

Genetic variability and Phylogenetic relationship among some Bamboo species of North-East India by AFLP analysis

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ABSTRACT

The commercial importance of bamboo calls for precise documentation of these plants using molecular parameters since flowering based classical taxonomic grouping of bamboo plants within this group have often been questioned. In the present report, AFLP markers has been used with an aim to providing better understanding of taxonomic grouping of nine bamboo species of Manipur state of North Eastern India that is home to a large population with wide diversity of bamboo plants. The experimental material consisted mainly species of *Bambusa* and *Dendrocalamus* genus for which reports on even DNA fingerprinting studies (albeit few) appear confusing. Two other genus viz. *Mellocanna* and *Thyrostachys* were also included in the study. AFLP analysis using six pair primer combinations on nine species belonging to four genera of bamboo, generated total 1620 alleles among which 1229 alleles were polymorphic while 391 alleles were monomorphic. The average similarity matrix was used to generate a tree for cluster analysis using software NTSys v 2.1. All the species under *Bambusa* genus form a distinct cluster except *Bambusa balcooa* which formed a separate cluster with *Thyrostachys siamensis*. All species under *Dendrocalamus* genus have clustered together sharing a common origin with *Bambusa* cluster. Another species viz. *Mellocanna baccifera* also originated from this common root but exhibited distinct existence suggesting independent evolution.

Keywords: DNA fingerprinting, Amplified Fragment Length Polymorphism, Bamboo, Genetic diversity, Molecular Systematics.

INTRODUCTION

Bamboos are giant members of the grass group of plants belonging to family Poaceae. The unique hard and strong stem (culms), varying in height in different species, provide structural props and building material. One of the species *Bambusa balcooa* has been identified by FAO as the strongest among all bamboos; stems and culms of this species have highest specific gravity compared to all other species of *Bambusa* genus. In addition to other uses of bamboo, this species also has huge demand in paper pulp industry. The use of bamboo as an ingredient in biofuel is also reported [1]. A total of about 100 species (including 13 cultivated) of bamboo belonging to the 19 genera have been reported from India [2]. Of this, the North Eastern states have over 50% of the genotypes representing about 16 genera covering 63 species [3] [4]. With this large population representing commendable biodiversity, it is important that the bamboo plant genetic resource of this region be precisely Characterized and Documented. Recognized for its importance universally bamboo constitutes an important entity in world trade the value for which is expected to reach about US \$ 20 billion by 2015. In this trade China's share (with its rich population and diversity of bamboo) alone is to the tune of 50% [5]

Due to the unusually long sexual cycle and thus late flowering, characterization of bamboo plants through conventionally used flower characters have often caused confusion in taxonomic grouping and determination of proper evolutionary interrelationship between the groups of subtribe Bambusinae. Other parameters often used in classical taxonomy viz. vegetative characters that are subject to variation by environmental factors [6], are also not considered reliable for systematic studies of Bamboo [7]. The confusion often encountered in conventional bamboo taxonomy thus calls for development of molecular taxonomy through use of DNA characterization of genomes.

AFLP analysis for DNA characterization that allows extensive comparison between taxa provide precision in establishing genetic distances and phylogenetic relationships even between closely related taxa including infra-specific variation [8] [9]; such studies should be useful for resolving taxonomic confusion. AFLP polymorphisms have also been used for systematic study of several plant groups [10-14]. In the present report, AFLP analysis was conducted on 18 different landraces of bamboo belonging to nine species under four genera of the classically recognized tribe Bambuseae collected from Manipur, using an automated DNA sequencer. The molecular markers generated in this study may be used for characterization of individual taxa and for assessing phylogenetic relationships among them.

MATERIALS AND METHODS

Plant Material:

The plant materials used in this study were collected from different districts of Manipur state in India. There are total 18 samples belonging to four different genera and nine species (Table-1). All the plant samples, involved in this study, are authentically identified by Botanical Survey of India (BSI). Voucher specimens of all samples are preserved at the Central National Herbarium (CAL), Botanical Survey of India, Howrah, for future reference.

