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Phylogenetic relationships among accessions of bamboos encountered in North Bengal, India based on RAPD and ISSR markers

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Bamboo is a nature's wonderful gift associated with the rural people since ages. In the present study, genetic relationships among the 29 accessions of bamboo using RAPD and ISSR markers were accessed. The RAPD decamers resulted in 326 loci ranging 187-1875 bp, of which only two were monomorphic. The percentage of polymorphism was found to 99.39%. Dendrogram showed that the most of *Bambusa* species were close to each other, while the four accessions of *Dendrocalamus* and two accessions of *Drepanostachyum* were distantly placed from each other. Based on the similarity indices, being varieties of the same species, *B. vulgaris*, 'Vittata' and 'Wamin'; *B. multiplex*, 'Alphonse-Karr' and 'Rivierorum', formed a cluster sharing a node at 88.3% and 91.1%, respectively. The ISSR primers produced 244 amplified bands, of which all were polymorphic (band size 137-2017 bp). The dendrogram was similar to RAPD analysis. Dendrogram based on the combined data sets of RAPD and ISSR showed considerable similarity with that obtained from individual RAPD and ISSR, except that in both the individual analysis, three accessions were segregated from the cluster and evolved as independent distinct clade, *B. pallida* being common. In the combined analysis, only *B. pallida* was found to diversify from the main clade.

Keywords: Bamboo, ISSR, North Bengal, phylogenetics, RAPD

Introduction

Bamboo is the name given to a group of perennial evergreen plants that represent one out of the 6-7 main natural groups of the grass family Poaceae[1]. There has always been a debate amongst the taxonomists regarding the number of species and genera of bamboo owing to the plant's often long flowering cycles. Several authors have estimated the number of genera and species of bamboo over time[2-4]. Geographically, India has the highest area under bamboo followed by China. However China has more genetic variation in bamboo[5]. Bamboo is found in almost all the parts of the country, except Jammu and Kashmir[6]. This difference in distribution of bamboo is probably due to the bioclimatic factors. In India, there are about 136 indigenous and exotic species found growing naturally and/or under cultivation[7]. Northeastern states like Assam, Arunachal Pradesh, Manipur, Meghalaya, Mizoram, Nagaland and Sikkim along with the state of West Bengal (North Bengal, Himalaya) houses over 50% of the total bamboo species recorded in India[6]. North Bengal has the potential to house a large diversity of bamboo because of the varied type of forest cover[8]. Basic knowledge in the biology and genetics of bamboo is lacking due to its unusual life cycle with the vegetative phase ranging 1-120 years[9]. Taxonomic studies of Bambusoideae that rely on floral morphology are in a state of flux, since the morphological features are often influenced by the environment factors[10]. Thus, phylogenetic study using molecular markers can be useful to genetically classify cultivars or varieties of a species. Review of literature shows that different molecular markers have been widely used in the plant genetic diversity analysis either in the nuclear genome[11-18] or nuclear r-RNA gene sequence[19-26] and bamboo is no exception. In bamboos, RAPD (random amplified polymorphic DNA) analysis has been attempted by several authors to study genetic relationships among them,[13-15,17,18]; however there is only a single report on the use of ISSR (inter simple sequence repeats) to access the phylogenetic and genetic diversity of bamboos[16]. Thus keeping this in mind, in the present study, both RAPD and ISSR markers have been employed to study the phylogenetic analysis of 29 accessions of bamboos encountered in North Bengal, India.

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Materials and Methods

Plant Material

Twenty-nine accessions of bamboo belonging to 13 different genera encountered in North Bengal were used in the present study (Table 1) after proper authentication by the bamboo taxonomist. The germplasm is planted in the "Bambusetum' at Kuresong Research Range, Sukna, Darjeeling between 26°47′26.94″ N Latitude and 88°21′47.41″ E Longitude with an elevation of 532 feet. Fresh leaf materials were used for DNA isolation.

