restriction enzymes

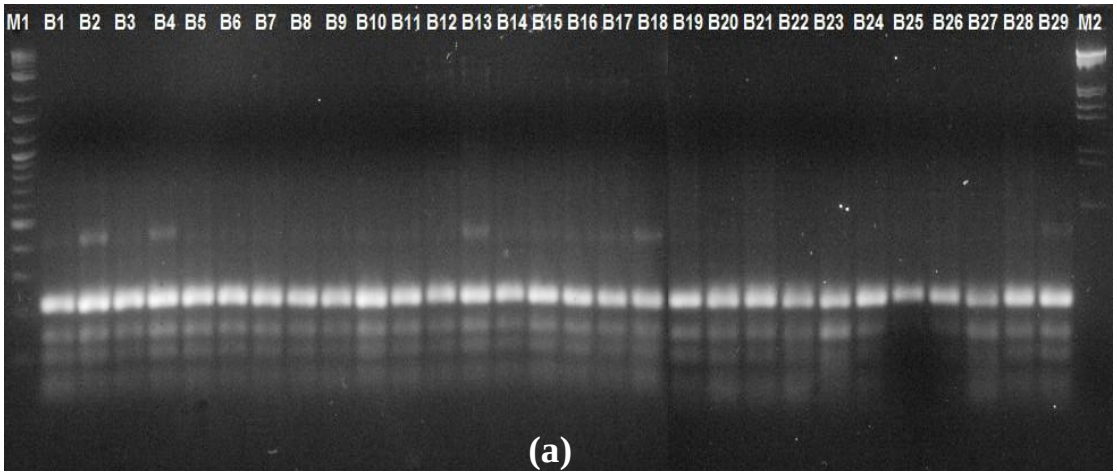
Restriction enzyme	Optimum temperature	No. of cuts	No. of polymorphic bands	Percentage of polymorphism	Band size
*Taq*I	65ºC	5	1	20%	354-740
*Alu*I	37 ºC	10	7	70%	333-1986
*Hinf*I	37 ºC	4	1	25%	200-453
Total		**19**	**9**	**47.37%**	

the polymorphism percentage was found to be 25%.

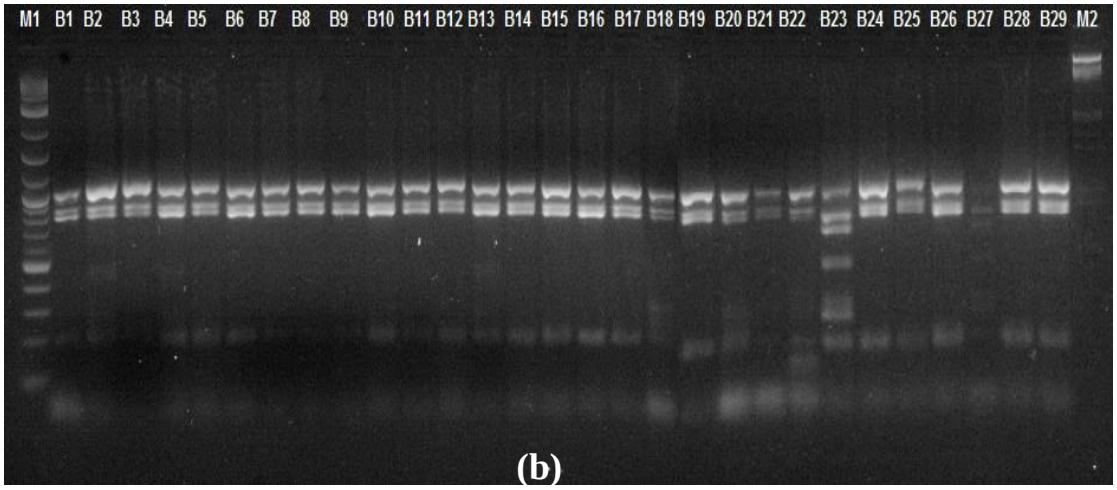
4.3.5.3 PCR-RFLP data analysis

A total of 19 scorable bands were produced by the various restriction digestion enzymes ranging in between 200-1986 bp. Of the 19 cuts 9 were polymorphic. The number of polymorphic bands ranged from one in *Taq*I and *Hinf*I to seven in *Alu*I. These clear and distinct bands were scored and used for further analysis. The percentage of polymorphism ranged from 20% to 70%. Restriction enzyme *Taq*I revealed 20% polymorphism, while *Hinf*I showed 25% polymorphism and *Alu*I generated 70% polymorphism. The overall polymorphism was found to be 47.37% (table 4.8).

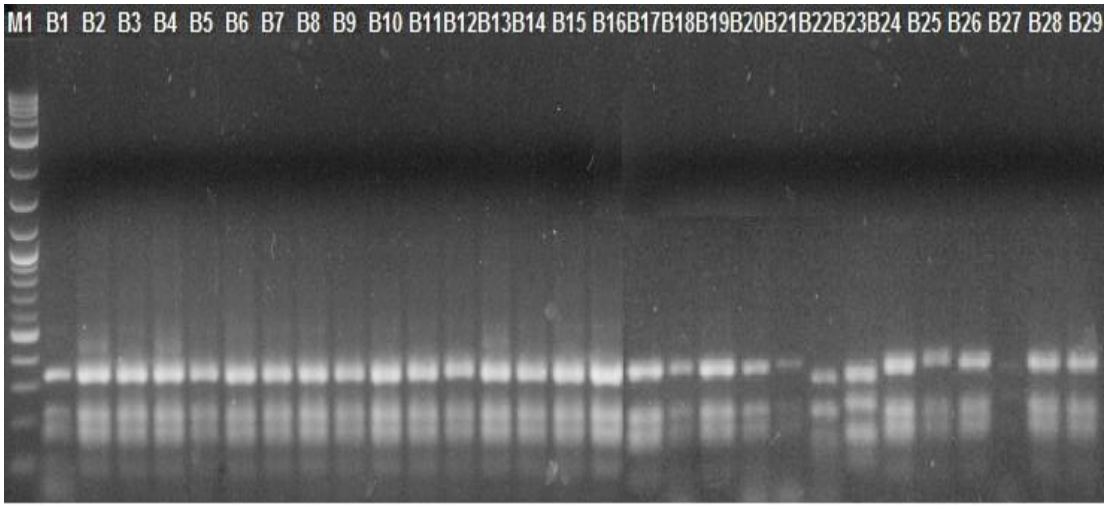
A dendrogram constructed on the basis of similarity estimates using the unweighted pair group method with arithmetic average (UPGMA) using NTSYSpc version (2.0) (Rohlf, 1998) is depicted in fig 4.13. The dendrogram was split into two clusters. *Phyllostachys nigra* was totally isolated and similarly *Melocanna baccifera* and *Shibataea kumasaca* were segregated from all other species of bamboo. The first cluster housed a total of 21 accessions of bamboo which was further divided into two groups. The first group consisted of 18 species, including 10 species from *Bambusa* of the total 12 under study, all the four species of *Dendrocalamus* along with *Gigantochloa* spp., *Pleioblastus argenteostriatus*, *Drepanostachyum intermedium* and *Sassaela ramosa*. The second group consisted of 2 species *Himalayacalamus hookerianus* and *Pseudosasa japonica* with a genetic similarity of TrnL-F region of 94.7% (table 4.9). The second cluster consisted of five species in which *Bambusa multiplex* „Alphanso Karr", *B. multiplex* „Rivierorum" and *Cephalostachym latifolium* clustered in the same sub-group. Similar type of closeness among these three species of bamboo was found in both the RAPD and ISSR analysis. *Drepanostachyum khasianum* and Chinese bamboo formed the other part of the second cluster. Both the 2D and 3D plot (fig 4.14) of the correspondence analysis of the RFPL data corroborated the dendrogram. The above analysis showed no major and well supported incongruences in the TrnL-F region of the chloroplast genome of different bamboo species. The probable reason for this is that the plastid DNA is generally non-recombining and



(a)



(b)



(c)

Figure 4.12: Restriction digestion products of TrnL-TrnF region of chloroplast genome. (a) *Taq*I; (b) *Alu*I and (c) *Hinf*I. Lane M1: 0.1-10 kb DNA ladder; Lane B1-B29: Different species of bamboo as listed in table 4.1 and M2: λ DNA/*Eco*RI/*Hin*dIII double digest DNA ladder

Table 4.9: The similarity matrix obtained using Dice coefficient of similarity among the 29 accessions of bamboo based on RFLP analysis of trnL-trnF

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14	B15	B16	B17	B18	B19	B20	B21	B22	B23	B24	B25	B26	B27	B28	B29
B1	1.000																												
B2	0.895	1.000																											
B3	0.947	0.842	1.000																										
B4	0.895	1.000	0.842	1.000																									
B5	1.000	0.895	0.947	0.895	1.000																								
B6	1.000	0.895	0.947	0.895	1.000	1.000																							
B7	1.000	0.895	0.947	0.895	1.000	1.000	1.000																						
B8	1.000	0.895	0.947	0.895	1.000	1.000	1.000	1.000																					
B9	1.000	0.895	0.947	0.895	1.000	1.000	1.000	1.000	1.000																				
B10	1.000	0.895	0.947	0.895	1.000	1.000	1.000	1.000	1.000	1.000																			
B11	1.000	0.895	0.947	0.895	1.000	1.000	1.000	1.000	1.000	1.000	1.000																		
B12	1.000	0.895	0.947	0.895	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000																	
B13	0.895	1.000	0.842	1.000	0.895	0.895	0.895	0.895	0.895	0.895	0.895	0.895	1.000																
B14	1.000	0.895	0.947	0.895	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.895	1.000															
B15	1.000	0.895	0.947	0.895	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.895	1.000	1.000														
B16	1.000	0.895	0.947	0.895	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.895	1.000	1.000	1.000													
B17	1.000	0.895	0.947	0.895	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.895	1.000	1.000	1.000	1.000												
B18	0.895	0.895	0.842	0.895	0.895	0.895	0.895	0.895	0.895	0.895	0.895	0.895	0.895	0.895	0.895	0.895	0.895	1.000											
B19	0.947	0.842	1.000	0.842	0.947	0.947	0.947	0.947	0.947	0.947	0.947	0.947	0.842	0.947	0.947	0.947	0.947	0.842	1.000										
B20	1.000	0.895	0.947	0.895	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.895	1.000	1.000	1.000	1.000	0.895	0.947	1.000									
B21	0.895	0.789	0.842	0.789	0.895	0.895	0.895	0.895	0.895	0.895	0.895	0.895	0.789	0.895	0.895	0.895	0.895	0.895	0.842	0.895	1.000								
B22	0.895	0.789	0.842	0.789	0.895	0.895	0.895	0.895	0.895	0.895	0.895	0.895	0.789	0.895	0.895	0.895	0.895	0.895	0.842	0.895	0.895	1.000							
B23	0.737	0.737	0.684	0.737	0.737	0.737	0.737	0.737	0.737	0.737	0.737	0.737	0.737	0.737	0.737	0.737	0.737	**0.632**	0.684	0.737	0.632	0.632	1.000						
B24	1.000	0.895	0.947	0.895	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.895	1.000	1.000	1.000	1.000	0.895	0.947	1.000	0.895	0.895	0.737	1.000					
B25	0.947	0.842	0.895	0.842	0.947	0.947	0.947	0.947	0.947	0.947	0.947	0.947	0.842	0.947	0.947	0.947	0.947	0.842	0.895	0.947	0.947	0.842	0.684	0.947	1.000				
B26	1.000	0.895	0.947	0.895	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.895	1.000	1.000	1.000	1.000	0.895	0.947	1.000	0.895	0.895	0.737	1.000	0.947	1.000			
B27	0.789	0.684	0.737	0.684	0.789	0.789	0.789	0.789	0.789	0.789	0.789	0.789	0.684	0.789	0.789	0.789	0.789	0.789	0.737	0.789	0.895	0.789	0.737	0.789	0.842	0.789	1.000		
B28	1.000	0.895	0.947	0.895	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.895	1.000	1.000	1.000	1.000	0.895	0.947	1.000	0.895	0.895	0.737	1.000	0.947	1.000	0.789	1.000	
B29	0.947	0.947	0.895	0.947	0.947	0.947	0.947	0.947	0.947	0.947	0.947	0.947	0.947	0.947	0.947	0.947	0.947	0.947	0.895	0.947	0.842	0.842	0.684	0.947	0.895	0.947	0.737	0.947	1.000

For details on sample ID (B1– B29) please refer table 4.1 in the results and discussion section