Table 1- Name (Scientific), site of collection with GPS data and Voucher numbers of bamboo species collected for AFLP analysis

Name of Bamboo Species	Site of collection	Latitude	Longitude	Altitude (m)	Voucher No.
<i>Dendrocalamus manipureanus</i>	Bishnupur District	24°30'15.4''	93°45'49.5''	791	IBSD/BN/09/B01
<i>Dendrocalamus manipureanus</i>	Thoubal District	24°42'50.9''	93°57'70.0''	798	IBSD/TH/09/B01
<i>Dendrocalamus manipureanus</i>	Thoubal District	24°37'0.99''	93°53'26.0''	918.6	IBSD/IW/09/B14
<i>Bambusa tulda</i>	Imphal West District	24°46'23.9''	93°55'45''	803	IBSD/IW/09/B08
<i>Bambusa tulda</i>	Imphal West District	24°48'23.8''	93°55'5.3''	796	IBSD/IW/09/B02
<i>Bambusa tulda</i>	Bishnupur District	24°34'05''	93°44'54''	819	IBSD/BN/09/B04
<i>Bambusa oliveriana</i>	Imphal West District	24°46'23.9''	93°55'45''	819	IBSD/IW/09/B12
<i>Bambusa oliveriana</i>	Thoubal District	24°42'50.9''	93°57'70.0''	798	IBSD/TH/09/B02
<i>Dendrocalamus latiflorus</i>	Thoubal District	24°42'39.3''	93°57'93.0''	798	IBSD/TH/10/B07
<i>Bambusa vulgaris</i>	Imphal West District	24°46'44.4''	93°54'55''	822	IBSD/IW/09/B06
<i>Dendrocalamus latiflorus</i>	Imphal West District	24°46'23.9''	93°55'45''	819	IBSD/IW/09/B13
<i>Melocanna baccifera</i>	Imphal West District	24°48'24.6''	93°54'59''	796	IBSD/IW/09/B04
<i>Thyrostachys siamensis</i>	Bishnupur District	24°32'04''	93°42'33''	819	IBSD/BN/09/B02
<i>Bambusa teres</i>	Imphal West District	24°11'10''	93°22'39''	784	IBSD/IW/09/B01
<i>Bambusa teres</i>	Imphal West District	24°46'17.2''	93°55'25.8''	821	IBSD/IW/09/B07
<i>Bambusa tulda</i>	Bishnupur District	24°30'15''	93°44'12''	820	IBSD/BN/09/B03
<i>Bambusa vulgaris</i>	Imphal West District	24°46'44.4''	93°54'55''	822	IBSD/IW/09/B05
<i>Bambusa balcooa</i>	Bishnupur District	24°29'55''	93°14'15''	827	IBSD/BN/09/B06

DNA extraction:

DNA was extracted from young leaves using the N-cetyl-N, N, N-trimethylammonium bromide (CTAB) method [15] with modifications. 200 mg of fresh leaf material ground in liquid nitrogen. 1ml of preheated extraction buffer [2% CTAB (w/v) 1% PVPP (w/v), 100mM Tris-HCl (pH 8.0), 50mM EDTA, 1.4M NaCl] were then added to the ground plant material. The DNA pellet was resuspended in 20–30 µl of Tris-EDTA (0.1mM EDTA and 10mM Tris-HCl). The quality of extracted DNA is determined by agarose gel electrophoresis (0.8%) using a standard containing 25ng/µl genomic λ DNA. The purity and concentration was also calculated from 260/280 and 260/230 O.D value. All DNA samples taken for AFLP study have their 260 /280 and 260/230 O.D value in the range of 1.75-1.90.

Fingerprinting by AFLP:**AFLP fingerprinting**

AFLP electropherograms were produced for each variety using the ABI prism fluorescent dye labeling and detection technology (Perkin-Elmer). AFLP analysis was performed using the kit supplied by Applied Biosystems (USA) and was used according to manufacturer instructions.