Isolation of Genomic DNA

The genomic DNA was isolated using the standard protocol of Doyle and Doyle[27] with minor modifications. Tender leaves (~ 5 g) were ground into a fine powder with the help of liquid nitrogen. The pulverized material was mixed with 15 mL of prewarmed (at 65°C) CTAB extraction buffer (100 mM Tris pH 8.0, 20 mM EDTA, 1.4 M NaCl, 2% CTAB, 1% PVP, 0.3% β-mercaptoethanol) and incubated in a water bath for 1 h at 65°C with occasional mixing by gentle swirling. To it equal volume, chloroform:isoamyl alcohol (24:1) was mixed, centrifuged for 15 min at 6,500 rpm (5,000 × g) at 24°C, and the supernatant was carefully transferred to a fresh tube. Then 0.6 volume of ice cold isopropanol was added to the final supernatant. Upon gentle swirling the DNA-CTAB complex precipitated as a whitish network and was spooled out using a bent Pasteur pipette. It was then washed in 70% ethyl alcohol and allowed to air dry and finally dissolved in 500 µL of 1× TE buffer (pH 7.4). The dissolved DNA was extracted with an equal volume of equilibrated phenol (pH 8.0) and centrifuged at 10,000 rpm (10,000 × g) for 15 min. The upper aqueous phase was taken in a fresh tube and extracted with an equal volume of chloroform:isoamyl alcohol (24:1) and then centrifuged at 10,000 rpm (10,000 × g) for 15 min at room temperature (RT). The upper aqueous phase was taken in a fresh tube and to it 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volume of absolute ethyl alcohol was added and precipitated at 4°C for 30 min in a cooling centrifuge at 12,000 rpm (13,500 × g). The pellet obtained was washed in 70% ethyl alcohol, dried and dissolved in 500 µL of 1× TE buffer (pH 7.4). RNA free DNA was obtained by treating the sample with RNase enzyme. RNaseA was added to the genomic DNA dissolved in 500 µL of 1× TE buffer (pH 7.4) and it was incubated at 37°C for 1 h in a dry bath. An equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed properly and centrifuged at 10,000 rpm (10,000 × g) for 15 min at RT. The aqueous phase was then transferred to a fresh microcentrifuge tube. To the aqueous phase, 0.1 volume of 3 M sodium acetate (pH 5.2) and double volume of absolute ethyl alcohol was then added for DNA precipitation. It was centrifuged at 12,000 rpm (13,500 × g) for 30 min at 4°C. The DNA pellet obtained was washed in 70% ethyl alcohol, air dried and finally dissolved in 100 µL of 1× TE (pH 7.4) buffer. The quality and quantity of the isolated genomic DNA was estimated using two methods. First by gel analysis method where the DNA samples were run in 0.8% agarose gel using λ DNA/*Eco*RI/*Hin*dIII double digest as mol wt

Table 1—List of bamboo species encountered in North Bengal

Sample ID	Scientific name
B1	*Bambusa vulgaris* Schrad 'Vittata'
B2	*B. multiplex* (Lour.) Raeusch. ex Schult. & Schult. f ' Alphanso-Karr'
B3	*B. bamboos* (L.) Voss
B4	*B. multiplex* (Lour.) Raeusch. ex Schult. & Schult. f 'Rivierorum'
B5	*B. balcooa* 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	*P. argenteostriatus* (Regel) Nakai
B25	*Pseudosasa japonica* (Steud.) Makino
B26	*Sasaella ramosa* (Makino) Makino
B27	*Shibateae kumasaca* Nakai
B28	*Yushania maling* (Gamble) R.B. Majumder
B29	Chinese bamboo (unidentified)

marker and second by spectrophotometrically recording the optical density (OD) at 260 and 280 nm.