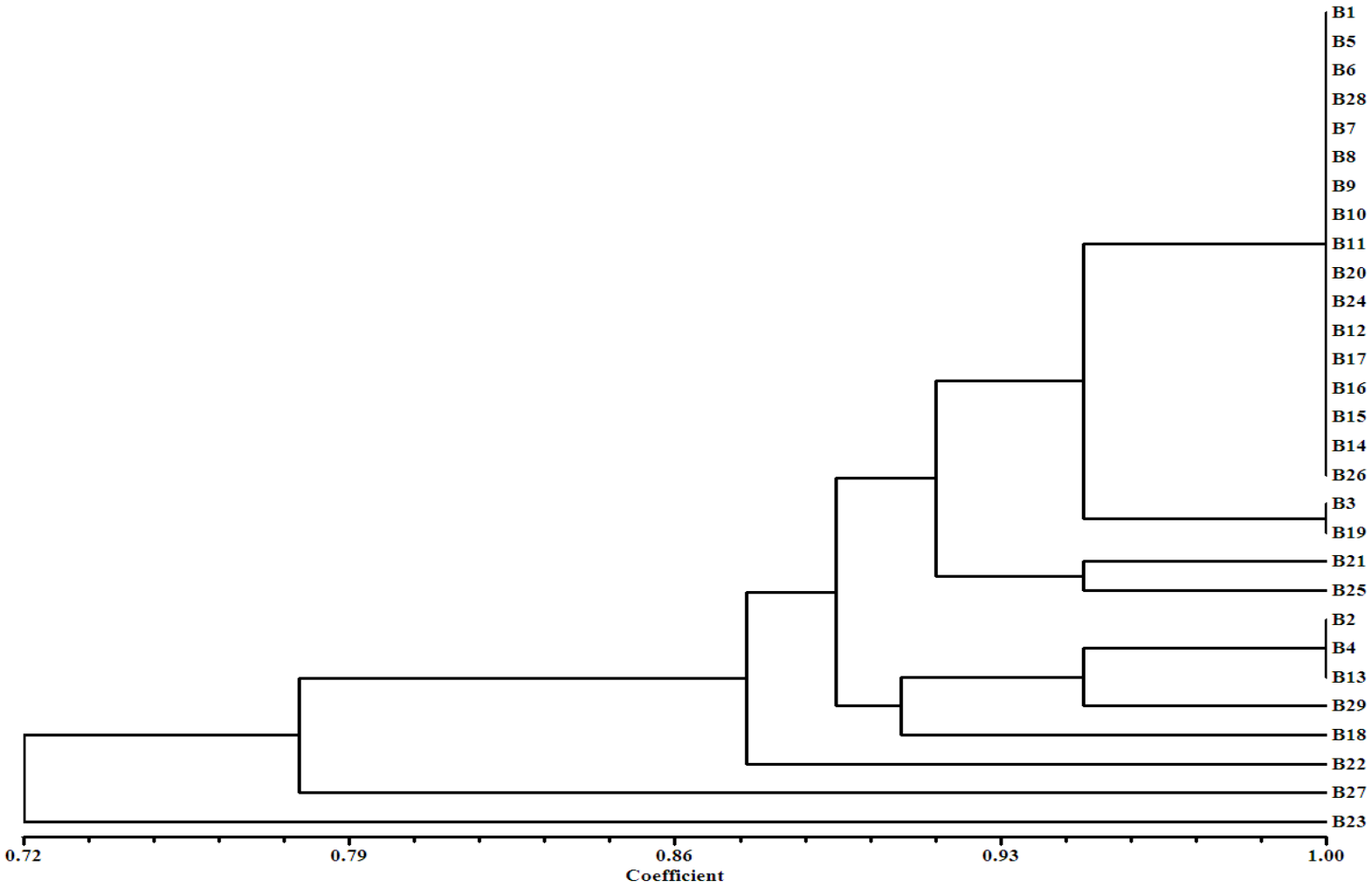


Figure 4.13: Dendrogram based on the restriction digestion products data of the TrnL-TrnF region of different bamboo genome

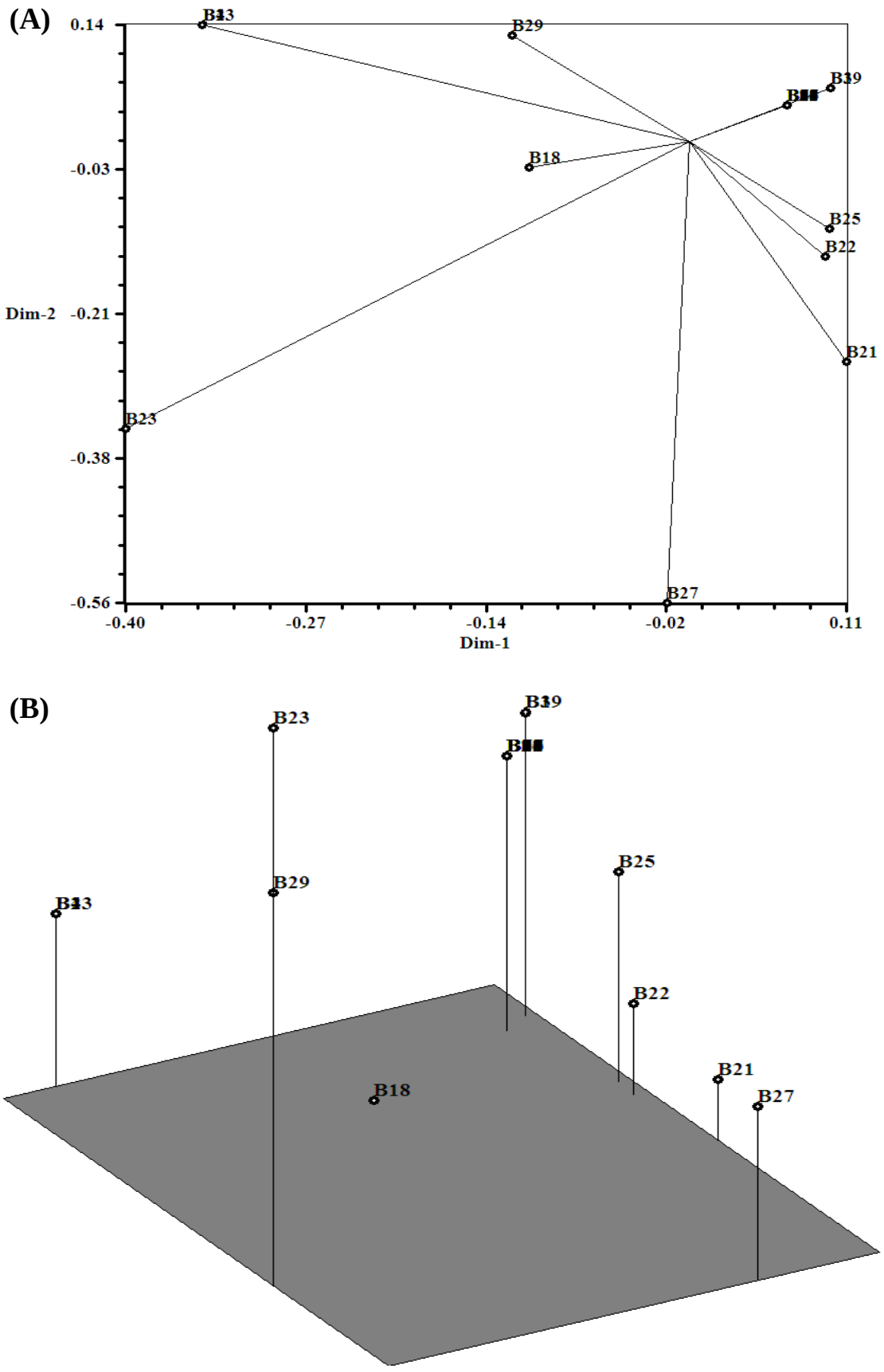


Figure 4.14: Principal coordinate analysis of 29 accessions of bamboo based on restriction digestion products of TrnL-TrnF region of chloroplast genome. (A) 2-dimensional plot and (B) 3-dimensional plot

maternally inherited in most angiosperms. However, from this study it is clear that the genus *Bambusa* is closely related to *Dendrocalamus* and *Gigantochloa* which is at par with the morphological taxonomy (Li and Xue, 1997; Li, 1998; Li and Stapleton, 2006). Similar observation was also made by Sungkaew and his co workers (2009) while assessing the phylogenetic relationships among the bamboos taking into account five plastid DNA regions.

4.3.5.4 Sequencing of PCR amplification product, performing BLAST and submission

A total of 13 samples (each representing one genera of bamboo) were sequenced from Chromous Biotech Pvt. Ltd, Bangalore for both the forward and reverse primers individually. The sequencing resulted in an average of 790bp for each reaction. In the present study the nucleotide BLAST was performed for each of the sequence obtained to find out the homology with the sequences already present in the GenBank. The nucleotide BLAST showed 95 to 100% identity with the bamboo sequence already available in the GenBank. After authentication of the sequences were submitted to the GenBank (fig 4.17). The list of different species of bamboo along with their GenBank accession number is given in table 4.10.

4.3.6 Exploration of 16S rDNA of chloroplast genome

4.3.6.1 PCR product agarose gel analysis

In this study the genomic DNA of all the 29 accessions of bamboo were subjected to PCR amplification using a primer pair consisting of forward and reverse primers to target the 16S rDNA region of the chloroplast DNA of bamboo. The primer pair successfully amplified the 16s rDNA region of the chloroplast genome. When the PCR product was resolved in agarose gel, the primer pair targeting the 16s rDNA region amplified a band of about 563bp (fig 4.15).

4.3.6.2 PCR product restriction digestion and agarose gel analysis

The PCR products were subjected to restriction digestion using different restriction enzymes like *Hae*III, *Hpa*I and *Msp*I to examine the degree of genetic variation among different species and varieties of bamboo. Different restriction enzyme resulted in different banding pattern and generated bands of different base pairs. The restriction enzyme *Hae*I produces

Table 4.10: List of bamboo species with the GenBank accessions for TrnL-TrnF region

Sl. No.	Plant species	GenBank accession number
1.	*Bambusa vulgaris* „Vittata" (TabC)	KC404810
2.	*Bambusa vulgaris* „Vittata" (TabF)	KC404822
3.	*Cephalostachyum latifolium* (TabC)	KC404812
4.	*Cephalostachyum latifolium* (TabF)	KC404809
5.	*Dendrocalamus hamiltonii* (TabC)	KC292015
6.	*Dendrocalamus hamiltonii*(TabF)	KC292013
7.	*Drepanostachyum khasianum* (TabC)	KC404811
8.	*Drepanostachyum khasianum* (TabF)	KC404814
9.	*Gigantochloa* spp. (TabC)	KC404799
10.	*Gigantochloa* spp. (TabF)	KC404825
11.	*Himalayacalamus hookerianus* (TabC)	KC404819
12.	*Himalayacalamus hookerianus* (TabF)	KC404818
13.	*Melocanna baccifera* (TabC)	KC404808
14.	*Melocanna baccifera* (TabF)	KC404817
15.	*Phyllostachys nigra* (TabC)	KC404815
16.	*Phyllostachys nigra* (TabF)	KC404803
17.	*Pleioblastus argenteostriatus* (TabC)	KC292011
18.	*Pleioblastus argenteostriatus*(TabF)	KC292014
19.	*Pseudosasa japonica*(TabC)	KC292016
20.	*Pseudosasa japonica*(TabF)	KC292018
21.	*Sasaella ramosa*(TabC)	KC292017
22.	*Sasaella ramosa*(TabF)	KC292012
23.	*Shibataea kumasaca* (TabC)	KC404807
24.	*Shibataea kumasaca* (TabF)	KC404801
25.	*Yushania maling* (TabC)	KC404821
26.	*Yushania maling* (TabF)	KC404798

bands between 304-675 bp, *Hpa*I 344-612 bp and *Msp*I 337-613 bp. The result obtained from restriction digestion with the enzyme *Msp*I is depicted in figure 4.16. However the chloroplast DNA of the different samples analyzed showed very little variation among them. Therefore the statistical analysis has not been performed.

4.3.7 Comparative account of the DNA fingerprinting study

A detailed study of DNA fingerprinting showed that among various techniques used in this study like RAPD, PCR-RFLP (trnL-trnF gene and 16s rDNA gene) and ISSR markers, ISSR marker proved to the most efficient molecular marker revealing 100% polymorphism among the twenty nine accessions of bamboo under study. This was followed by the RAPD technique which showed polymorphism of about 99.39% among the different species and cultivars of bamboo. RAPD

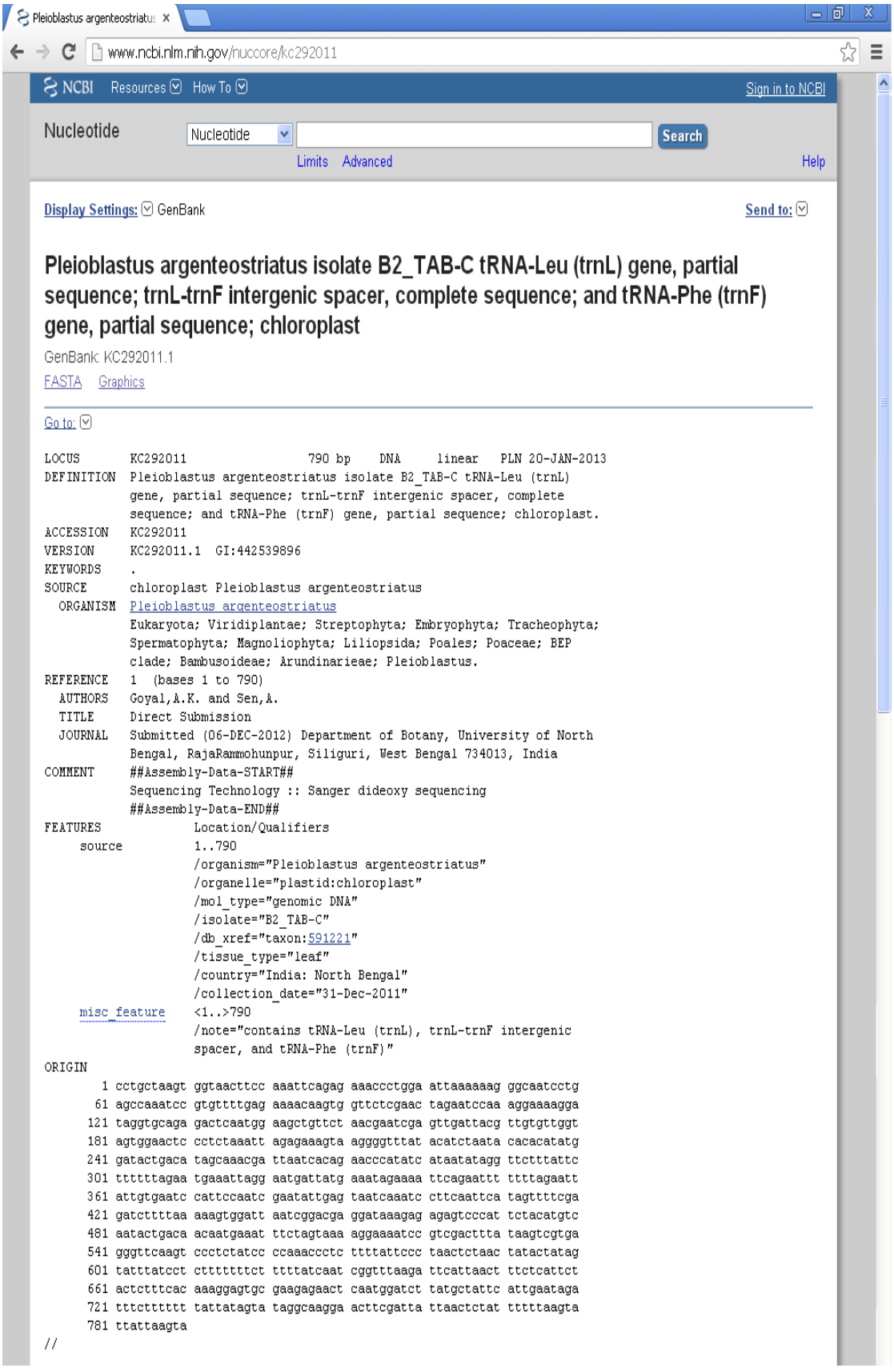


Figure 4.17: Snapshot of partial sequence of *Pleioblastus argenteostriatus* chloroplast genome (TrnL–F region) with Genbank accession number.