High-quality genomic DNA (500 ng) was digested with 1 U *MseI* and 5 U *EcoRI* restriction endonucleases. *EcoRI* and *MseI* adaptors were ligated with 1 U T4 DNA ligase (all enzymes were from New England Biolabs, Beverly, MA, USA). Restriction and ligation were done simultaneously [9] in a single step by incubating at 37°C for 2 h in a thermocycler (Applied Biosystems). Pre-amplification and selective amplification were carried out according to instructions provided in the kit. Pre-amplifications were evaluated running pre-amplified samples on a 1.5% agarose gel. A smear of product from 100-1500 bp was clearly visible. Selective amplification was carried out using six primer pairs for all the landraces belonging to nine species and four genera of the tribe Bambuseae. The amplified products were mixed with Size Standard Gene Scan 500 ROX, and the samples were then analyzed on an automated DNA sequencer (ABI Model 3130 XL genetic analyzer, Applied Biosystems).

Data analysis:

Fragment analysis was carried out for peaks in the range of 35-500 bp. For diversity analysis, peaks were scored as present (1) or absent (0) to form a raw data matrix. A square symmetric matrix of similarity was then obtained with Jaccard's [16] similarity coefficient $[a/(n-d)]$, where *a* is the number of fragments in common between two cultivars, *n* is the total number of fragments scored and, *d* is the number of fragments absent in both cultivars [17]. Genetic diversity estimates (GDEs) were then calculated as 1 minus Jaccard's similarity coefficient and used for cluster analysis using the unweighted pair group method of arithmetic averages (UPGMA) technique. The average similarity matrix was used to generate a tree for cluster analysis by using software NTSys v 2.2.

Table 2: Selective-Primer pairs used for the AFLP analysis; number of total, polymorphic and monomorphic alleles and also the percent of polymorphism for each set of primer pair used in this study (*-Fluorescent dye tagged)

Name of selective AFLP primers	No of total alleles	No of polymorphic alleles	No of monomorphic alleles	Percent of polymorphism
EcoRI-ACA*+MseI- CTG	257	190	67	73.92 %
EcoRI-ACT*+MseI- CAT	345	256	89	74.20 %
EcoRI-ACT*+MseI- CTC	322	266	56	82.60 %
EcoRI-ACA*+MseI- CTC	243	165	78	67.90 %
EcoRI-AAG*+MseI- CTA	198	152	46	76.76 %
EcoRI-AAC*+MseI- CTA	255	200	55	78.43 %
Total	1620	1229	391	

RESULTS

AFLP analysis using six pairs of primer combinations of the eighteen landraces of bamboo comprising of nine species under four genera revealed 1620 alleles, out of which 1229 alleles appeared polymorphic and 391 alleles appeared monomorphic (Table-2). Representative AFLP fingerprinting pattern of some species of bamboo was shown in Fig.1,2,3,4,5,6,7. Similarity matrix developed on AFLP binary matrix was used to determine phylogenetic relationship between the species using UPGMA cluster analysis represented in Fig 8. The dendrogram consisted of two major (Cluster I and Cluster II) and one minor cluster (Cluster-III). Cluster I, consisting of two sub-clusters i.e. cluster-Ia and cluster-Ib, contained ten landraces, belonging to four different species of the *Bambusa* genus. Cluster Ia contained four landraces, among them two landraces were of the species *Bambusa teres* and other two were of the species *Bambusa vulgaris*. *Bambusa teres* and *Bambusa vulgaris* species showed 87.4% similarity between them, whereas the landraces of *Bambusa teres* showed 88 % and landraces of *Bambusa vulgaris* shows 95 % similarity between them respectively. Cluster Ib contained six genotypes, among them four landraces were of the species *Bambusa tulda* and rest two landraces were of *Bambusa oliveriana*. According to the dendrogram, *Bambusa tulda* and *Bambusa oliveriana* showed 87% similarity between them. The landraces of *Bambusa tulda* showed 92.5% similarity among them whereas the landraces of *Bambusa oliveriana* showed 87% similarity. It was interesting to note that the genotype of *Bambusa balcooa* was not included in the Cluster-I in which all other species of *Bambusa* genus had clustered. *Bambusa balcooa* formed a separate cluster with *Thyrostachys siamensis* (Cluster-III). Cluster II contained total five genotypes, all belonging to the genus *Dendrocalamus*. The landraces of the species *Dendrocalamus manipureanus* were 91.3% similar among them and formed a distinct cluster (Cluster-IIa) whereas

