Phylogenetic analysis of Bamboo species using Internal Transcribed Spacer sequence

Ashiq Hussain Khanday
Sant Gadge Baba Amravati University
Prashant A. Gawande
Sant Gadge Baba Amravati University
Irfan Ashraf Badroo
Sant Gadge Baba Amravati University
Nasir Aziz Wagay (nasir.wagay1989@rediffmail.com)
Government Degree College Sopore
Gholamreza Abdi
Persian Gulf Research Institute, Persian Gulf University
Maryam Abbasi Tarighat
Persian Gulf University

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Abstract

Need for the use of molecular technique in Bamboo is very useful for better resolution of phylogenetic studies. In this study the present investigator has used (ITS1, 5.8S rRNA and ITS2) region of Bamboo species to construct the phylogenetic tree of 18 tropical bamboo species. Ribosomal internal transcribed spacer (ITS) sequences are commonly used for phylogenetic reconstruction because they are highly reiterated as components of rDNA repeats, and hence are often subject to rapid homogenization through concerted evolution. All Bamboo species studied were collected from different regions of India and were further subjected for the process of DNA sequencing. A phylogenetic analysis of the 18 Bamboo species under six different genera were performed using neighbor-joining and maximum-likelihood methods. These findings are further correlated with the morphological character based dendrogram of these species. The implications of the findings are discussed. Present study has shown a close relation between the two genera *Dendrocalamus* and *Bambusa*. However, dendrogram based on the morphological characters showed the intergenic and interspecific clustering of Bamboo species. Keeping in view all the possible outcomes the current research has focused to produce some useful conclusion of the two data sets. Molecular data was used to give the resolution for anomalies encountered which prompted the refinement of morphological character analysis. Although the molecular data helped to resolve the phylogenetic issues in Bamboos still the dependability of combined data sets (molecular and morphological) would be of very importance. This work has focused to reveal the phylogenetic relation among Bamboo species.

1. Introduction:

Bamboos are tall woody perennial, arborescent or shrubby, fastest growing grasses of subfamily (Bambusoideae) which is one of the thirteen currently recognized subfamilies within the grass family (Poaceae) (GPWG, 2001; Sanchez-Ken et al. 2007; Bouchenak-Khelladi et al. 2008). India is the second largest producer of Bamboo in the world next only to China and also has a very high diversity of Bamboos with total of 148 species and 4 varieties in 29 genera grow in India, both wild and cultivated (Sharma and Nirmala, 2015).

The identification depending on the reproductive structure is difficult in Bamboos (Bhattacharya et al. 2006). Consequently, the focus on identification of Bamboo has shifted from reproductive to vegetative characters (Bhattacharya et al. 2006; Sharma and Borthakur, 2008). Since, vegetative characteristics can be influenced by environmental conditions (Shalini et al. 2013; Yeasmin et al. 2015). Therefore, need of molecular based assessment is required.

Molecular markers can be used in Bamboos for dual purpose, first of all genotype identification of Bamboo and next genetic variation assessment within species irrespective of factors responsible to phenotypic variability or the geographic location. Literature cited revealed that number of molecular markers have been extensively used in Bamboo genetic diversity and phylogenetic analysis. Nuclear DNA markers ITS1, 5.8S rRNA and ITS2 spacer sequences has been proved as one of the best molecular markers to resolve taxonomical disputes in Bamboo at the lower taxonomic level (Sun et al. 2005; Yang et al. 2008). ITS1, 5.8S rRNA and ITS2 spacers are widely used in plant taxonomy for the individualization or barcoding of a taxon (Kress *et al*., 2005) and molecular phylogenetic studies because of their ubiquitous nature (Hribova *et al*., 2011). Phylogenetic studies in *Thamnocalamus* and *ITS* associated groups based on the *ITS* data sets divulged inconsistency in the classification of Bamboos based on the morphological parameters (Guo et al. 2002).