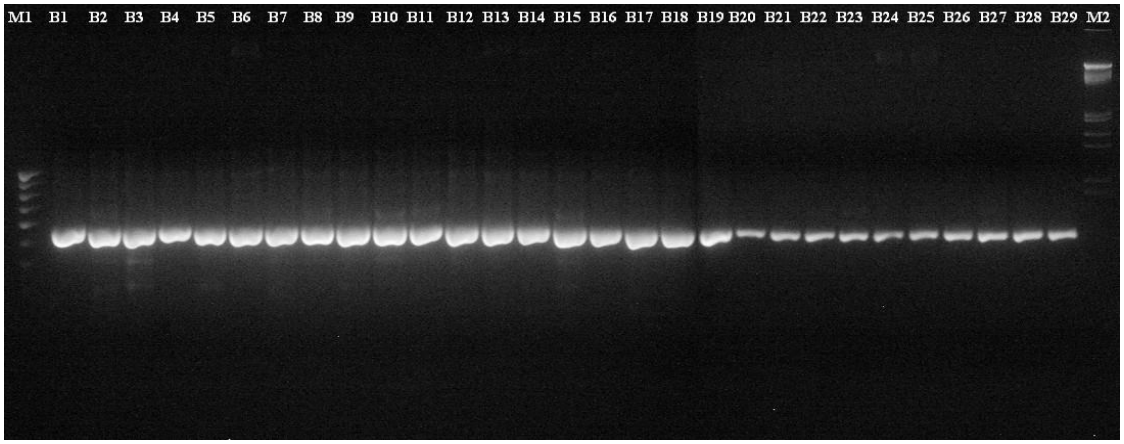


Figure 4.15: PCR amplification product of 29 accessions of bamboo for 16S rDNA region of the chloroplast genome. Lane M1: 100bp DNA ladder; Lane B1-B29: Different species of bamboo as listed in table 4.1 and M2: λ DNA/*Eco*RI/*Hin*dIII double digest DNA ladder

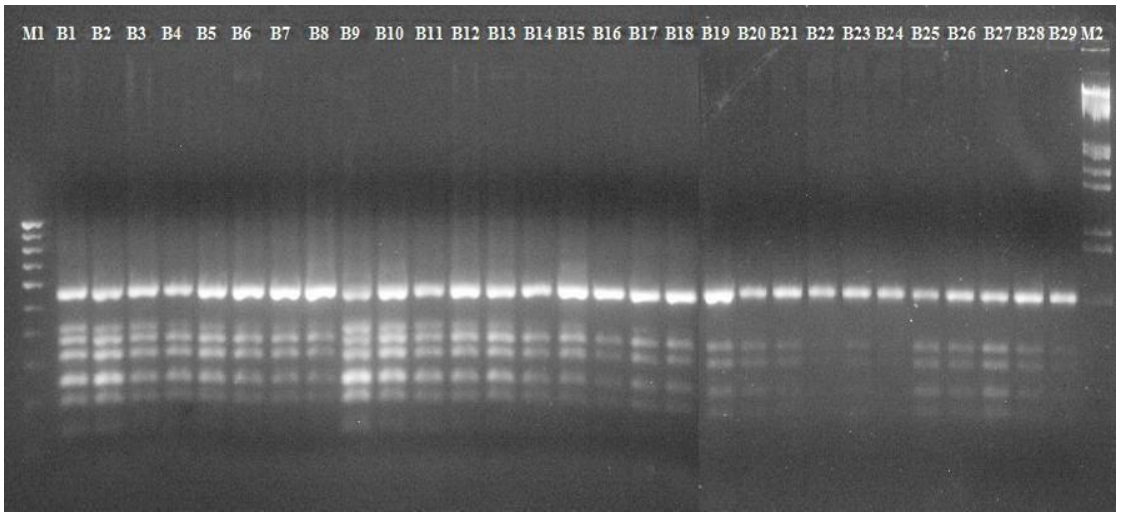


Figure 4.16: Banding pattern of 16S rDNA PCR product of different bamboo under study after restriction digestion with restriction enzyme *Msp*I. Lane M1: 100bp DNA ladder; Lane B1-B29: Different species of bamboo as listed in table 4.1 and M2: λ DNA/*Eco*RI/*Hin*dIII double digest DNA ladder

technique revealed a quick and effective means to establish the genetic relationships between the bamboo species without any prior knowledge of genomes or the use of polyacrylamide gels and radioactivity for resolution. Different species and cultivars can be conserved on the basis of their molecular differences under the light of the statistical analysis using newly developed software like NTSYSpc. The sixteen RAPD primers (table 4.3) and nine ISSR primers (table 4.5) revealing 99.39 % and 100 % polymorphism respectively may prove to be the most efficient markers obtained from this study. These markers may provide a cheap, rapid and effective means to evaluate the genetic diversity among a different

bamboo and help devise sampling strategies to compliment classical morpho-agronomic descriptors. PCR-RFLP technique using trnL-trnF region revealed a polymorphism of 47.37% among the bamboos under study. The only disadvantage of this method is that it requires locus specific primers for studying various gene locations in the genome and also it requires very pure DNA for restriction digestion studies. However, work done on PCR-RFLP of trnL-trnF region have provided us with a very good data i.e., sequences of 13 (thirteen) species of bamboo representing thirteen genera encountered in North Bengal and their GenBank accession numbers has been obtained which can be helpful in future for conservation of bamboo germplasm and will also help various workers working in this field. PCR-RFLP of 16s rDNA region of the 29 accessions of bamboo could not reveal any good amount of polymorphism among them, thus it may be concluded that 16S rDNA of the chloroplast genome may not be a good candidate for the study of bamboo diversity. Thus, overall DNA fingerprinting study of bamboos have generated lots of new markers for identification of bamboo and can prove to be effective and promising marker in assessing genetic variations among the bamboo species found in North Bengal.

4.4 *In vitro* regeneration of *Dendrocalamus strictus*

4.4.1 Plant material

In this experiment single nodal segments were used as explants for *in vitro* regeneration. The nodal segment was opted since they have active meristems and has the potential to produce axillary buds which in turn can develop into plantlets. This property of nodal culture forms the basis of vegetative propagation. However using tissue culture techniques the rate of shoot multiplication can be increased to many folds. The phytohormone (cytokinins and auxins) accelerates the event. It is due to the continuous availability of the cytokinin in the growth medium that the nodal segment gives rise to axillary bud which grows into shoot. Thus the nodal segment proves to be suitable explants for *in vitro* regeneration (Aruna *et al*., 2009; Ramadevi *et al*., 2012).

4.4.2 Culture initiation

Contamination by fungi and bacteria was the main problem that posed during the early stage of the culture initiation. To get rid of this, various pre -disinfection treatments (extran, 70% ethanol, cetrimide and mercuric

chloride) were employed but it could not eliminate the contaminants totally but however was effective in reducing the rate of bacterial and fungal infection. Extran in combination with $HgCl_2$ has been used in the tissue culture study of *Thamnocalamus spathiflorus*, the Asian bamboo by Bag and his co-workers (2000) previously. Mercuric chloride and 70% ethanol have been used to disinfect the explants of *Bambusa wamin* and *Dendrocalamus farinosus* (Arshad *et al.*, 2005; Hu *et al.*, 2011) respectively. Other than these different chemicals have been used by different workers in tissue culture to get rid of contaminations in several bamboo species belonging to different genera (Arya, 1999; Ndiaye *et al.*, 2006; Arya *et al.*, 2008; Mehta *et al.*, 2010; Bejoy *et al.*, 2012; Devi *et al.*, 2012). The contamination of the culture was observed parallel to the separating of the sheaths and sprouting of the explants. It can be inferred from the above observation that the contaminants are housed within the layers of the sheaths and just mere surface sterilization failed to remove them. Pre-treatment of the explants with 0.1% $HgCl_2$ for 5 min could help to eliminate bacterial infection but to eradicate the fungal infection, the duration of $HgCl_2$ treatment was increased to 10 min. These explants remained green and healthy growth and proliferation of the axillary shoots were observed.

4.4.3 In vitro bud breaking and shoot initiation

Only 20% of the explants sprouted when cultured in basal medium. The percentage of bud breaking frequency was increased by supplementing the basal medium with plant growth regulators like BAP and Kinetin. Regeneration of the plantlets also depended upon the type of medium used. MS medium was found to suitable in comparison with the wood plant medium (WPM) for bamboo. Since the WPM was not found to be effective it was not used anymore and only MS was employed for further regeneration. This indicates that that some of the essential component required by bamboo for its regeneration is not available in WPM. Highest sprouting rate (49%) was found to be in MS medium with 4mg/l BAP. Further increase in the hormone concentration resulted in reduction of bud sprouting. Of the two hormones BAP was found to be more suitable (table 4.11). The data pertaining the

effect of BAP and Kn individually on the shoot multiplication of *D. stictus* is depicted in table 4.11. It was observed that different concentration of BAP had a significant effect on shoot multiplication. The maximum rate of shoot multiplication (3.68 shoots per explant) was observed on medium supplemented with 4 mg/l BAP which was significantly higher than other BAP treatments after 6 weeks. The effect of Kn on shoot multiplication was found to be moderate upto 5mg/l with highest (2.66 shoots per explant) at 4mg/l Kn after 6 weeks. The hormones also had considerable effect on the shoot length. The highest shoot length after 6 weeks (3.11 cm) was noted with 5mg/l BAP followed by 3.07 cm with 5mg/l Kn while decrease in the shoot length was noted with other treatments. The new shoots were excised from the *in vitro* developed shoots for subculture. The subculture was done every 2 weeks. The increase in shoot number was observed in the first two subsequent subcultures in all the treatments with BAP (i.e 1-5 mg/l) and then reduced thereafter (table 4.12). However the best multiplication of 3.86 shoots per explant was recorded during the 2nd subculture in MS medium supplemented with 4mg/l BAP (table 4.12). Both the cytokinins behaved differently in the present investigation with BAP being more effective compared to Kn. BAP is most commonly used cytokinin mainly due to two fold reasons, firstly it is cheap and secondly it can be autoclaved (Thomas and Blakesley, 1987). In the present study BAP was found to be effectual for shoot multiplication. These findings are in accordance with the earlier work on *in vitro* propagation of different bamboo species like *Bambusa arundinacea* (Ansari et al., 1996), *Dendrocalamus asper* (Arya and Arya, 1997), *Bambusa ventricosa* (Huang and Huang, 1995), *Bambusa nutans and Dendrocalamus membranaceus* (Yasodha et al., 1997), *Bambusa bambos* (Arya and Sharma, 1998), *Bambusa glaucescens* (Shirin and Rana 2007), *Bambusa balcooa* (Mudoi and Borthakur, 2009), *Dendrocalamus hamiltonii* (Arya et al., 2012) where BAP has been widely used and was found to be effective. The less effectiveness of Kn on *in vitro* propagation of *Dendrocalamus strictus* was also noted by Nadgir and his co-workers (1984) and Das and Rout (1991).

4.4.4 In vitro rhizogenesis and acclimatization

Table 4.11: Effect of BAP and Kinetin on bud breaking frequency, shoot bud proliferation and shoot length

Hormone	mg/l	Bud break frequency (%)	No of primary shoots per explant			Shoot length after 6 weeks (cm)
			2 weeks	4 weeks	6 weeks	
BAP	1	32	1.25±0.31	1.38±0.20	1.46±0.07	1.67±0.11
	2	36	1.93±0.24	1.99±0.11	2.23±0.21	1.89±0.18
	3	45	2.32±0.49	2.46±0.24	2.89±0.20	2.04±0.14
	4	49	3.18±0.12	3.40±0.17	3.68±0.37	2.61±0.14
	5	46	2.88±0.27	3.11±0.54	3.46±0.32	3.11±0.41
Kinetin	1	27	1.06±0.13	1.22±0.19	1.46±0.16	1.74±0.11
	2	33	1.69±0.10	1.84±0.13	2.14±0.21	1.96±0.18
	3	38	2.02±0.09	2.16±0.15	2.45±0.17	2.13±0.09
	4	36	2.31±0.43	2.43±0.32	2.66±0.29	2.58±0.30
	5	32	2.16±0.27	2.31±0.18	2.48±0.21	3.07±0.28

Table 4.12: Effect of BAP on shoot proliferation in the subsequent subculture

Hormone	mg/l	No of primary shoots per explant (after 2 weeks)	Subculture (shoots per explant after 2 weeks)		
			1st	2nd	3rd
BAP	1	1.25±0.06	1.32±0.09	1.49±0.11	1.33±0.15
	2	1.93±0.11	2.03±0.08	2.18±0.17	2.29±0.08
	3	2.32±0.09	2.54±0.19	2.78±0.22	2.72±0.10
	4	3.18±0.17	3.41±0.22	3.86±0.19	3.72±0.13
	5	2.88±0.10	3.19±0.27	3.77±0.21	3.74±0.19

The results of root induction among the *in vitro* regenerated plantlets achieved by culturing on either MS medium or half strength MS medium supplemented with different auxins like IBA and NAA either singly or in combination is depicted in table 4.13 and 4.14. About 87% of the shoots rooted in MS medium supplemented with either IBA or NAA or combination of both (1-3 mg/l). The highest number of root (1.36) was regenerated on full strength MS medium supplemented with 3 mg/l NAA (table 4.13), while maximum length of 1.64 cm of roots was recorded with combination of 1 mg/l IBA and 3 mg/l NAA (table 4.14). The half strength MS medium was found to be less effective for *in vitro* rooting in comparison to full strength MS medium. This result was in accordance with previous reports (Rout *et al.*, 1999; Kumaria *et al.*, 2012). The

Table 4.13:Effect of IBA and NAA on various parameters of *in vitro* rooting in *D. strictus*

Rooting media	Hormone	(mg/l)	No of primary roots per explant	Root length (cm)
MS	IBA	1	0.32±0.01	0.88±0.03
	IBA	2	0.78±0.03	1.12±0.02
	IBA	3	1.24±0.09	1.56±0.07
	NAA	1	0.29±0.01	0.64±0.01
	NAA	2	0.81±0.02	0.98±0.09
	NAA	3	1.36±0.04	1.44±0.07
½ MS	IBA	1	0.21±0.01	0.58±0.02
	IBA	2	0.69±0.03	0.95±0.08
	IBA	3	0.98±0.04	1.09±0.05
	NAA	1	0.18±0.01	0.36±0.01
	NAA	2	0.56±0.04	0.69±0.02
	NAA	3	0.74±0.07	0.88±0.04

Table 4.14:Combined effect of IBA and NAA on *in vitro* rooting

Rooting media	Hormone (mg/l)		No of primary roots per explant	Root length (cm)
	IBA	NAA		
MS	1	1	0.54±0.01	1.02±0.03
	1	2	0.87±0.01	1.21±0.06
	1	3	1.32±0.03	1.64±0.03
	2	1	0.62±0.01	0.88±0.01
	2	2	1.11±0.06	1.16±0.02
	2	3	1.06±0.02	1.29±0.04

plantlets with well developed roots after transplantation to potting mixture containing perlite, soil and farm yard manure with a ratio of 1:1:1 (by volume) exhibited 70% survival rate and grew in the greenhouse (fig 4.18). After a month these acclimatized plants were transferred to the field.