landraces of *Dendrocalamus latiflorus* showed 94.4% similarity between them and included together in Cluster-IIb. Cluster I and Cluster II have a common root, suggesting same origin with later evolutionary divergence of the genus *Bambusa* and *Dendrocalamus*. The genus *Melocanna* represented by *Melocanna baccifera* in this study originated from the common root of Cluster I and Cluster II. This genotype showed 85.5% similarity with *Bambusa* cluster (Cluster I) and 88.2% similarity with *Dendrocalamus* cluster (Cluster II).

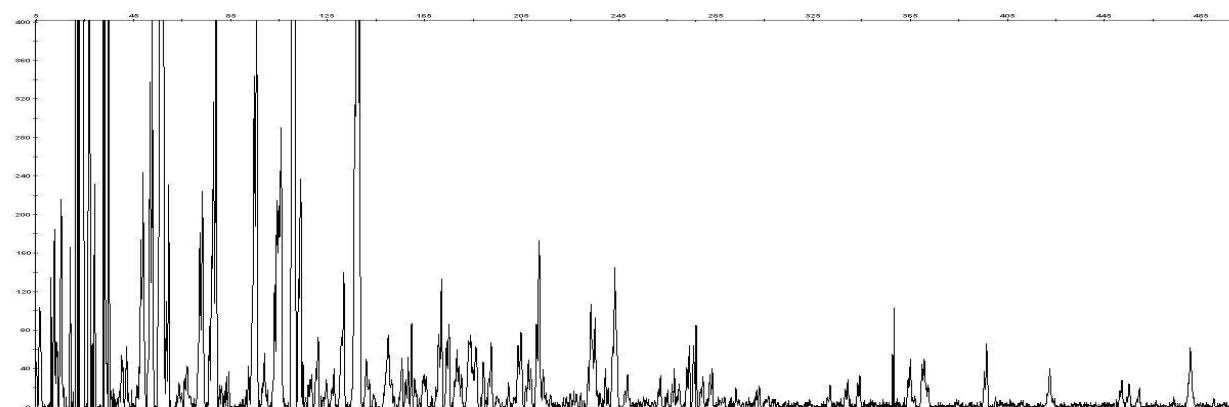


Figure 1: AFLP pattern of *Bambusa balcooa* using primer pair EcoRI-ACA*+MseI-CTC X-axis- base pair of alleles Y-axis- Intensity of alleles *-Fluorescent dye tagged

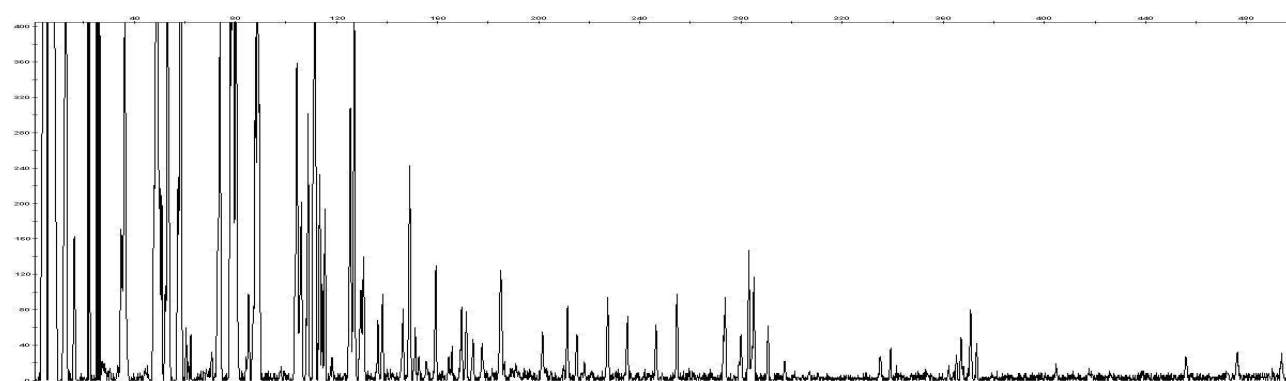


Figure 2: AFLP pattern of *Thyrostachys siamensis* using primer pair EcoRI- ACA*+MseI-CTC. X-axis- base pair of alleles Y-axis- Intensity of alleles *-Fluorescent dye tagged