PCR Amplifications

Initially a total of 30 RAPD and 15 ISSR markers were used for screening. PCR reaction was carried out in a volume of 25 µL containing 2 µL (25 ng DNA). The reaction buffer for RAPD contained 12.5 µL of PCR master mix (GeNei^{TM} Cat# 610602200031730 Pl. No. MME22), 1.25 µL primer and Pyrogen free water to a final volume of 25 µL. PCR amplification was performed on a Perkin-Elmer Thermocycler 2400 with the following specifications: initial denaturation at 94°C for 4 min, followed by 44 cylces of denaturation at 94°C for 1 min, primer annealing at 37°C for 1 min, primer extension at 72°C for 2 min with final extension at 72°C for 10 min. In case of ISSR, the reaction mixture was made similar to RAPD, however the amplification was carried out with the following specifications: initial denaturation at 94°C for 5 min, followed by 34 cycles of denaturation at 94°C for 45 sec, primer annealing at 50-52°C (depending upon the primer) for 1 min, primer extension at 72°C for 1 min with the final extension at 72°C for 10 min. The amplified products using both RAPD and ISSR markers were resolved on 1.8% (w/v) agarose gel containing ethidium bromide solution (0.5 µg/mL) run in 0.5× TBE (Tris-borate EDTA) buffer. The fragment size was estimated using 0.1-1 kb DNA ladder and λ DNA/*Eco*RI/*Hin*dIII double digest as mol wt marker.

Data Analysis

Each polymorphic band was regarded as a binary character and was scored as 1 (presence) or 0 (absence) for each sample and assembled in a data matrix. A similarity matrix on the basis of band sharing was calculated from the binary data using Dice coefficient[28]. Similarities were graphically expressed using the group average agglomerative clustering to generate dendrograms. The analysis was done using the software package NTSYSpc (version 2.0)[29]. Correspondence analysis (2D and 3D plot) of right vectors from the binary data was performed to graphically summarize associations among the varieties. Analysis was done through a batch file following the software package NTSYSpc.

Results and Discussion

Cluster Analysis using RAPD Markers

RAPD analysis using 30 different primers, each of 10 mers in length were used for the 29 accessions of bamboo. Of the 30 primers screened, 16 resulted in producing distinct and scorable bands (Table 2). The amplification profiles of the total genomic DNA from the 29 accessions of bamboo using 16 primers resulted in the production of 326 fragments, ranging

Table 2—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	% 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	AGGGGTCTTG	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	

187-1875 bp, of which only 2 were monomorphic, while rest were polymorphic (Table 2). The percentage of polymorphism was found to be 99.39%. The number of bands generated by each decamers ranged 12 (OPA17) to 34 (OPA07). A representative of RAPD profile of the 29 accessions of bamboo generated using OPA07, where all the bands generated were polymorphic, and OPG19, where only one monomorphic band was seen, are presented in Fig. 1. Similarity coefficient[28] among the 29 accessions ranged 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 *B. multiplex* 'Rivierorum'. The dendrogram constructed on the basis of the data obtained from RAPD analysis is depicted in Fig. 2. In the present study, two aspects were taken into consideration to evaluate the genetic diversity and relationships in the 29 different accessions of bamboo. First the comparison was made among genera of bamboo and second among bamboo species and varieties. The dendrogram showed that, based on the similarity indices, two varieties of the same species, *Bambusa vulgaris* 'Vittata' and 'Wamin' on one hand, and *B. multiplex* 'Alphonse-Karr' and '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[13] using RAPD analysis, where *B. multiplex* and *B. multiplex* var. Silver Stripe clustered together sharing a node.

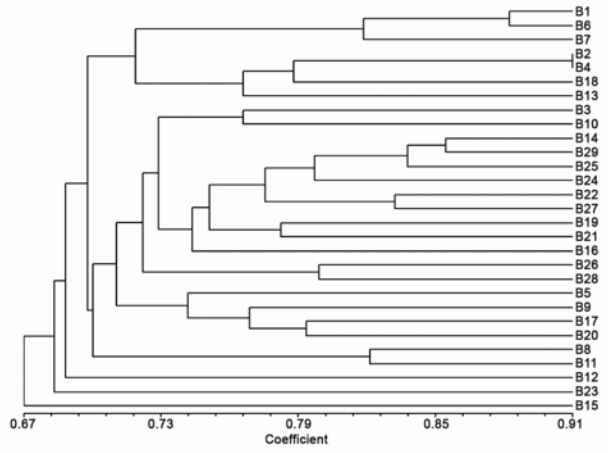

Fig. 2—Dendrogram derived from UPGMA cluster analysis of RAPD markers illustrating the genetic relationships among the 29 accessions of bamboo.