4.4.5 Somaclonal variation among the **in vitro** *raised plantlets*

In bamboo micropropagation through axillary bud proliferation where no intermediary callus formation occurs has been largely attempted (Saxena, 1990; Saxena and Bhojwani, 1993; Arya *et al*., 1999; Jimenez *et al*., 2007). The *in vitro* raised plants are expected to be genetically identical to the mother plant (used as explants), but however possibility of having some genetic variations cannot be ignored. Somaclonal variation is thought be a common event in the *in vitro* raised plants which include an array of genetic and epigenetic variations (Peredo *et al*., 2006). In this study an attempt was made to screen the *in vitro* raised *Dendrocalamus strictus* plantlets for somaclonal variations (if any) by employing both RAPD and ISSR

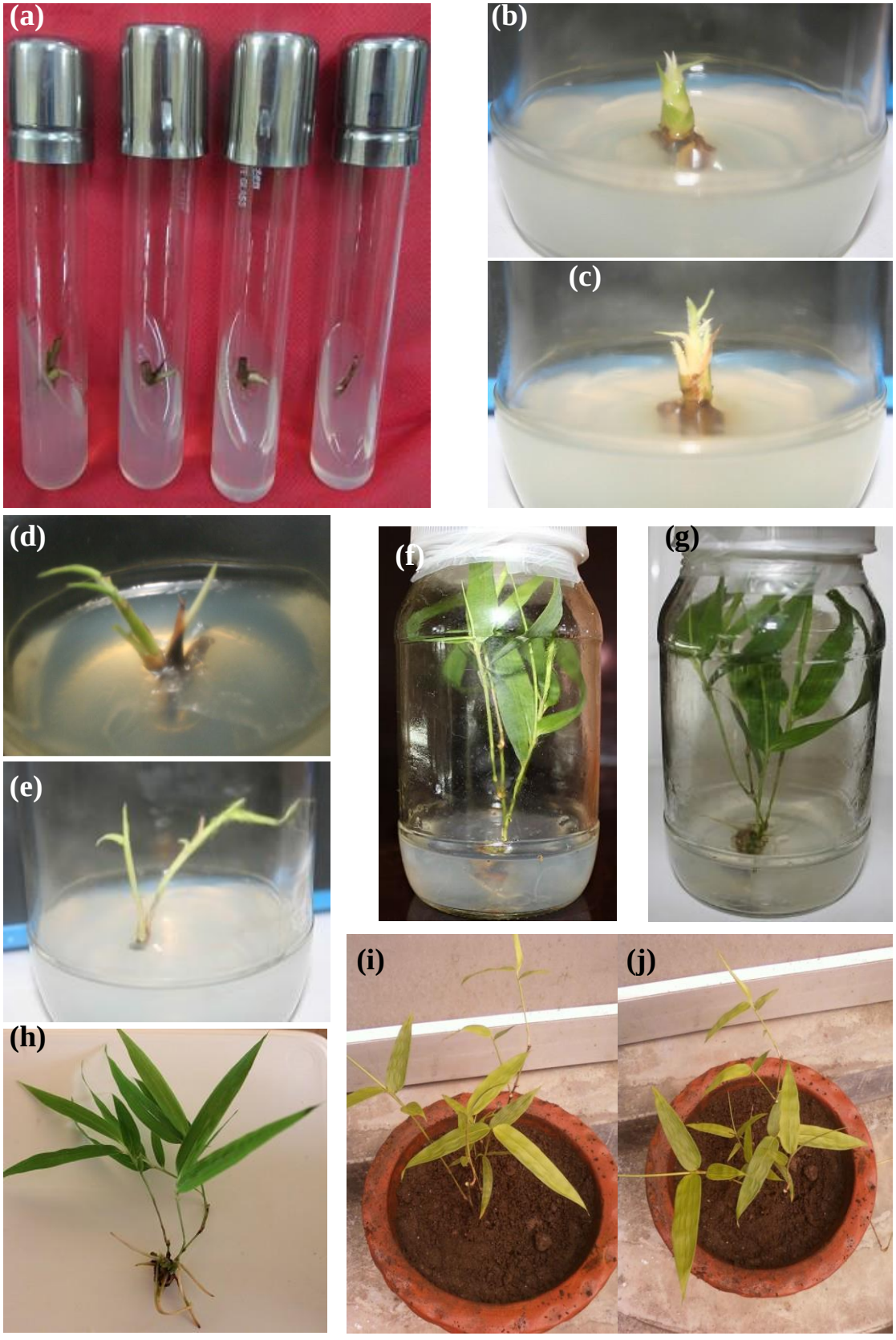


Figure 4.18: Stages in development of *in vitro* cultured *Dendrocalamus strictus.*
(a) Axillary bud breaking. (b) Healthy axillary bud increasing its volume. (c & d) Multiple shooting. (e) Development of leaves. (f &g) Root induction in rooting medium. (h) Plantlet with well developed roots. (i & j) Acclimatized plant in the green house.

markers analysis. A total of 19 primers (which already successfully amplified the genomic DNA of all the 29 accessions of bamboo) where used to screen somaclonal variations, out of which 10 were RAPD and rest 9 were ISSR primers. The amplification using all the 10 RAPD primers resulted in 58 scorable bands where as the ISSR primers resulted in producing 66 distinct and scorable bands (table 4.15). The number of bands varied from 3 to 11 in case of RAPD primers and 5 to 11 in case of ISSR markers with an average of 5.8 and 7.33 bands respectively. All the bands generated were found to be monomorphic across all the *in vitro* raised plantlets and the parent plant analyzed irrespective of whether RAPD primers or ISSR markers were used. A representative of RAPD and ISSR profile is depicted in figure 4.19. The band size ranged in between 240-1455 bp and 183-1544 bp in case of RAPD and ISSR markers respectively. This uniformity in the banding pattern confirms the genetic fidelity of the *in vitro* raised *Dendrocalamus strictus* plantlets. Detection of clonal fidelity in *in vitro* raised bamboo have been attempted by Das and Pal (2005a) and Negi and Saxena (2010). In *Bambusa balcooa* and *Bambusa tulda*, Das and Pal (2005b) used merely 5 RAPD decamers to establish the genetic stability among the micropropagated plantlets. Later in 2010, Negi and Saxena made an attempt to access the genetic fidelity among the regenerants of *Bambusa balcooa* employing 15 ISSR markers. Though both RAPD primers and ISSR markers helps in detecting polymorphism, ISSR markers

Table 4.15: PCR amplicons obtained from RAPD and ISSR markers in *in vitro* raised *Dendrocalamus strictus*

Primer ID	Total bands	No. of monomorphic bands	Band size (bp)
OPA03	7	7	240-1145
OPA07	6	6	245-892
OPA11	4	4	545-1455
OPA20	6	6	344-667
OPF09	5	5	435-710
OPG19	11	11	249-893
OPH04	3	3	451-864
OPN04	7	7	409-851
OPN13	6	6	358-1375
OPN19	3	3	315-1444
Total bands	**58**	**58**	
UBC810	8	8	309-845
UBC815	9	9	190-1554
UBC818	11	11	137-1011
UBC822	5	5	587-1214
UBC824	7	7	305-1237
UBC825	7	7	330-1152
UBC841	9	9	312-1163
UBC856	5	5	183-1108
UBC873	5	5	294-857
Total bands	**66**	**66**	

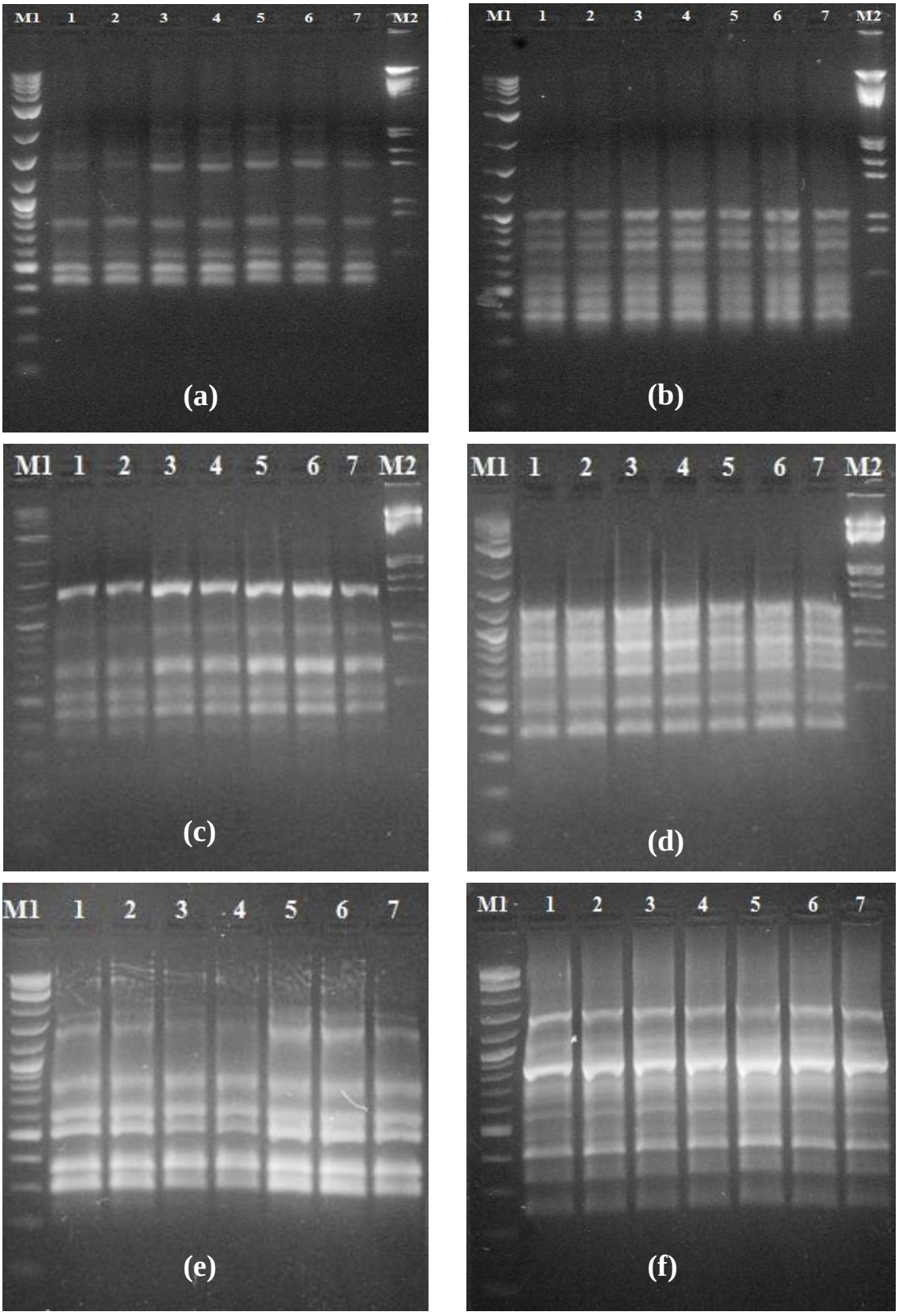


Figure 4.19: DNA fingerprinting patterns of *in vitro* raised *D. strictus*. ISSR primers, (a) UBC824; (b) UBC810; (c) UBC822 and RAPD primers (d) OPH04; (e) OPN04 and (f) OPG19, among *in vitro* regenerated plantlets compared with the donor plant: Donor plant (lane1), micropropagated plants (lanes 2-7) and molecular weight markers 0.1-10 kb DNA ladder (M1) and λ DNA/*Eco*RI/*Hin*dIII double digest (lane M2).

dominates the RAPD primers. ISSR markers showed high polymorphism and have good reproducibility because of the presence of large number of SSR region as compared to RAPD (Ray *et al*., 2006). Moreover, ISSR markers are much larger in size than RAPD which are decamers and hence has higher annealing temperature. Higher annealing temperature is considered to result in greater consistency where as lower annealing temperature might produce artefact amplicons because of non-specific amplification (Bornet and Branchard, 2001). Both the fingerprinting techniques have their own advantages and disadvantages. Thus, keeping this in mind both RAPD primers and ISSR markers were used to screen the clonal stability of *Dendrocalamus strictus*. This analysis showed that there was virtually no variability among the micropropagated plantlets of *Dendrocalamus strictus* and thus can be concluded that the *in vitro* raised plants avoided the genomic aberrations and did not lead to any somaclonal variation.

4.5 Antioxidant potential of bamboo leaf

Despite the growing interest in the bamboo not only as food additive but also as medicine because it is considered to be a good source of natural antioxidants, there is still scarcity of information related to the chemical constituents of many bamboo species. So here is an attempt to evaluate the *in vitro* antioxidant activities of six bamboo species encountered in North Bengal *viz*. *Bambusa vulgaris* „Vittata", *Bambusa pallida*, *Bambusa balcooa*, *Dendrocalamusstrictus*, *Dendrocalamus sikkimensis* and *Dendrocalamus hamiltonii*.