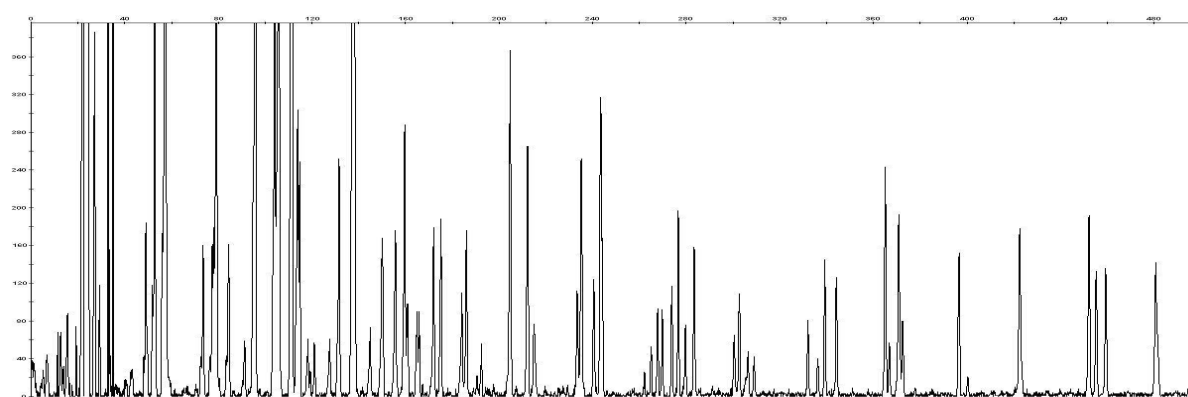


Figure 3: AFLP pattern of *Dendrocalamus latiflorus* using primer pair. EcoRI-ACA*+MseI-CTC. X-axis- base pair of alleles Y-axis- Intensity of alleles *-Fluorescent dye tagged

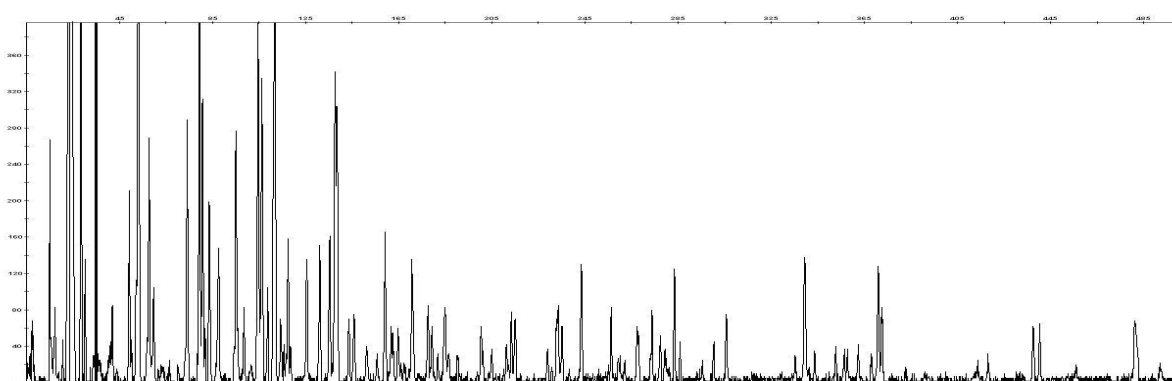


Figure 4: AFLP pattern of *Bambusa oliveriana* using primer pair EcoRI-ACA*+MseI-CTC
X-axis- base pair of alleles Y-axis- Intensity of alleles *-Florescent dye tagged