Molecular approach can be used over morphological character-based analysis for phylogenetic relationship assessment of Bamboos. Molecular phylogenies are not in congruence with morphological character-based classification system, direct to intricacy in understanding the evolution of morphological traits in various bamboo clades (Triplett et al, 2014; Attigala et al.2016). For example, no morphological synapomorphies were identified for tribe Arundinarieae which have been divided into 12 or 13 major lineages based on nuclear or plastid DNA markers (Attigala et al. 2016; Ma et al. 2014). The problems are more severe at generic level where inadequate morphologically informative characters and introgression contribute to the lack of concurrence between morphological based classification system and recent molecular phylogenies (Zhang et al.2012; Yang et al.2013).

2. Materials And Methods:

2.1 Plant materials:

Healthy and fresh leaf samples of 18 Bamboo species were collected from the germplasm mentained at Central forest Nurcery Wadali Amravati Maharashtra India. The identification of species was performed with the help of identification keys, floras, experts and literature available.

2.2. Isolation of PCR-compatible genomic DNA:

Prior to the DNA isolation the young leaf samples collected were surface sterilized with 2 % (w/v) sodium hypochlorite (NaClO) for 15 min, then rinsed with 70% (v/v) absolute alcohol and finally washed with double distilled water 2-3 times. 200 mg of young surface sterilized leaves from plant sample were weighed. These leaves were grinded in liquid nitrogen using mortar and pestle into fine powder and the powder was transferred into a microcentrifuge tube. For immediate use the samples were kept on ice. Further, GeneEluteTM Plant Genomic DNA Miniprep Kit was used for the isolation of purified DNA. Extracted DNA samples were quantified using UV-VIS Spectrophotometer (Shamidzu).

2.3.PCR amplification of ITS1, 5.8S rRNA and ITS2 sequences:

An initial amplification was performed in a 20 µl reaction volume containing 10X Taq DNA buffer with 20mM MgCl2, 1.2µl dNTP 10 mM each, 1.3µl of 25 mM $MgCl_2$, 0.25µl of 5U/ µl Taq DNA polymerase, 0.4µM each ITS4 and ITS5 primers (White *et al.,* 1990) were used to amplify the whole ITS region, 1µl BSA(Bovine Serum Albumin)20mg/ml, 1µl DMSO, approximately 25 ng DNA template and the volume was make up with nuclease free water. Thirty-six cycles of amplification were performed with the following temperature profile: initial denaturation at 94°C for 4 min; then 36 cycles of 94°C for 45 s, 51°C for 45 s, 72°C for 1 min; and a final extension step at 72°C for 7 min.

All the PCR amplifications were analysed on 1.2% agrose gel prepared by adding 1.2 g of agarose in 100 ml of 1X TAE buffer. The agarose was dissolved in buffer using micro-oven for 2-3 minutes. 3-5 µl of Ethadium bromide was added to the mixture in order to visualize the DNA. The gel was run at 100V for 90 minutes in a Gel Electrophoresis Unit. After the run time was over the DNA bands were visualized by placing the Gel plate in gel documentation chamber. Photographs of gel were taken for the records. The quality of band was judged by the presence of single compact desired band at the corresponding position to the 100bp DNA ladder. The quantity of the DNA was estimated by comparing the sample DNA with the control by eye.

2.4. Sequencing of the amplified products:

The bi-directional sequencing of all the PCR products performed on ABI prism 3500 Genetic Analyser (Applied Biosystems Thermo Fisher Scientific USA) using ABI Big Dye ^{TM} Terminator v3.1 cycle were obtained from Chromos Biotech and a raw chromatogram file (abi file) was provided by them. These files were analyzed in DNA sequence analysis software Sequencher 5.2.4 for the quality checking of sequences. End trimming of low quality sequences was performed for quality improvement.