4.5.1 Plant yield

Efficiency of extraction is the important factor in studying the antioxidant potential of a plant. The percentage yield in different bamboo species ranged between 3.37% to 9.04% in water, 3.33% to 6.98 % in methanol and 2.79% to 5.57% in acetone (table 4.16).

4.5.2 Total phenol content

The total phenol content of crude extracts (aqueous, methanolic and acetonic) of different species of bamboo leaf was obtained from the regression equation (R) for the calibration curve of gallic acid and expressed as gallic acid equivalent (GAE). The phenolic content of all the six bamboo species (*Bambusa vulgaris* „Vittata", *Bambusa pallida*, *Bambusa*

Table 4.16: Plant yield of different bamboo leaf extracts

Plant yield (%)			
Species Name	**Water**	**Methanol**	**Acetone**
Bambusa vulgaris „Vittata‟	3.37	3.33	2.94
B. pallida	6.26	4.78	4.51
B. balcooa	3.74	4.21	2.79
Dendrocalamus strictus	8.55	5.93	5.57
D. sikkimensis	5.93	5.07	4.29
D. hamiltonii	9.04	6.98	4.86

Table 4.17: Total phenol contents of different bamboo leaf extracts

Phenol Content in mg GAE/g			
Species Name	**Water**	**Methanol**	**Acetone**
Bambusa vulgaris „Vittata‟	345.91±0.071	220.69±0.084	325.63±0.051
B. pallida	583.53±0.053	399.69±0.019	194.53±0.021
B. balcooa	367.90±0.016	378.55±0.030	219.96±0.026
Dendrocalamus strictus	112.103±11.2	68.942±6.89	36.158±3.61
D. sikkimensis	351.40±0.007	344.87±0.012	144.53±0.004
D. hamiltonii	285.26±0.01	392.13±0.06	178.38±0.02

balcooa, Dendrocalamus strictus, Dendrocalamus sikkimensis and *Dendrocalamus hamiltonii*) leaf extract in different solvent is depicted in table 4.17. There was a large dispersion in the concentration of phenols in the examined extracts ranging from 36.158±3.61 to 583.53±0.053 mg GAE/g. The higher concentrations of total phenol content was found to be in the aqueous extract irrespective of the species of bamboo, this was followed by methanol and acetone, except for *Bambusa vulgaris* „Vittata‟ where acetonic extract had higher phenol content compared to methanol. The highest phenol content was found in aqueous extract of *Bambusa pallida* leaf extract, while the lowest was recorded in the acetonic extract of *Dendrocalamus strictus* leaf extract. This difference in the phenolic content with respect to solvent type might be due to difference in polarity of the solvent depending upon which selective phenolic compounds percolate in the extract. Thus it can be inferred that the content of phenol in the plant extract depends upon the

polarity of the solvent used for extraction.

4.5.3 Total flavonoid content

The concentration of flavonoids of crude extracts (aqueous, methanolic and acetonic) of different species of bamboo leaf was expressed in terms of qercetin equivalent (QE). The summary of the total flavonoid content in the tested extracts are depicted in table 4.18. The concentration of flavonoids in the experimented samples differed greatly with respect to the solvent type ranging from 155.2±0.001 to 918.2±0.035 mg QE/g. The higher flavonoid content was measured in methanolic extracts followed by the acetonic and aqueous. *Bambusa vulgaris* „Vittata" leaf extract however behaved differently showing flavonoid content as acetonic> methanolic> aqueous. Higher level of flavonoids in methanolic extracts can be attributed to the fact that methanol is less polar than water and thus has the potential to release the bound flavonoids and polyphenols from the cell wall of the plant (Lapronic *et al*., 2005). This suggests that for flavonoids isolation the solvents of moderate polarity are reliable and methanol proved to be the best amongst the tested solvents.

4.5.4 Total flavonol content

The flavonol content of the aqueous, methanolic and acetonic extracts of the leaves of different bamboo species is elucidated in table 4.19. Flavonol was abundant in the methanolic extract compared to acetonic and aqueous extracts. Great variation was observed in the flavonol contents of different bamboo species ranging from 20.02±0.01 µg QE/g in *Dendrocalamus sikkimensis* methnolic extract to 0.153±0.001 µg QE/g in *D. sikkimensis* aqueous extract. Thus it can be said that the flavonols are potent methanol soluble antioxidants which prevent oxidative cell damage and is responsible for the pharmacological activity of the bamboo leaves.

4.5.5 Total proanthocyanidin content

Table 4.18: Total flavonoid contents of different bamboo leaf extracts

	Flavonoid Content in mg QE/g		
Species Name	**Water**	**Methanol**	**Acetone**
Bambusa vulgaris „Vittata"	155.2±0.001	159.80±0.047	289.2±0.008
B. pallida	179.2±0.003	912±0.037	303.2±0.006
B. balcooa	227.2±0.007	918.2±0.035	355.2±0.014
Dendrocalamus strictus	199.2±19.92	619.2±61.92	175.2±17.2
D. sikkimensis	223.2±0.007	895.2±0.006	161.2±0.004
D. hamiltonii	277.2±0.003	827.2±.007	187.2±0.001

Results obtained in the present study revealed that the level of proanthocyanidin was considerable in the methanolic extract compared to the aqueous (water) or acetonic extract irrespective of the species of bamboo (table 4.20). Among the aqueous extracts, *Dendrocalamus hamiltonii* showed the highest amount of proanthocyanidin with 53.59±0.03 µg Cathechin E/g while least was noted in *D. sikkimensis* with just 6.99±0.007 µg Cathechin E/g. The amount of proanthocyanidin varied in between 298.24±0.011 µg Cathechin E/g in *B. balcooa* to 108.04±0.009 µg Cathechin E/g in *Dendrocalamus strictus* in the methanolic extract. The acetonic extract didnot show much variation in the proanthocyanidin content irrespective of the bamboo species. The content was found between 38.45±0.011 µg Cathechin E/g in *B. balcooa* to 49.51±0.04 µg Cathechin E/g in *D. hamiltonii*. The presence of considerable amount of proanthocyanidin in bamboo leaf extract might be responsible for its antioxidant activity. Since higher amount of proanthocyanidin is found in the methanolic extract, it can be said that methanol proved to be suitable for solvent for extraction compared to other solvent types.

4.5.6 Total Antioxidant Activity

4.5.6.1 DPPH (2,2-Diphenyl-1-picrylhydrazyl) scavenging activity

This assay is based on the ability of DPPH (a protonated radical), to decolourise in the presence of antioxidants having the characteristic absorption maxima at 517nm. Thus, DPPH is extensively used in determining the radical scavenging activity of plants including bamboo (Jun *et al*., 2004). DPPH scavenging ability by the leaf extract of six bamboo species, screened in water, methanol and acetone solvent is depicted in fig. 4.20. The highest scavenging activity of 92.84 % was noted in the methanolic extract of *Bambusa balcooa* followed by 89.84% and 88.97% in *Dendrocalamus strictus*

Table 4.19: Total flavonol contents of different bamboo leaf extracts

	Flavonol Content in µg QE/g		
Species Name	**Water**	**Methanol**	**Acetone**
Bambusa vulgaris „Vittata“	3.14±0.002	4.14±0.01	2.31±0.001
B. pallida	0.51±0.000	14.18±0.007	1.73±0.001
B. balcooa	1.80±0.001	12.29±0.006	3.62±0.002
Dendrocalamus strictus	0.62±0.007	10.3±0.003	1.57±0.001
D. sikkimensis	0.153±0.001	20.02±0.01	1.12±0.001
D. hamiltonii	0.204±0.01	9.41±0.14	1.02±0.07

Table 4.20: Total proanthocyanidin contents of different bamboo leaf extracts

Proanthocyanidin content in µg Cathechin E/g			
Species Name	**Water**	**Methanol**	**Acetone**
Bambusa vulgaris „Vittata"	39.61±0.003	197.2±0.024	47.18±0.015
B. pallida	8.16±0.003	111.26±0.001	40.08±0.017
B. balcooa	20.39±0.009	298.24±0.011	38.45±0.011
Dendrocalamus strictus	9.63±0.002	108.04±0.009	42.31±0.006
D. sikkimensis	6.99±0.007	144.46±0.007	41.65±0.002
D. hamiltonii	53.59±0.03	233.58±0.02	49.51±0.04

and *B. vulgaris* „Vittata". Among the aqueous extracts the DPPH scavenging activity ranged in between 47.59% to 66.15%, among acetonic extracts between 20.19% to 61.63%; while it ranged from 79.84% to 92.84% in case of methanolic extracts. The scavenging activity was also found to increase with the increase in concentration of the extracts irrespective of the bamboo species. Overall the methanolic extract was found to be most effective followed by water, acetonic extract exhibited the lowest scavenging activity. This may be because of elevated levels of the active compounds together with the polyphenols in both the aqueous and the methanolic extracts which scavenge DPPH radicals and thus attribute to their higher antioxidant activity (Chueng *et al*., 2003; Goyal *et al*., 2011).

4.5.6.2 Ferric reducing power (FRP) assay

The reductive capability of the various bamboo leaf extracts and fractions compared to ascorbic acid is depicted in fig 4.21. The reducing power of various extracts of bamboo leaves was found to be notable, which increased gradually with a rise in concentration. The reductive capability is determined by the transformation of Fe^{3+} to Fe^{2+} in presence of either the extract or the ascorbic acid used as standard (Akinpelu *et al*., 2010). The aqueous extract was found to possess higher reductive capabilities in all cases except (*Dendrocalamus hamiltonii* where methanol showed highest). Compared to methanolic extracts, acetonic extracts of *Bambusa vulgaris* „Vittata", *B. pallida* and *Dendrocalamus strictus* had slightly higher reductive potentiality. From the

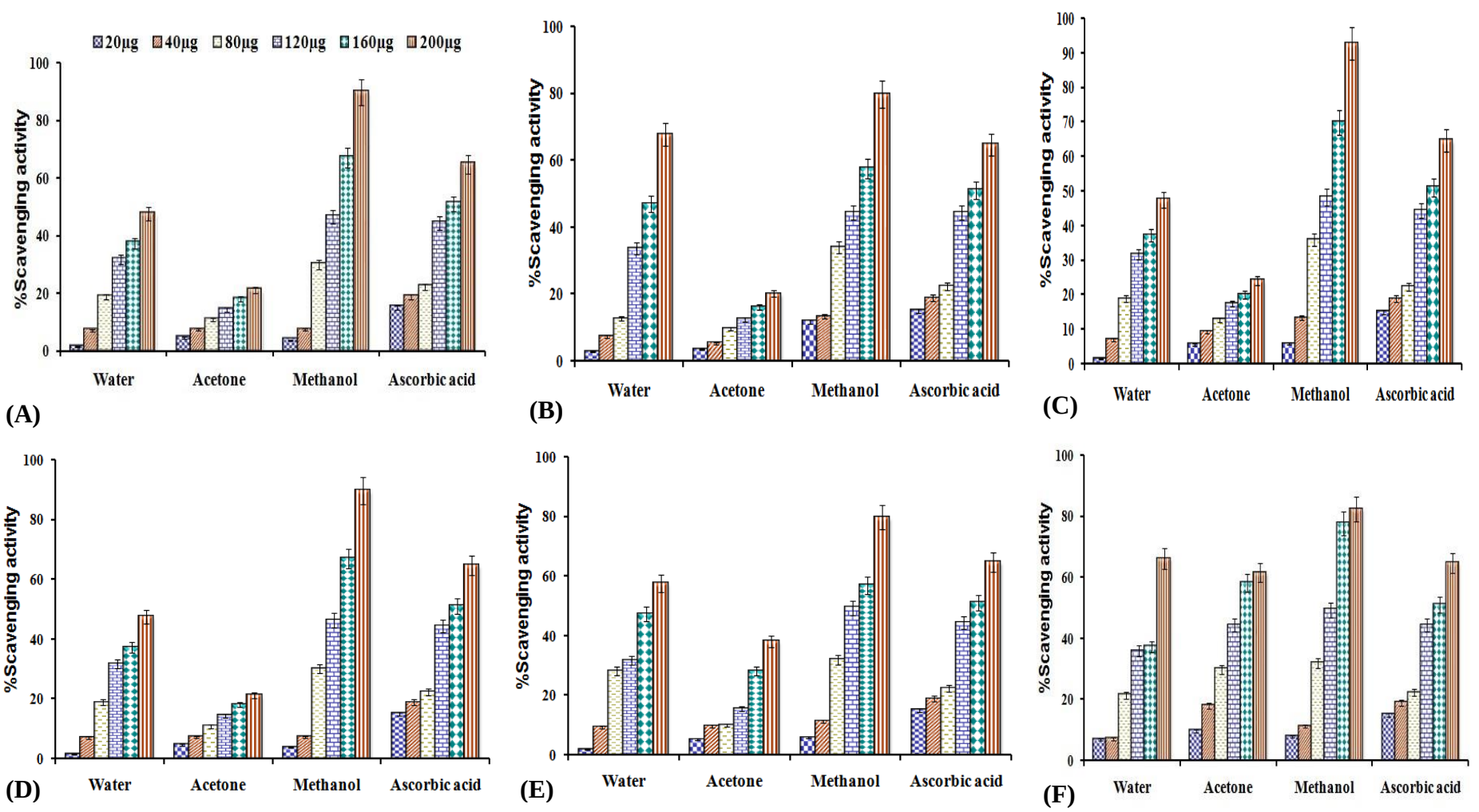


Figure 4.20: DPPH scavenging activity of bamboo leaf extracts . (A) *Bambusa vulgaris* „Vittata"; (B) *B. pallida*; (C) *B. balcooa*; (D) *Dendrocalamus strictus*; (E) *D. sikkimensis* and (F) *D. hamiltonii*

figure it is clear that at many events irrespective of the solvent type, the reducing capacity of the bamboo extracts complemented with the standard ascorbic acid.