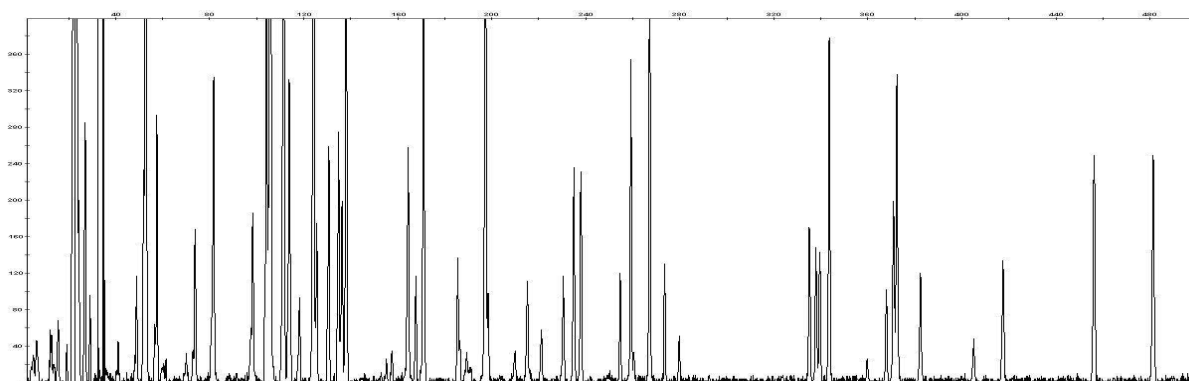


Figure 5: AFLP pattern of *Dendrocalamus manipuranus* using primer pair EcoRI-ACA*+
MseI-CTC. X-axis- base pair of alleles Y-axis- Intensity of alleles. *-Florescent dye tagged.

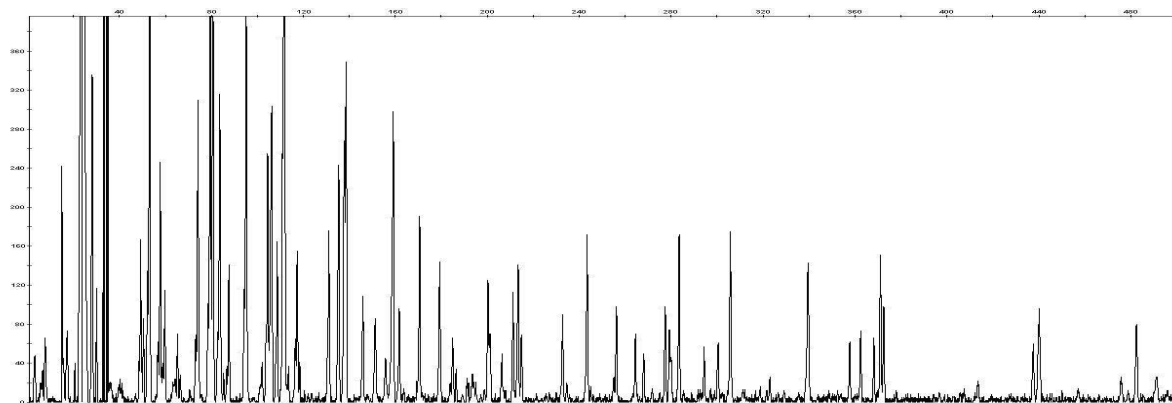


Figure 6: AFLP pattern of *Bambusa vulgaris* using primer pair EcoRI-ACA*+MseI- CTC.
X-axis- base pair of alleles Y-axis- Intensity of alleles *-Florescent dye tagged