2.5.DNA sequence contig formation and editing of sequence:

The forward and reverse sequences were aligned and checked for the proper matches of chromatogramic peaks using SeqMan pro DNASTAR. Lasergene.v 7.1. Improper matches were edited by visual examination. The contig files were saved in FASTA format as a separate file.

The contig sequences were compared (in NCBI-BLAST) with other available bamboo-ITS sequences in the GenBank (http://www.ncbi.nlm.nih.gov/GenBank) and percentage of similarity and INDELs were recorded. The contig sequences were subsequently saved in FASTA format for further analysis. Molecular evolutionary genetic analysis (MEGA-X) version 10.0.4 (Kumar *et al*., 2018) was used for sequence analysis.

2.6. Construction of phylogenetic trees:

Phylogenetic trees of 18 ITS (ITS1, 5.8S rRNA, and ITS2) sequences were constructed using (MEGA-X) version 10.0.4 (Kumar *et al*., 2018). The ClustalW (Larkin et al. 2007) of Molecular Evolutionary Genetics Analysis (MEGA-X) was employed to align the FASTA formats of these 19 sequences. Gaps were treated as missing data or as the fifth base and multistate characters as uncertain. In phylogenetic analysis sequences of other species from GenBank maintained by NCBI were also included. All characters were equally weighted. Phylogenetic tree was prepared using MEGA-X version 10.0.4. The evolutionary history was inferred by using the Maximum Likelihood (ML) method and Neighbor-joining (Nj) method based on the Jukes-Cantor method (Jukes and Cantor, 1969). Retrieved FASTA format sequences were assembled and subjected to MEGA X for multiple sequence alignment. The aligned sequences were used to generate the phylogenetic tree using Neighbor-joining (Nj) method and Maximum Likelihood (ML) method.

Bootstrap values were taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions were reproduced and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test was shown next to the branches (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value (Gascuel, 1997). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. In Nj the tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969) and are in the units of the number of base substitutions per site. All ambiguous positions were removed for each sequence pair.

Pairwise distance was computed using MEGA X to generate distance matrix. The numbers of base substitutions per site from between sequences were shown. Standard error estimates were shown above the diagonal. Analyses were conducted using maximum composite likelihood model (Kumar et al. 2004).

2.7. Submission of sequence to Gene Bank:

All the contig sequences of Gene and Intergenic spacer were submitted to the GeneBank via Bankit an online submission tool. Prior to the submission all the gene sequence are checked for the translation to find the exact frame shift. The translates were found using the following sites: https://web.expasy.org/translate/, http://www.fr33.net/translator.php, http://www.bioinformatics.org/sms2/translate.html.

3. Observation And Results:

3.1. Sequence variation in ITS region:

Analyzed for the conserved sites, variable sites and parsimoniously informative sites of representative intergenic spacers. Alignment sequences with MEGA X generated the readings for phylogenetic analysis, the compositional features of DNA sequences were useful to understand the evolution, structure and function of genome. Alignment length of sequence of *ITS*1 and ITS2 was 765bp and conserved sites (C), variable sites (V) and parsimoniously informative sites (P) of representative gene are shown in (Table I).

Table I: Nucleotide sequence composition containing conserved sites (C), variable sites (V) and parsimoniously informative sites (P) of representative gene and intergenic spacers.

Sr. no.	Gene region	Total sites	C	V	Pi	S
1.	*ITS*1and *ITS*2	765	478	266	170	92

3.2. Phylogenetic analysis:

Phylogenetic analysis using *ITS*1, 5.8S rRNA and *ITS*2 sequences involved a total of 19 nucleotide sequences with total of 792 positions in the final dataset. Eighteen Bamboo species were placed in five different clusters and each cluster representing different taxon. *Brachyelytrum erectum* was used as an outgroup specie to determine the position of the root. Maximum Likelihood method yielded a tree with both medium and high boorstrap values. The tree with the highest log likelihood (-4112.17) was obtained. Three different species *Guadua angustifolia, Melocanna baccifera* and *Dinochloa andamanica* representing three genera belonged to Cluster I. Cluster II represented *Dendrocalamus* with species *Dendrocalamus membranaceus* and *Dendrocalamus strictus.* Cluster III was sheared by *Bambusa bambos* and *Gigantichloa albociliata.* Cluster IV is the major clade divided into sub clusters. Subcluster I consisted of five species under genera *Bambusa* such as (*Bambusa ventricosa, Bambusa balcooa, Bambusa vulgaris, Bambusa burmanica, Bambusa tulda*) and one species from *Dendrocalamus (D.longispathus)*.