4.5.6.3 Hydrogen peroxide scavenging activity

As shown in figure 4.22, different bamboo extract exhibited hydrogen peroxide reducing activity in a concentration manner. The H_2O_2 scavenging activity was found to be between 50.58 to 99.35% in all the solvent types. The higher values were however shown by the extracts prepared using methanol as solvent in all the cases followed by water. The values for methanolic extract were in between 79.36% (*Dendrocalamus strictus*) to 99.35% (*Bambusa baclooa*) and that of water between 57.92% (*D. strictus*) to 94.89% (*B. baclooa*). However, the H_2O_2 scavenging was drastically reduced in the acetonic extracts. Hydrogen peroxide being a weak oxidizing agent has the potential to inactivate a few enzymes, by oxidation of essential thiol groups. It also has the potential to form hydroxyl radical either by reacting with Fe^{2+} or Cu^{2+}ions once inside the cells and thus cause toxicity (Halliwell and Gutteridge, 1993). To avoid such toxicity the accumulation of H_2O_2 needs to be restricted. Scavenging of H_2O_2 by the different fractions of different bamboo leaf extract may be ascribed to their phenolics, which donate electron to H_2O_2, thus reducing it to water.

All the six species of bamboo under study viz. *Bambusa vulgaris* „Vittata", *Bambusa pallida*, *Bambusa balcooa*, *Dendrocalamus strictus*, *Dendrocalamus sikkimensis* and *Dendrocalamus hamiltonii* exhibited considerable antioxidant activity irrespective of the solvent type used for the preparation of the leaf extract. Among the three solvent types, methanol was found to be more suitable compared to water and acetone. The present study though confirms that the bamboo leaves as store house of natural antioxidants and are rich sources of polyphenols that could have great importance as therapeutic agents in preventing or slowing the progress of aging and age associated oxidative stress related degenerative diseases. However, further investigation with respect to identification of the compounds is of absolute necessity.

Similar observations were also made by Macwan *et al*. (2010) while

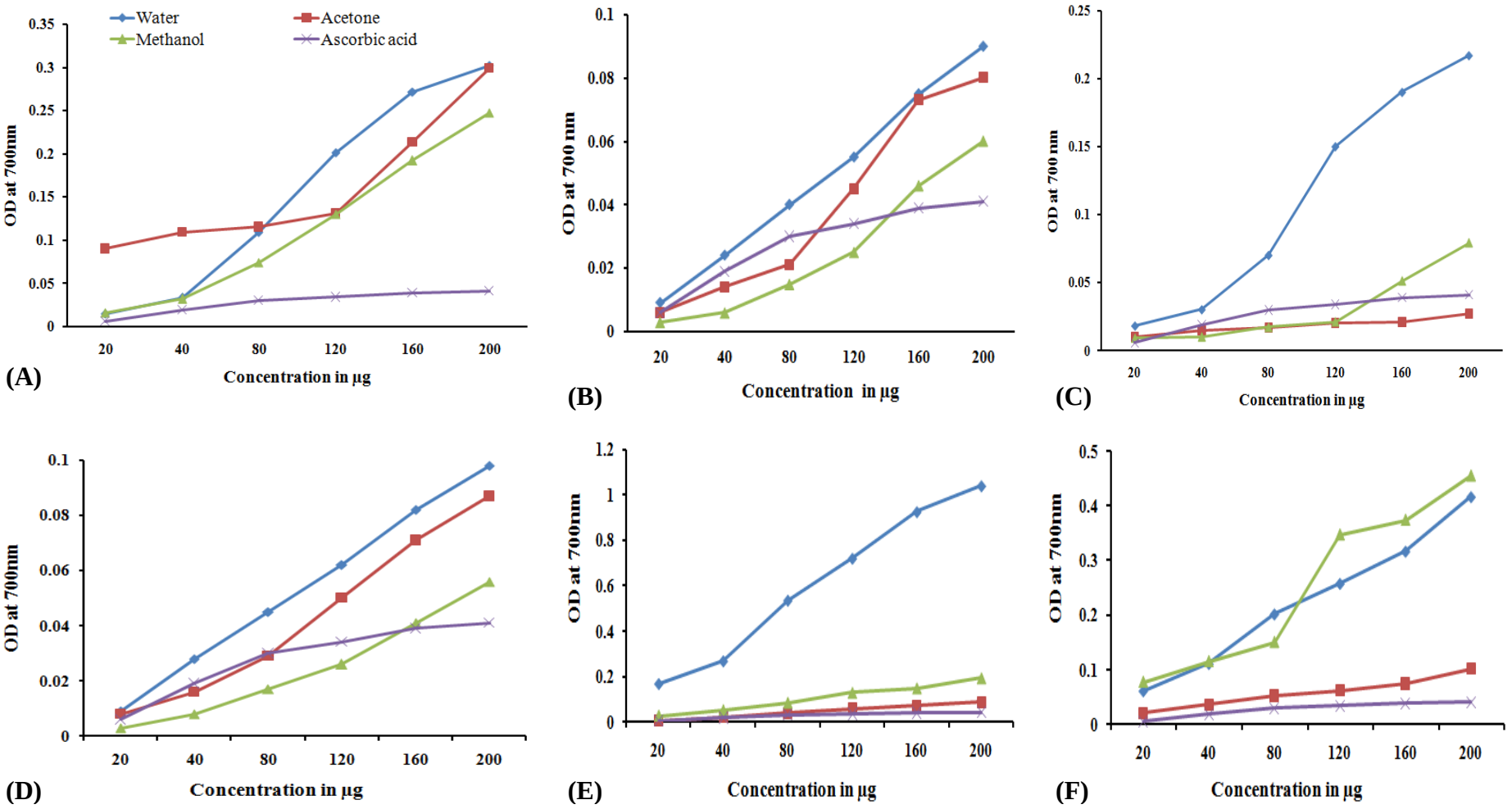


Figure 4.21: Ferric reducing power activity of bamboo leaf extracts . (A) *Bambusa vulgaris* „Vittata"; (B) *B. pallida*; (C) *B. balcooa*; (D) *Dendrocalamus strictus*; (E) *D. sikkimensis* and (F) *D. hamiltonii*

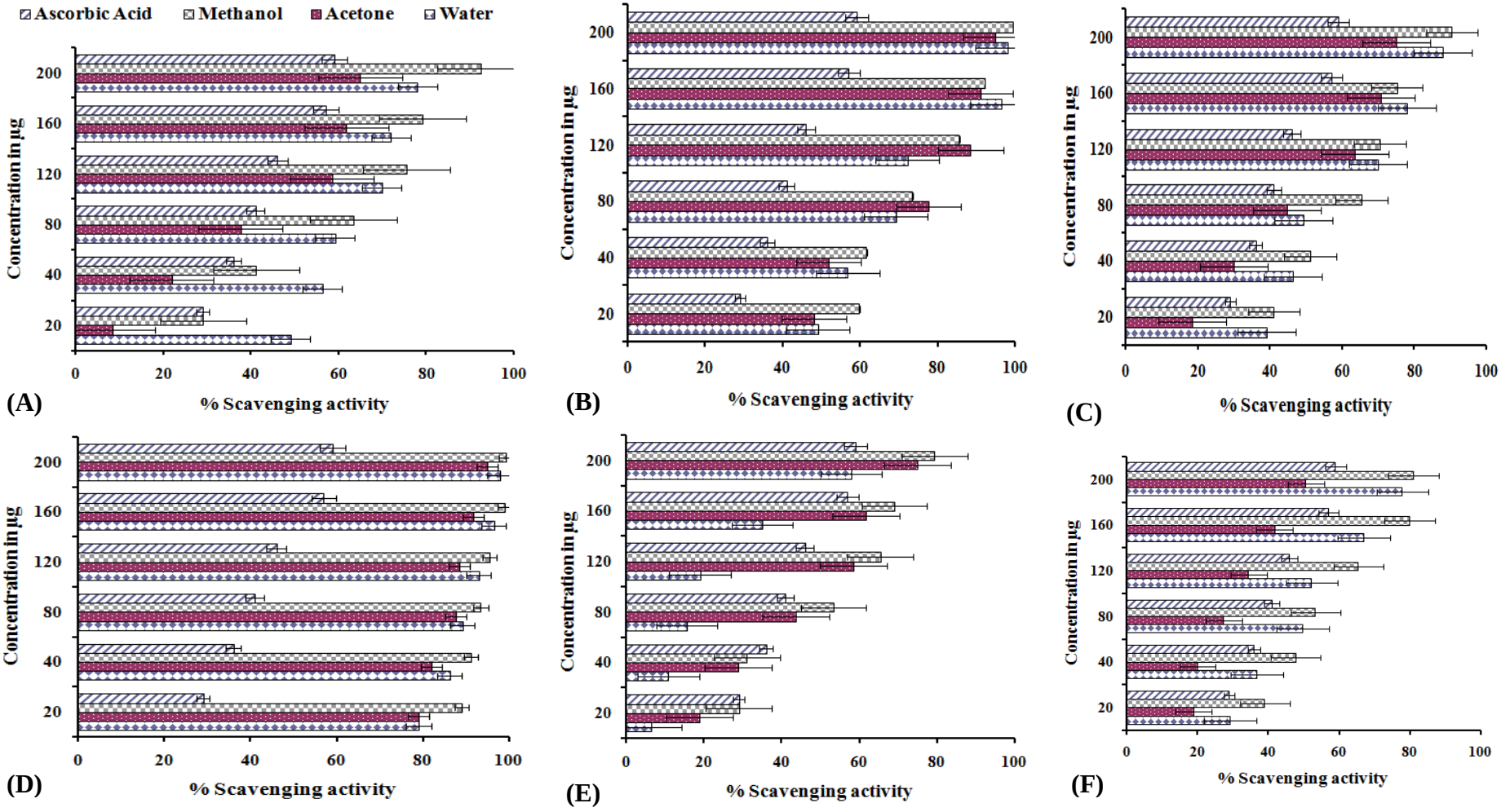


Figure 4.22: H_2O_2 scavenging activity of bamboo leaf extracts . (A) *Bambusa vulgaris* „Vittata"; (B) *B. pallida*; (C) *B. balcooa*; (D) *Dendrocalamus strictus*; (E) *D. sikkimensis* and (F) *D. hamiltonii*

evaluating the antioxidant potential of *Bambusa arundinacea* leaf using three different types of solvents, water, methanol and butanol. They also reported that compared to water and butanol, methanolic extract had considerable higher amount of antioxidant activity and thus proved to be suitable candidate for preparing the extracts.

4.5.7 HPLC analysis of **Bambusa balcooa** *aqueous leaf extract*

Bamboo, because of its nutrients content is regarded as the most valuable natural plants (Pathak, 1979; Rajebhosale *et al*., 1998). The use of bamboo in traditional medicine dates back to some 2500 years and has been well documented in some Chinese medicinal books. In China the antioxidants from bamboo leaves has already been approved as novel additive and is included in the list of "natural product serving as both food and medicine" by the Ministry of Health, People‟s Republic of China (Ministry of Health, 2003). A large number of functional polyphenols are present in bamboo. Polyphenols are classified as flavonoids, phenolic acid derivatives, stilbenes or lignins based on their structures (Harborne, 1988). Many different natural compounds have been isolated from different bamboo species which include homoorientin, isovitexin, orientin, vitexin, quercetin, luteolin, rutin, caffeic acid, *p*-coumaric acid, chlorogenic acid and tricin (Zhang *et al*., 2002b) but still much is left to be explored. In the present study aqueous extract of *Bambusa balcooa* leaf was considered for HPLC analysis primarily due to two fold reasons. One being the presence of moderate amount of antioxidants and the other being its traditional use to treat diabetes by the Moran folk in Tinsukia district of Assam (India) (Kalita and Phukan, 2010) in the same form. The chromatogram tested at 254nm is depicted in fig 4.23a. The retention time for standard rutin, gallic acid and β sitosterol was 1.85, 2.61 and 6.27respectively (fig 4.23b). The HPLC estimation of aqueous fraction of *B. balcooa* leaf showed the presence of different phytoconstituents. However, only three phytoconstituents were identified as rutin, gallic acid and β sitosterol having retention time of 1.94, 2.74 and 6.35 respectively. The amount of rutin, gallic acid and β sitosterol per gram of *B. balcooa* leaf was found to be 1.03 mg, 0.007 mg

and 0.065 mg respectively. Thus the HPL chromatogram of the aqueous extract of *B. baclooa* leaf confirms the presence of biomarkers like rutin, gallic acid and β sitosterol. Rutin is the glycosidic form of quercetine. The pharmacological effect of rutin is well documented and finds its application in varieties of ways like antitumor (Deschner *et al*., 1991), anti inflammatory (Aleksandrov *et al*., 1986), antidiarrheal (Di Carlo *et al*., 1993), antimutagenic (Bear and Teel, 2000), myocardial protecting (Pozin *et al*., 1996), immunomodulator (Chen *et al*., 2000), hepatoprotective activities (Janbaz *et al*., 2002) and antidiabetic (Kamalakkannan *et al*., 2006). The presence of rutin in the bamboo extract can induce the β cells to produce the insulin and/or protect the β cells from deterioration and thus help in reducing diabetes. Similarly, the antioxidant property of gallic acid is also reported (Hsu and Yen, 2007) and the property of gallic acid to enhance the insulin receptor sensitivity might be responsible for its anti hyperglycaemic activity (Huang *et al*., 2005). Moreover rutin was higher in content compared to gallic acid and hence rutin may be responsible for the antidiabetic activity and the gallic acid in turn may act as adjuvant with rutin and enhance the pharmacological action of *B. balcooa* leaf extract. The third compound detected is the β sitosterol, which is already established as an active phytosterol having multiple pharmacological properties (Mutai *et al*., 2009) like antiatherosclerosis (Choi *et al*., 2002; Vivancos and Moreno, 2008), anticancer (Awad *et al*., 1996; Awad *et al*., 1998), anti-inflammatory (Park *et al*., 2001) and antidiabetic (Lau *et al*., 2005) activities. The presence of β sitosterol in the plant extract might also be responsible for the antidiabetic activity of *B. balcooa* leaf extract. The possible reason might be that it elevates insulin secretion either thorough antioxidant activity (Vivancos and Juan, 2005) or regeneration of β cells of Islets of Langerhans (Gupta *et al*., 2011).