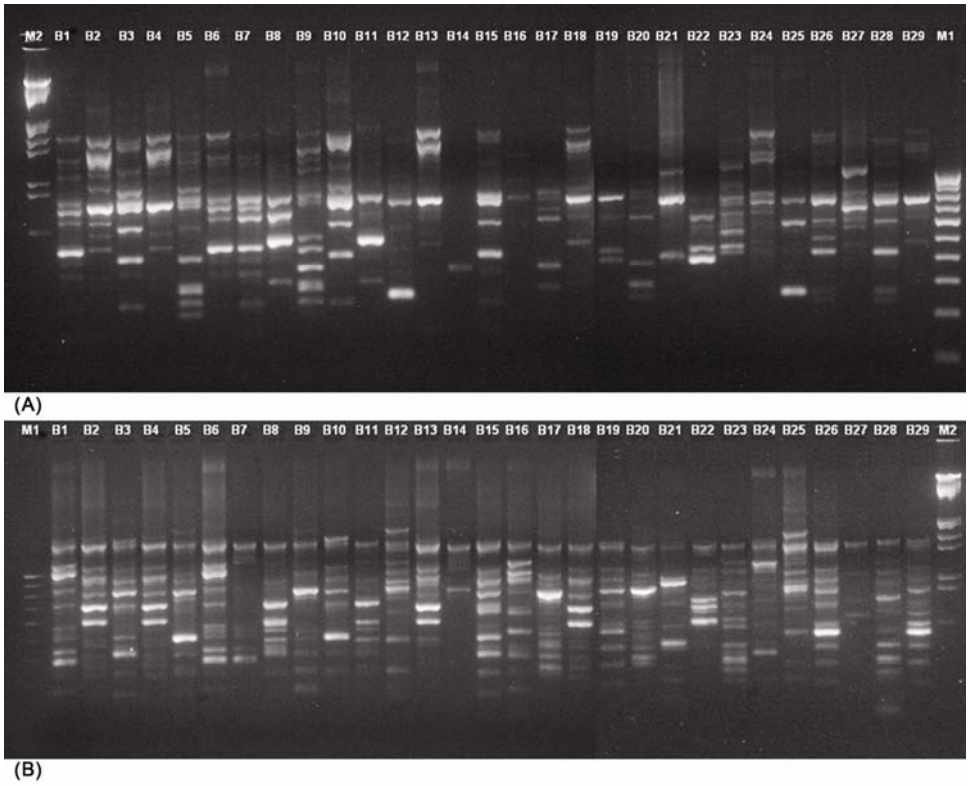

Fig. 1 (A & B)—A representative RAPD profile of 29 accessions of bamboo amplified with (A) OPG19 and (B) OPA07 primers. [Lane M1, 100 bp mol marker; Lanes B1-B29, 29 different accessions of bamboo under study (For the species and varieties names refer Table 1); Lane M2, λ DNA/*Eco*RI/*Hin*dIII double digest DNA ladder]

Das *et al*[15] also using RAPD analysis found that *B. striata* and *B. wamin* shared a node, while as per Mukherjee *et al*[16] *B. vulgaris* and *B. vulgaris* var. Vittata showed the 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 *B. balcooa* and *B. oliveriana*, which clustered in different clade, and *B. atra* and *B. tulda*, which clustered in a minor clade showing coefficient similarity of 82.2%. Of all the species and varieties of *Bambusa* under study, *B. 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[30], RAPD primers[13,15], ISSR and EST based primers[16]. The four accessions of *Dendrocalamus* studied here showed considerable divergence among themselves in contrast to traditional taxonomy. Similar pattern of clustering were noted by Loh *et al*[30] between *D. giganteus* and *D. brandissi* using AFLP analysis and by Nayak *et al*[13] between *D. strictus* and *D. giganteus*. The two species of *Drepanostachyum*, *viz.*, *D. khasianum* and *D. intermedium*, were distantly placed in the dendrogram showing 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*, and *D. 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[31] (Fig. 3). 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.