whereas, subcluster II consist of five species in which two species belong to *Bambusa* such as *Bambusa multiplex, Bambusa polymorpha*) and three to *Dendrocalamus* to which *Dendrocalamus hamiltonii, Dendrocalamus asper, Dendrocalamus giganteus*. The cluster IV represented by the species under *Dendrocalamus* and *Bambusa*. the cluster V represented by only one species *Gigantichloa albociliata* and it was found to be a separate lineage.

Phylogenetic analyses inferred using NJ tree was in congruence with the ML tree except the species of *Bambusa polymorpha* in NJ tree showed complete divergence from rest of *Bambusa* and *Dendrocalamus* species with high bootstrap value. *Gigantochloa ablociliata* and *Bambusa bambos* clustered into two different clades with comperatively higher bootstrap value (Fig 1). Phylogenetic analysis of 18 tropical Bamboo species belonged to subfamily Bambusoideae based on ITS1, 5.8S rRNA and ITS2 sequences by ML and NJ methods yielded trees with approximately similar topologies. Each taxon in all clusters revealed inter species variation which articulates their genetic diversity (Fig 1 to 2). The mixing of *Dendrocalamus* and *Bambusa* in the ITS sequence based phylogram was found to be significant.

Pairwise distance estimates of ITS1, 5.8S rRNA and ITS2 sequences using maximum composite likelihood model provides major of distance between each pair of sequences. All the Bamboo sequences are more alike to each other than each is to *Brachyelytrum erectum* an outgroup species which showed resemblance between (0.9277-1.0540). The measure of distance between D*. asper* and *D. giganteus* (0.0000), *D. longispathus* and *D. strictus* (0.0014), *D. longispathus* and *B. balcooa* (0.0028), *D. asper* (0.0044) and *D. hamiltonii* (0.0088) was found to be significant. Hence, these species with lower coefficients showed more closeness. However, besides *Brachyelytrum erectum* species with higher coefficients between *B.bambos* and *Dinochloa andamanica* (0.3178), *B. bambos* and *Guadua a angustifolia* (0.3147), *Guadua aangustifolia* and *B. balcooa* (0.3002), *Dinochloa andamanica* and *B. balcooa* (0.2906), *Guadua aangustifolia* and *D. hamiltonii* (0.28700) and *B. bambos* and *Melocanna baccifera* (0.2864), were less closer species (Table II).

Table II: Distance matrix of 19 species using ITS1, 5.8S rRNA and ITS2 spacer with upper diagonal showing standard error estimates.