Thus for the above analysis it can be concluded that all the tree compound detected have the potential to cure diabetes individually and in this case they might complement with each other and enhance the activity of *B. balcooa* to cure diabetes and thus justifies its traditional use by the

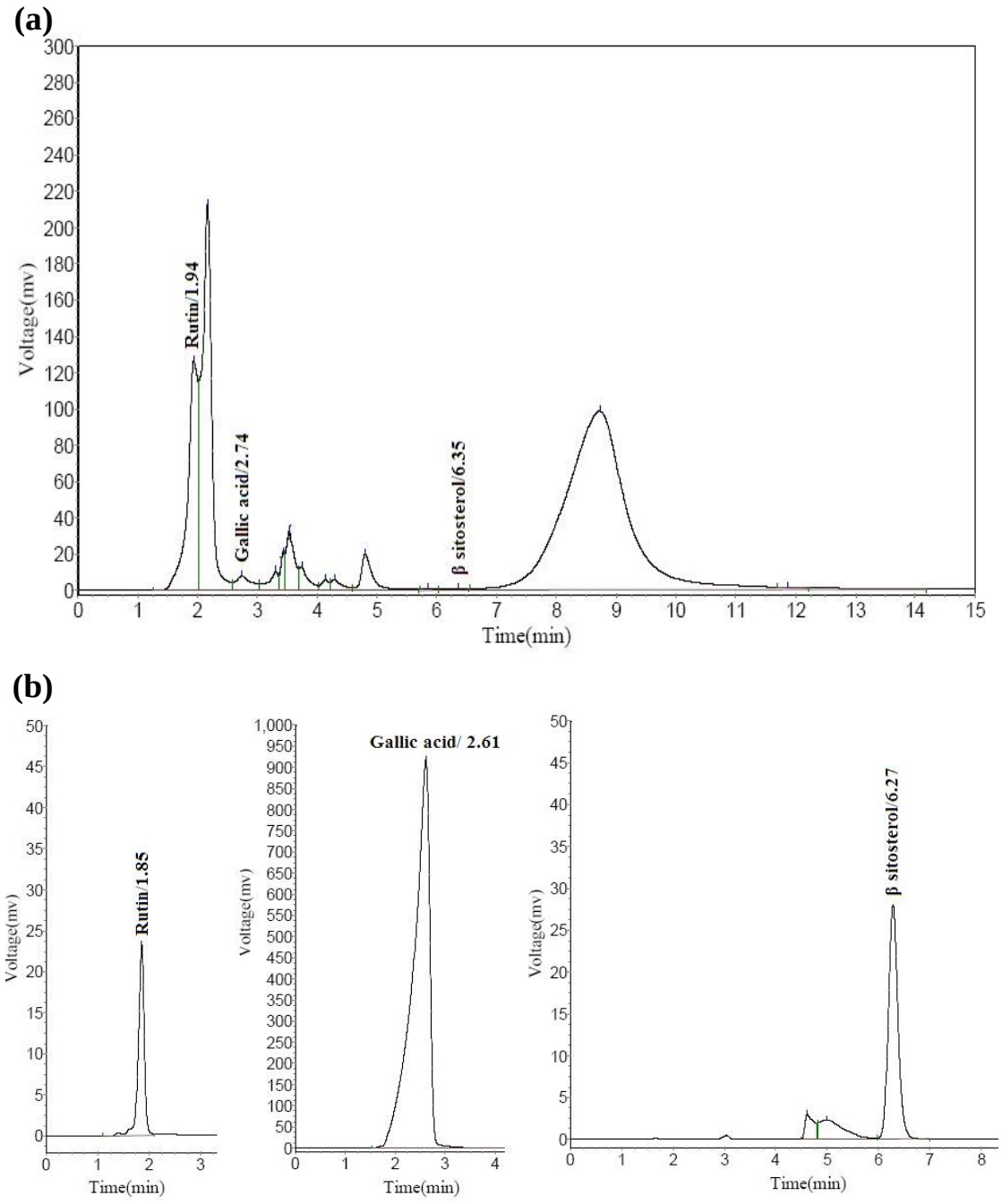


Figure 4.23: HPLC chromatogram. (a) *Bambusa balcooa* aqueous leaf extract and (b) Standards like rutin, gallic acid and β sitosterol

ethnic communities in India to conserve good health.

4.5.8 In vivo *anti-hyperglycemic activity*

Today there are innumerable *in vitro* reports on the use of plants as a source of dietary natural antioxidants and the list is ever increasing and the term „rich in antioxidant" has become a very common practice (Halliwell, 2007; Gutteridge and Halliwell, 2010), but their biological availability to animals and humans are not clearly established. Thus, before claiming that a particular

food or beverage is rich in antioxidant and is suitable for consumption, it is always necessary to evaluate whether they exhibit the same activity in both *in vitro* and *in vivo* systems (Halliwell, 2012) or not. In some cases the activity in both *in vitro* and *in vivo* systems are same (Lee *et al*., 2006; Wang *et al*., 2007) but more often it is not (Halliwell *et* al., 2005; Halliwell, 2009; Boomgaarden *et* al., 2010). In the present study an attempt has been made to study the anti-hyperglycemic effect of the aqueous leaf extract of *Bambusa balcooa* in alloxan induced diabetic rats. The aqueous leaf extract of *Bambusa balcooa* is being used by traditional practitioners to treat diabetes (Kalita and Phukan, 2010). Since the leaf extract is traditionally prepared using aqueous methods for the treatment of diabetes, so here also we used the aqueous method rather than organic to approximate the levels of compounds in the plant under study that might be consumed to help control diabetes.

4.5.8.1 Acute toxicity test

The primary sign of toxicity was noticed only after 10-12 hours of the administration of extract with decrease in locomotor activity and sense of touch. This was followed by reduced feed intake and prostration after about 18 hours. The median lethal dose ($LD_{50)}$ of the aqueous extract of BB was found to be 5.18 gm/kg body weight in mice and thus can be considered to be relatively safe (Lork, 1983).

4.5.8.2 Effect on fasting blood glucose, plasma insulin and glycated Hb

Table 4.21 interprets the fasting blood glucose (FBG), plasma insulin and glycated hemoglobin (glycated Hb) level in alloxan induced diabetic rats treated with 100 mg/Kg and 200 mg/ Kg of *Bambusa balcooa* extract, insulin, glienclamide and control groups. FBG increased up to three folds after alloxan induction compared to normal control rats. The higher dose (200mg/Kg BW) of *B. baclooa* leaf extract showed more significant reduction (50.86%) in FBG than lower dose (100mg/Kg BW) (35.64%) and glibenclamide treated (44.98%) as compared to the diabetic control rats. The possible mechanism by which *B. baclooa* leaf extract brings about its hypogycemic action might be that it possess insulin like effect either by promoting glucose uptake or inhibiting hepatic gluconeogenesis (Tanko *et al*., 2008) also it might act by eliciting either the pancreatic secretion of

insulin from the β- cells of Islets of Langerhans or its release from the bound form (Parmar *et al*., 2007; Middha *et al*., 2012). Similar to FBG level, the administration of extract at 100 and 200 mg/Kg BW and glibenclamide significantly reduced the glycated Hb level by 34.81%, 54.27% and 40.78% as compared to diabetic control rats. This decrease in the glycated Hb level might be due to increase in insulin secretion and reactivation of glycogen synthase enzyme system (Pandhare *et al*., 2011). The infusion of bamboo extract at both the doses significantly increased the plasma insulin level from 2.09±0.27 µU/ml in diabetic control to 7.08±0.41 µU/ml and 11.08±0.54 µU/ml at 100mg/Kg BW and 200mg/Kg BW respectively. These observation suggest that the hypoglycaemic activity of this bamboo may be mediated through enhancement of peripheral metabolism of glucose and an increase in insulin release.

4.5.8.3 Effect of B. balcooa *leaf extract on SOD, GPx and MDA*

In the current study, the activity of endogenous enzymatic antioxidants such as SOD (Superoxide dismutase) and GPx (Glutathione periodase) have been evaluated. SOD is an important antioxidant defence enzyme which protects tissue against oxygen free radicals by catalyzing the dismutation of superoxide radicals converting it into hydrogen peroxide and molecular oxygen (Arivazhagan *et al*., 2000; Chis *et al*., 2009). The reactive oxygen species of SOD is effective when its activity is followed by GPx. GPx is involved in detoxifying the hydrogen peroxide (H_2O_2) generated by SOD (Halliwell, 2001) and other organic hydroperoxides (ROOH) and thus

Table 4.21: Effect of *Bambusa balcooa* aqueous leaf extract on fasting blood glucose, plasma insulin and glycated Hb levels in control and alloxan induced diabetic rats

	Fasting Blood Glucose (mg/dL)	**Plasma Insulin (µU/ml)**	**Glycated Hb (%total Hb)**
NL	89.54±6.74	13.15±0.65	1.32±0.07
NL+BB	90.12±8.87	13.09±1.71	1.28±0.21
DC	289±3.87[a]	2.09±0.27[a]	5.86±0.74[a]
LB	186.61±8.7[b]	7.08±0.41[b]	3.82±0.27[b]
HB	142.86±7.17[b]	11.08±0.54[b]	2.68±0.42[b]
DG	159.0±9.47	9.83±0.47	3.47±0.54
DI	108.60±1.43[b]	12.08±0.46[b]	1.98±0.42[b]

NL, Normal; NL+BB, normal control rats treated with *Bambusa balcooa* leaf; DC, Diabetic Control; LB, Diabetic control rats treated with *B. balcooa* leaf (100mg/Kg BW); HB, Diabetic control rats treated with *B. balcooa* leaf (200mg/Kg BW); DG, Diabetic control treated with glibenclamide and DI, Diabetic insulin. Values are mean ± SE (n=6 animals/group) in experimental animals. [a]Significant as compared to normal control rats. [b]Significant as compared to allaxon induced diabetic rats

protects the membrane from lipid peroxidation. In diabetic rats, the activity of SOD was significantly decreased in the liver as depicted in figure 4.24. Supplementation of *B. balcooa* leaf extract exhibited an increase in the SOD activity in the liver by 9.49% and 28.98% at a dose of 100 mg/Kg BW and 200 mg/Kg BW. A similar trend was noticed in glibenclamide treated rats by 18.71%. Similarly, GPx activity was reduced in diabetic rats with respect to the control rats (figure 4.25). Treatment of diabetic rats with both low and high doses of extract up regulated the GPx activity by 9.96% and 16.06% respectively. Glibenclamide treated rats also attained an elevation of 12.51% in GPx activity. In insulin treated rats, the activity was restored to normal. These observations emphasises that the two enzymes are interconnected and lowering of their enzymatic activity leads to deposition of lipid peroxides which in turn increases the oxidative stress in the diabetic rats. Treatment with *B. balcooa* leaf extract increased the enzyme activity significantly and thus may help eliminate the free radicals generated during diabetes mellitus.

The concentrations of MDA (Malondialdehyde) in liver of normal and experimental rats are depicted in figure 4.26. MDA is one of the final products of polyunsaturated fatty acid peroxidation whose production increases with the increase in free radicals in the cells. MDA level is popularly designated as marker of oxidative stress (Gawel *et* al., 2004; Misra *et al*., 2009). In the present study, diabetic rats showed a significant increase in MDA compared to normal rats. Supplementation of low *B. balcooa* leaf extract supplementation lowered the lipid peroxidation by 47.44% whereas 49.36% reduction was observed in the rats fed with high *B. balcooa* leaf extract. The effect of glibenclamide treatment on MDA was in between the low and high dose and reduced MDA by 48.72%. Thus it can be inferred that this bamboo extract could be effective in reducing lipid peroxidation.

These results are in accordance with the other reports on the activity bamboo. Nzreen and his co-workers (2011) reported the hypoglycaemic activity of *Bambusa arundinacea* in streptozotocin induced diabetic rats. In their experiment they found a significant decrease in the blood glucose level in treated rats along with reduction in the glutathione and lipid

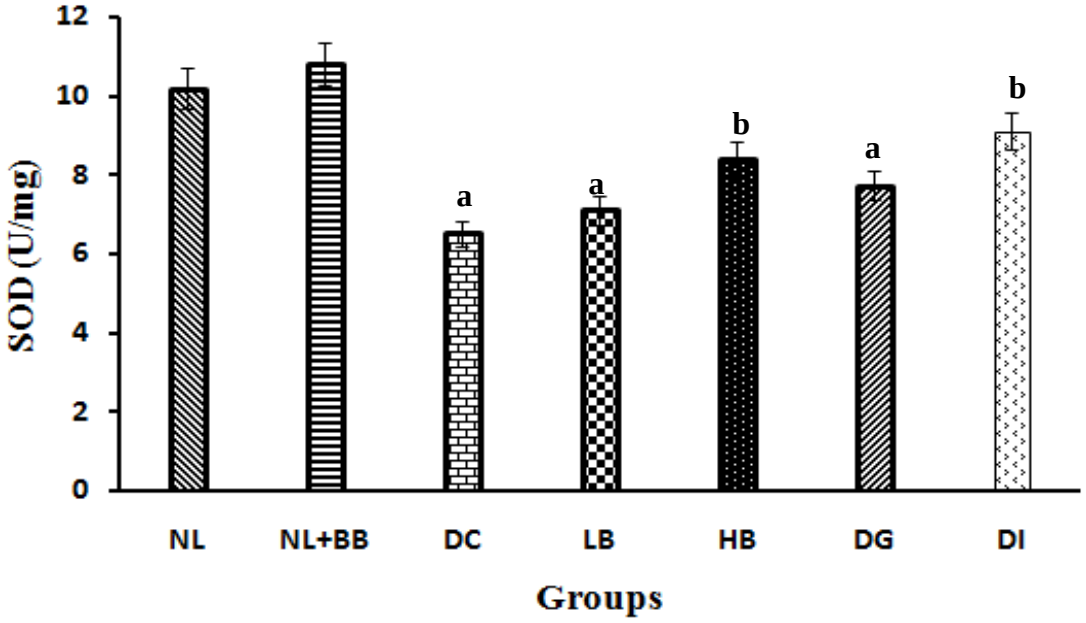


Figure 4.24: Effect of *B. balcooa* leaf extract supplementation on superoxide dismutase in liver of experimental rats. Values are means + S.E. (n=6 animals/groups) in liver of experimental animals. Results were considered significant between the groups at $p < 0.001$ in a comparative studies with DC. Those are not sharing the common letters (a & b) are significantly different.