Cluster Analysis using ISSR Markers

The phylogenetics among the 29 accessions of bamboo documented from North Bengal, India using

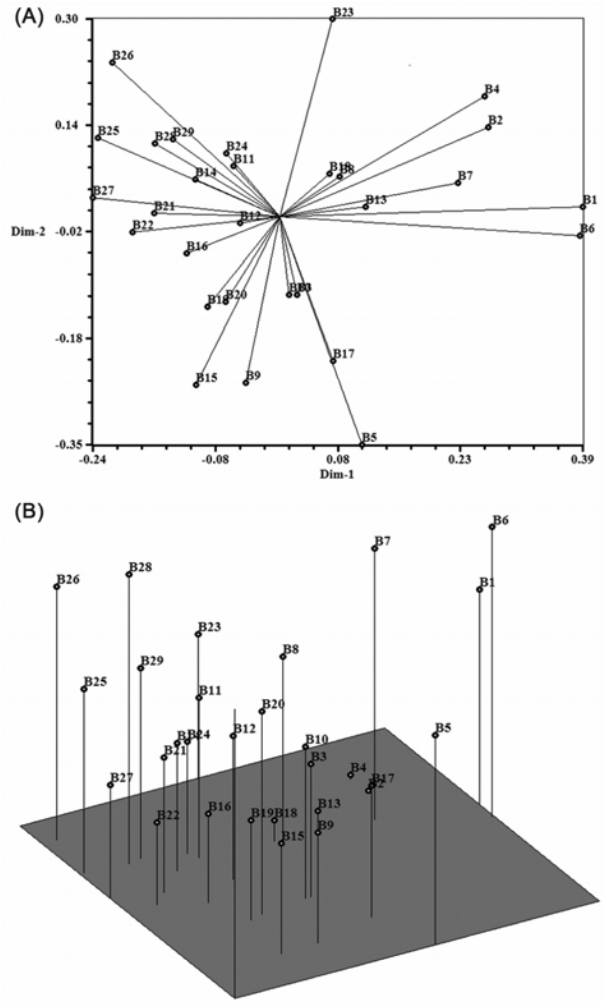

Fig. 3 (A & B)—Principal coordinate analysis of 29 species and varieties of bamboo based on RAPD analysis data: (A) 2-Dimensional plot; & (B) 3-Dimensional plot.

DNA based technique complemented with 9 ISSR primers. Fifteen ISSR primers were initially screened to generate polymorphic bands, of which only 9 primers were able to produce distinct, scorable bands (Table 3) 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 (33) of bands . On an average, each primer produced about 27.11 scorable, distinct bands. In total, 244 amplified bands were produced by the 9 primers, of which all the 244 were polymorphic; the frequency of polymorphism was 100% and the band size ranged 137-2017 bp. The representatives of ISSR profile of the 29 accessions of bamboo generated with UBC815 and UBC824 primers are depicted in Fig. 4. Nei's genetic similarity between each pair of species ranged 0.613-0.960. The lowest value was found between

Table 3—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	% 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
UBC41	(GA)8YC	25	25	100	243-1325
UBC856	(AC)8YA	29	29	100	143-1975
UBC873	(GACA)4	27	27	100	217-2017
	Total	244	244	100	