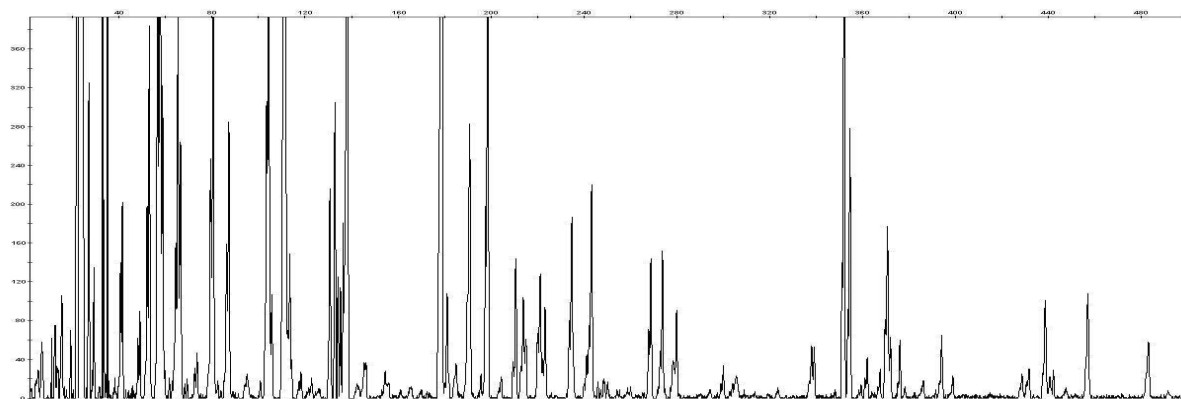


Figure 7: AFLP pattern of *Melocanna baccifera* using primer pair EcoRI-ACA*+MseI-CTC
X-axis- base pair of alleles Y-axis- Intensity of alleles *-Florescent dye tagged.

DISCUSSION

AFLP pattern (Fig-1,2,3,4,5,6,7) based dendrogram of nine species under four genera (viz. *Bambusa*, *Dendrocalamus*, *Melocanna* and *Thyrostachys*) shows polyphyletic origin of both *Bambusa* and *Dendrocalamus* genus (Fig-8). A comparison of this dendrogram with classical taxonomy based plant grouping shows that while in conventional taxonomy the four *Bambusa* species appear in one cluster only indicating closeness enough to warrant monophyletic origin, AFLP analysis reveals dissimilarity (among the genus) that is sufficient enough to be separated into two subclusters viz of Cluster-Ia (with *Bambusa teres* and *Bambusa vulgaris*) and Cluster Ib (with *Bambusa oliveriana* and *Bambusa tulda*) within Cluster I, thereby indicating polyphyletic origin of the genus *Bambusa*. Polyphyletic origin of *Bambusa* genus is also suggested by Loh *et al* (2000) [18] from his AFLP studies on *Bambusa* genus that shows 7 species of *Bambusa* genus distributed into three clusters, widely separated from each other. It may be noted that the pattern of species distribution in RAPD based dendrogram [19] also show that the studied 11 species under *Bambusa* genus are distributed in different subclusters (suggesting polyphyletic origin) while remaining within a single cluster. This is similar to the present study. The difference in phylogenetic relationship revealed by the two different methods used appears to be due to the precision in genotype analysis afforded by DNA fingerprinting as in molecular taxonomy over the morphological characterization based conventional taxonomy.

Dendrocalamus genus was divided into two groups based on vegetative characters, inflorescence morphology and flowering behavior [20] whereas on the basis of biochemical parameters viz. chromatographic separation of phenolic compounds and isozyme patterns Chou and Hwang (1985) [21] divided the same genus into four groups. Encountering such taxonomic confusion, Wong (1995) [20] concluded that *Dendrocalamus* genus requires further critical study using precise molecular biology methods as is discussed above for studies on *Bambusa*. Loh *et al* (2000) [18] through manual AFLP analysis showed 2 species (studied) of *Dendrocalamus* appear as distantly related entities. In our study the two species viz. *Dendrocalamus manipureanus* and *Dendrocalamus latiflorus* appeared in a single cluster (Cluster-II) diverging from the cluster-I (Fig-8). Three landraces of *Dendrocalamus manipureanus* have formed a subcluster (cluster IIa) and the landraces of *Dendrocalamus latiflorus* have clustered separately (cluster IIb) both remaining within Cluster II. The variation in data where Loh *et al* (2000) [18] finds separation of 2 species as separate entities whereas in the present report the 2 species appear within one group could be due to the different equipments used viz. AFLP in automated DNA sequencer coupled with sophisticated software based data analysis vis-à-vis AFLP on manual DNA sequencer. While the species under genus *Bambusa* and those under genus *Dendrocalamus* appear to have a common origin [22], each of the other three species studied here (*Melocanna baccifera*, *Thyrostachys siamensis* and *Bambusa balcooa*) appears to have evolved independently (Fig-8).