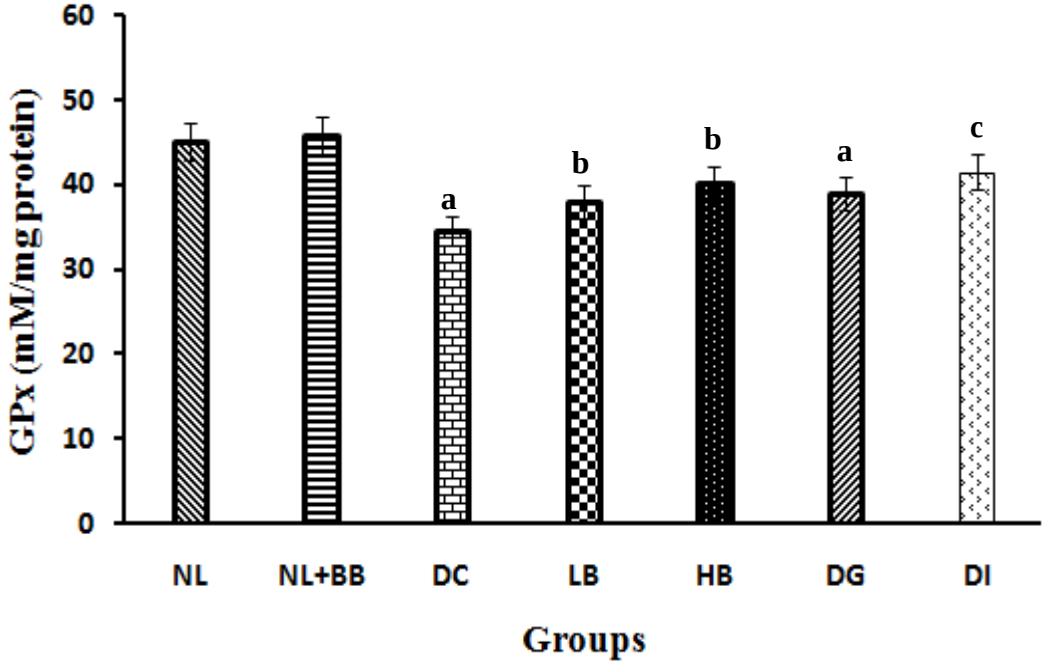


Figure 4.25: Effect of *B. balcooa* leaf extract supplementation on glutathione peroxidase in liver of experimental rats. Values are means + S.E. (n=6 animals/groups) in liver of experimental animals. Results were considered significant between the groups at $p < 0.001$ in a comparative studies with DC. Those are not sharing the common letters (a-c) are significantly different.

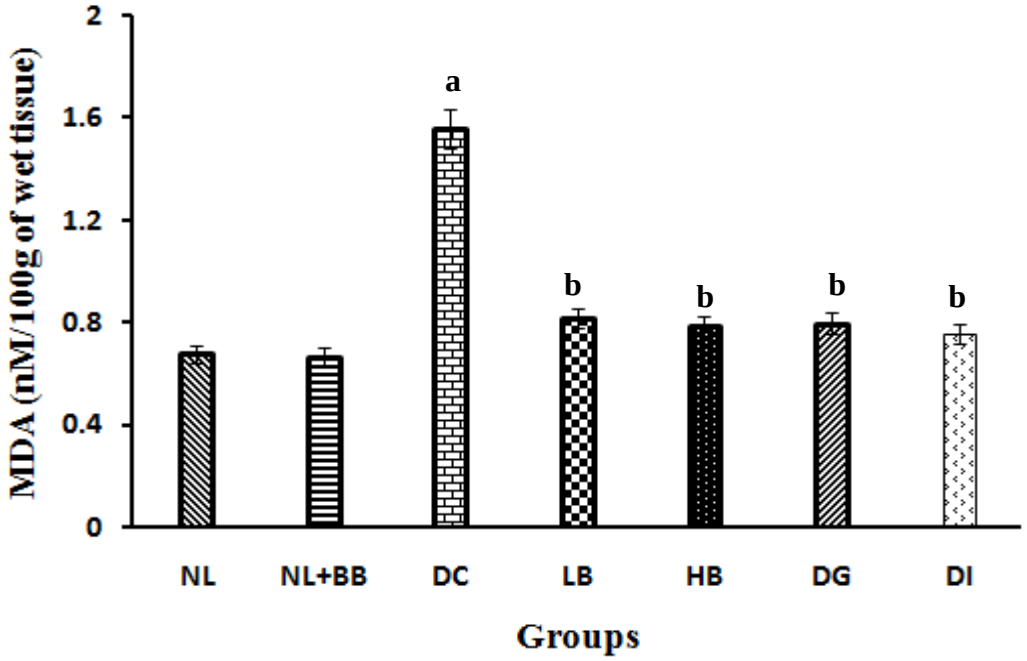


Figure 4.26: Effect of *B. balcooa* leaf extract supplementation on lipid peroxidation in liver of experimental rats. Values are means + S.E. (n=6 animals/groups) in liver of experimental animals. Results were considered significant between the groups at $p < 0.001$ in a comparative studies with DC. Those are not sharing the common letters (a & b) are significantly different.

peroxidation level and thus elevation in the enzymes activity. In another experiment on *Dendrocalamopsis oldhami* leaf extract, Lv *et al*. (2012) also observed significant decrease in the SOD and GPx activity in the liver when the animals were fed with the leaf extract. This study suggests that the diabetic animals are exposed to oxidative stress and the leaf extract of *B. balcooa* can partially reduce the imbalances between the events of generation of reactive oxygen species and the scavenging enzyme activity. Thus it can be inferred from the above study that *B. balcooa* could be a supplement as an antioxidant therapy due to its resemblance in activity with insluin and may prove to be beneficial in eliminating the hypergylcemia and preventing diabetic complications occurring due to lipid peroxiadtion and free radicals. However further studies on *B. balcooa* and its isolated compounds are essential, for better understanding the mechanism of action by which it modulates the oxidative stress in liver due to diabetes and thus develop a potential antidiabetic drug.

4.6 Creation of database

This part of the country is endowed with resplendent flora and bamboo is one of them. A curated database based on different species and varieties of

Figure 4.27: Snapshot of home page of bambooinfoline

bamboos growing in North Bengal called "Bambooinfoline: a database of North Bengal bamboos" have been developed, that can be accessed at http://www.bamboodb.ind.in/. The first page of the database is called the "home" page which gives a general introduction about bamboo and the need of developing "Bambooinfoline" (fig 4.27). This page has links to other pages of the database like bamboopedia, bamboo mining, bamboo click. Other than this information about the contributors, contact, data collection etc. are also linked. The "bamboopedia" page provides an idea about the different uses of bamboo along with graphs showing the distribution of bamboo worldwide, number of bamboo species/ live collections in different parts of India and the hyperlinked references (fig 4.28). The list of 34 different bamboo encountered in North Bengal

Figure 4.28: Snapshot of bamboopedia

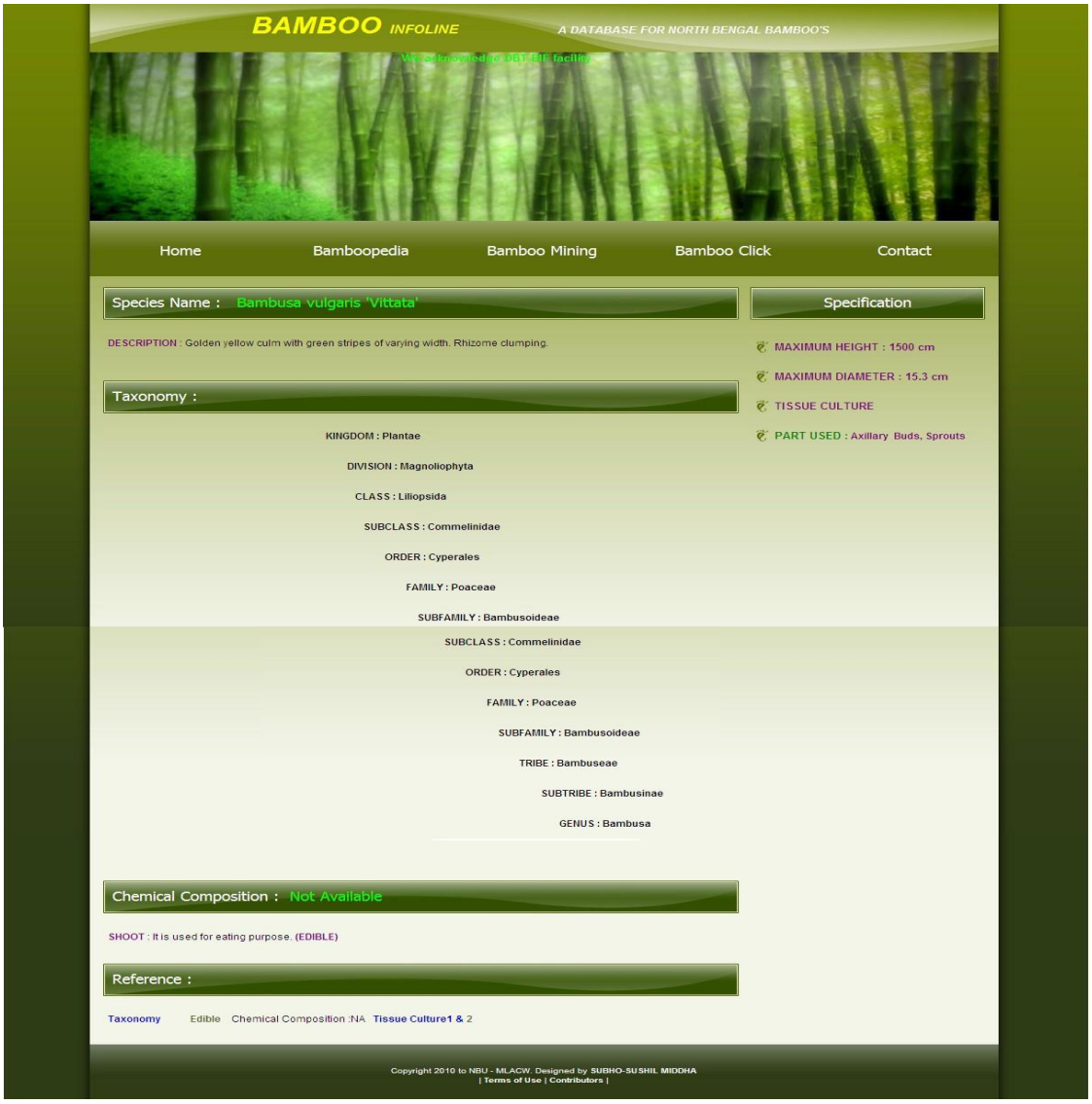


Figure 4.29: Snapshot of bamboomining

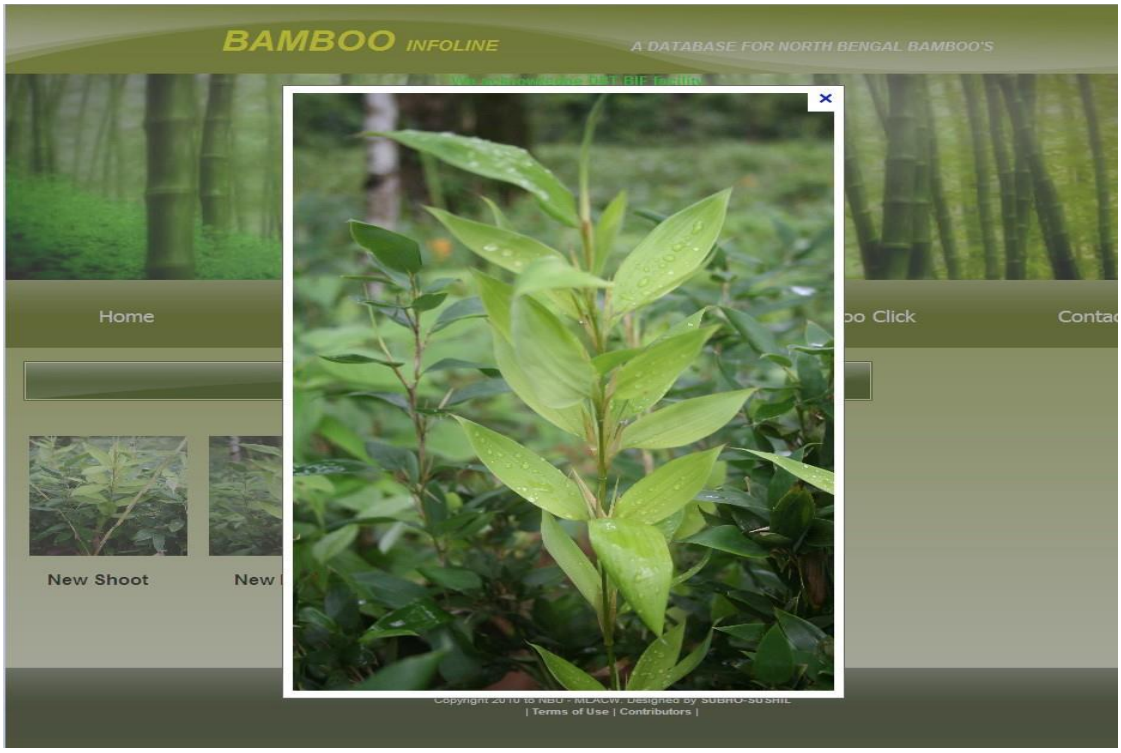


Figure 4.30: Snapshot of bambooclick

has been provided in "bamboo mining". Each scientific name of the bamboo is hyperlinked. The detailed information about the particular bamboo is being displayed in a new web page containing brief description, their specifications, taxonomy, chemical constituents (if any), whether the shoots are edible or not and the references which is also further hyperlinked (fig 4.29). In "bamboo click" original photographs of bamboo is displayed (fig 4.30). For each species multiple photographs representing different part of plants has been uploaded. "Bambooinfoline" is perhaps the first approach as a free accessible academic real time database for bamboo. More scientific information regarding bamboo species of this region will be incorporated in due course of time. Periodically continuous updates will be released to include other bamboo species. Using bioinformatics, an approach has been made to systematically compile the information related to this green gold in North Bengal which was unexplored till date. This knowledgebase based on bamboo would be an ideal source for information retrieval and would also be helpful in identifying areas for future research in providing a quick review on the number of bamboo species for the benefit of scientific community in general and bamboo lovers in particular.