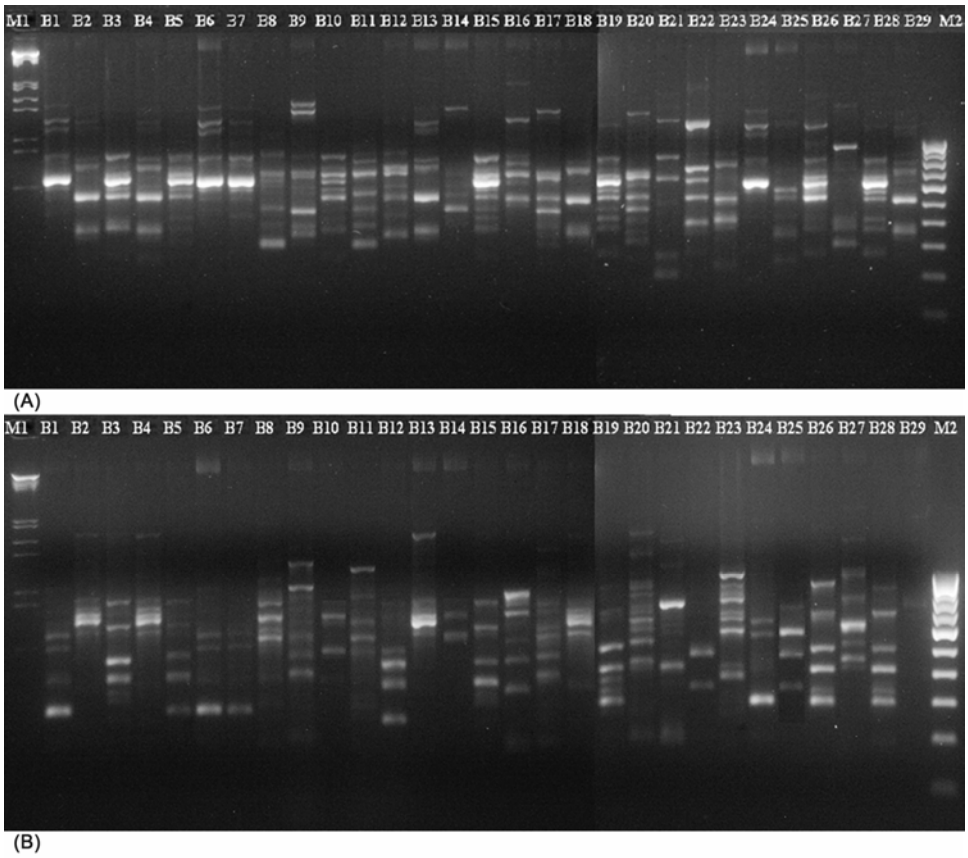

Fig. 4 (A & B)—ISSR banding patterns of 29 accessions of bamboo generated by (A) UBC824 and (B) UBC815 primers. [Lane M1, λ DNA/*Eco*RI/*Hin*dIII double digest DNA ladder; Lanes B1-B29, 29 different accessions of bamboo under study (For the species and varieties names refer Table 1); Lane M2:100 bp mol marker]

B. vulgaris 'Vittata' and *Bambusa pallida*, while the highest value was recorded between *B. multiplex* 'Alphonse-Karr' and *B. multiplex* 'Rivierorum'.

The dendrogram constructed based on the data from the ISSR random primers showed that most of the *Bambusa* species and varieties clustered together, except *B. atra* and *B. oliveriana*, which clustered in a minor clade showing coefficient similarity of 74.7% (Fig. 5). *B. 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 also held by other workers[15,16,30]. *B. multiplex* 'Alphonse-Karr' and *B. multiplex* 'Rivierorum' formed a cluster sharing a node at 96% similarity being varieties of the same species. This

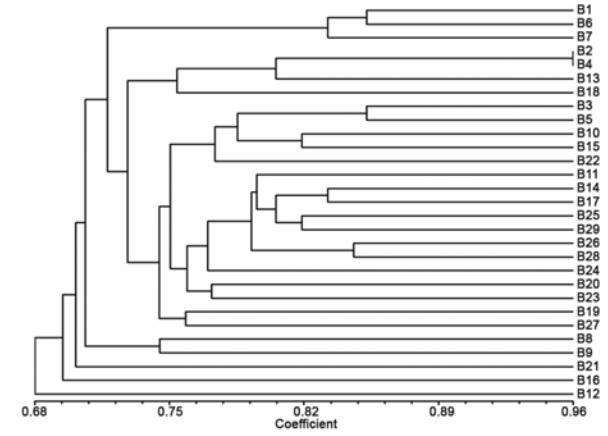

Fig. 5—Dendrogram generated from the cluster analysis of ISSR markers of 29 bamboo accessions.