One species under *Bambusa* genus viz. *Bambusa balcooa*, the universally recognized strongest Bamboo, instead of clustering with all the other *Bambusa* species, remains in a separate cluster (Cluster-III-Fig-8) along with a different genus viz *Thyrsotachys* (under the same subfamily) thus showing more genetic similarity with *Thyrsotachys siamensis* than the other species of *Bambusa* genus studied here. It is interesting to note that stems of *Thyrsotachys siamensis* is also very hard and thus suitable for furniture manufacture; this bamboo species is also being explored for its suitability for use as bio-fuel [23]. Although morphological parameters indicate that *Bambusa balcooa* and

Bambusa oliveriana are closely related, molecular characterization (viz. RAPD) show that these two species are distantly related [19], as is also evident in the AFLP based dendrogram presented here (Fig-8).

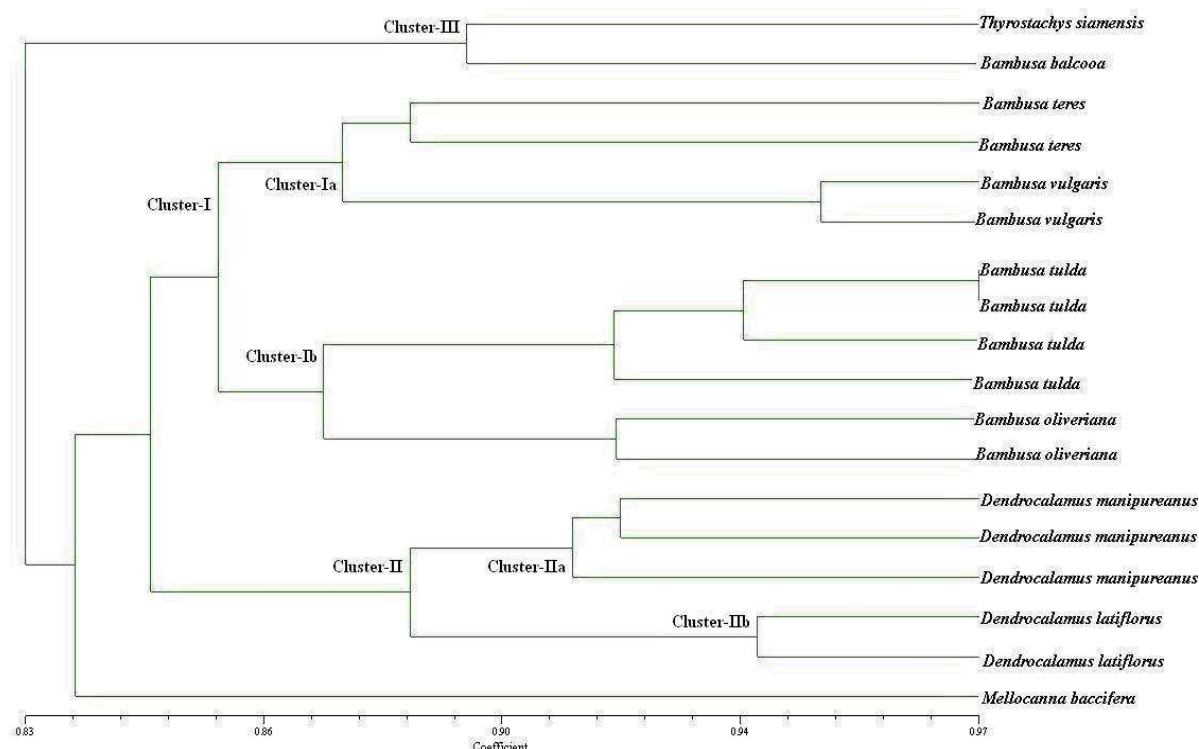


Figure 8: Cluster diagram of nine species of bamboo, collected from Manipur, India, showing phylogenetic relationship among them

CONCLUSION

Florescent AFLP analysis coupled with software based data analysis for DNA characterization in this study provide precision in establishing genetic distances and phylogenetic relationships between different species of bamboo where for a long time taxonomic confusion exists.

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