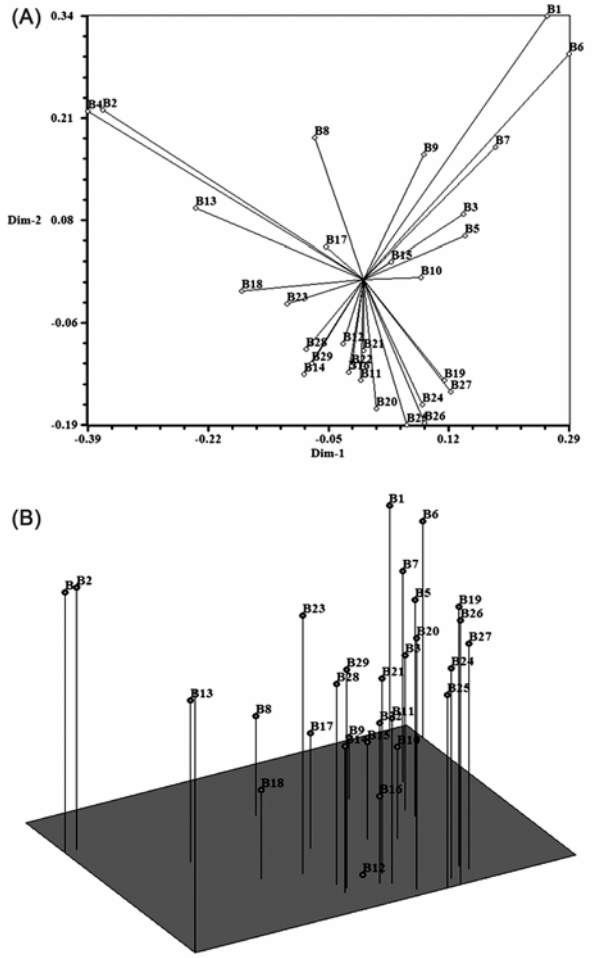

Fig. 6 (A & B)—Principal coordinate analysis of 29 species and varieties of bamboo based on ISSR analysis data: (A) 2-Dimensional plot; & (B) 3-dimensional plot.

was in accordance with the results obtained by Nayak and his coworkers[13] where *B. multiplex* and *B. multiplex* var. Silver Stripe were found to be very close to each other. Similarly *B. vulgaris* 'Vittata' and *B. 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[13,15,16]. Among all the 29 accessions, the least proximity (0.613) was found between *B. vulgaris* 'Vittata' and *B. pallida*. Of the 4 accessions of the genus *Dendrocalamus*, *D. strictus* and *D. hamiltonii* were close to each other and formed a cluster sharing the node at 83.3% similarity, while *D. sikkimensis* clustered with *B. sinospinosa*, whereas *D. 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[13]. This divergence in the genus *Dendrocalamus* has been previously encountered by Loh *et al*[30], where they found *D. giganteus* and *D. brandissi* forming different cluster using AFLP technique and both Nayak *et al*[13] and Mukherjee *et al*[16] found *D. strictus* and *D. giganteus* in different clade employing RAPD and ISSR marker analysis, respectively. The two species of *Drepanostachyum*, *i.e.*, *D. khasianum* and *D. intermedium*, were distantly placed in the dendrogram showing 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[31] (Fig. 6). Both the dendrogram and the principal coordinate analysis showed the similar cluster. Mukherjee *et al*[16] 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.

Cluster Analysis using Combined RAPD and ISSR Markers

The similarity coefficients of the 29 accessions of bamboo based on 326 RAPD and 244 ISSR loci ranged 0.629-0.934. The highest similarity was found between *B. multiplex* 'Alphonse-Karr' and *B. multiplex* 'Rivierorum' (0.934) like RAPD and ISSR analysis separately, while the lowest was noted

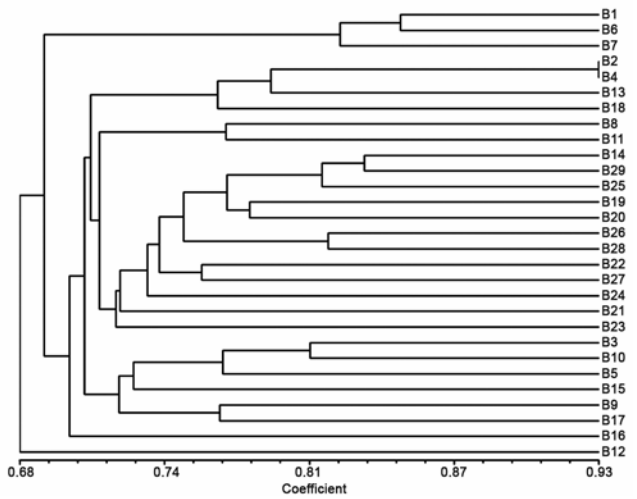

Fig. 7—Dendrogram constructed on the basis of data obtained from the combined RAPD and ISSR analysis.

between *B. vulgaris* 'Vittata' and *Himalayacalamus hookerianus* (0.629). Cluster analysis performed from the combined data sets of both RAPD and ISSR markers generated a dendrogram as illustrated in Fig. 7. The dendrogram gave clustering pattern similar to that of ISSR and RAPD analysis separately, except for that in both the individual analysis (ISSR & RAPD), 3 accessions were segregated from the cluster and evolved as independent distinct clade, *B. 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. 8) corroborated the cluster analysis results. However, the ISSR based cluster was found to be more similar as compared to RAPD based cluster.

Conclusion

In summary, it is apparent from the above results that with the use of molecular markers like RAPD and ISSR together with the morphological features, long-standing problem related to identification and systematic of bamboo can be resolved.

Acknowledgement

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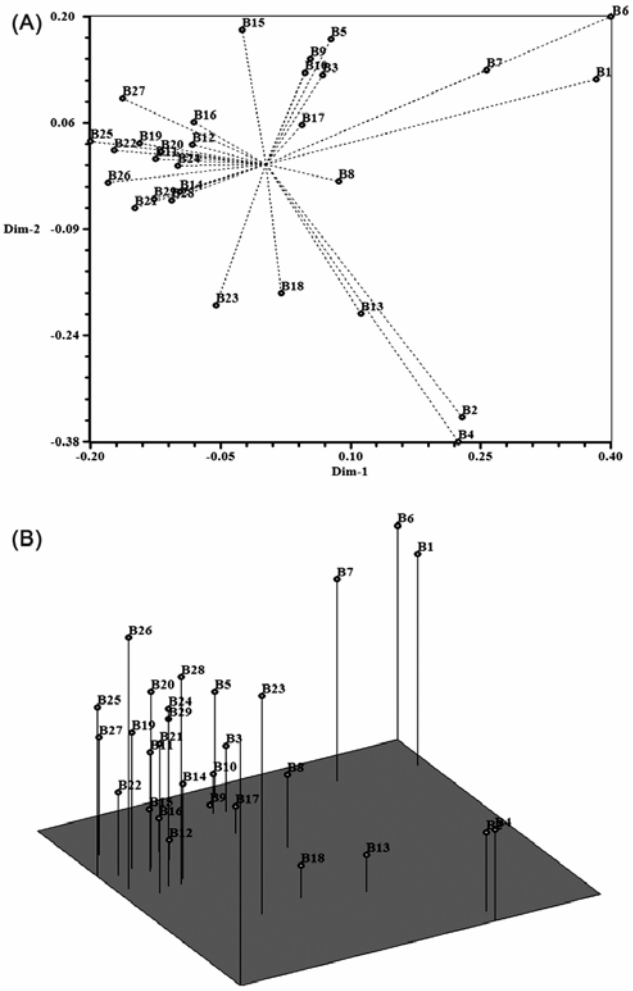

Fig. 8 (A & B)—Principal coordinate analysis of 29 accessions of bamboo based on combined RAPD and ISSR analysis data: (A) 2-Dimensional plot; & (B) 3-dimensional plot.

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