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	*Brachyelytrum_erectum*													
2	*Bambus_bambos*	1.039												
3	*Dendrocalamus_longispathus*	1.000	0.067											
4	*Guadua_angustifolia*	1.054	0.315	0.303										
5	*Melocanna_baccifera*	1.017	0.286	0.259	0.259									
6	*Bambusa_vulgaris*	0.989	0.065	0.029	0.162	0.124								
7	*Dendrocalamus_membranaceus*	0.928	0.054	0.057	0.238	0.214	0.047							
8	*Bambusa_multiplex*	0.958	0.063	0.047	0.263	0.226	0.041	0.046						
9	*Dinochloa_andamanica*	0.996	0.318	0.279	0.254	0.186	0.148	0.236	0.254					
10	*Bambusa_burmanica*	0.991	0.062	0.021	0.152	0.120	0.024	0.040	0.030	0.128				
11	*Dendrocalamus_asper*	0.968	0.039	0.027	0.127	0.093	0.029	0.025	0.011	0.108	0.031			
12	*Dendrocalamus_hamiltomii*	0.965	0.072	0.059	0.287	0.231	0.047	0.056	0.025	0.261	0.042	0.009		
13	*Bambusa_balcooa*	0.976	0.061	0.003	0.310	0.264	0.029	0.058	0.048	0.291	0.027	0.030	0.063	
14	*Gigantichloa_albociliata*	1.024	0.070	0.061	0.191	0.154	0.064	0.057	0.055	0.172	0.037	0.022	0.058	0.064
15	*Bambusa_tulda*	1.090	0.095	0.062	0.166	0.133	0.067	0.080	0.076	0.144	0.044	0.051	0.080	0.069
16	*Dendrocalamus_giganteus*	0.965	0.071	0.061	0.282	0.218	0.042	0.056	0.023	0.260	0.036	0.000	0.011	0.063
17	*Bambusa_polymorpha*	0.931	0.045	0.028	0.127	0.096	0.030	0.030	0.018	0.118	0.031	0.004	0.016	0.033
18	*Bambusa_ventricosa*	0.988	0.065	0.001	0.300	0.256	0.027	0.055	0.045	0.282	0.024	0.027	0.061	0.001
19	*Dendrocalamus_strictus*	0.943	0.063	0.063	0.285	0.247	0.047	0.005	0.050	0.286	0.040	0.025	0.054	0.064

The sequences of the *ITS* markers for 18 bamboo species, generated during the study, were deposited in NCBI GenBank with the following IDs in (Table III).

3.3. Morphological Data analysis:

Phylogenetic relation based on the morphological data of the Bamboo species Khanday et al. (2015) has revealed that interspecies and inter-generic relationship of Bamboos cannot be completely resolved on the basis of morphological characters only. The inter-generic and inter-specific clustering of Bamboo species under observation was also depicted in (Fig3).

Table III: The sequences of the ITS1, 5.8S rRNA and ITS2 markers for 18 bamboo species, generated during the study, were deposited in NCBI GenBank with the following IDs:

Genus	species	Accession no.
Bambusa	*Bambusa balcooa* Roxb	MH921473
	Bambusa burmanica Gamble	MH921470
	Bambusa bambos (L.) Voss	MH921479
	Bambusa tulda Roxb	MH921475
	Bambusa polymorpha Munro	MH921477
	Bambusa multiplex (Lour.) Raeush. ex. Schultes	MH921468
	Bambusa ventricosa McClure	MH921478
	Bambusa vulgaris Schard	MH921466
Dendrocalamus	*Dendrocalamus asper* (Schult.) Backer	MH921471
	Dendrocalamus hamiltonii Nees & Am. ex. Munro	MH921472
	Dendrocalamus membranaceus Munro	MH921467
	Dendrocalamus giganteus (Wall.) ex. Munro	MH921476
	Dendrocalamus longispathus (Kurz.) Kurz	MH916750
	Dendrocalamus strictus (Roxb.) Nees	MH921480
Melocanna	*Melocanna bacifera* (Roxb) Kurz	MH921465
Guadua	*Guadua angustifolia* Kunth	MH921464
Dinochloa	*Dinochloa andamanica Kurz*	MH921469
Gigantochloa	*Gigantochloa ablociliata* (Munro.) Kurz	MH921474

4. Discussion:

Ribosomal *ITS* sequences are generally used for phylogenetic reconstruction due to their ubiquitous nature and high copy number. Various studies in recent years have discovered that *ITS* polymorphism within individuals is quite common (Denduangboripant and Cronk, 2000; Mayol and Rossello, 2001; Bailey et al. 2003; Rossello et al. 2006, 2007; Zhang and Ge, 2007; Kim et al. 2008; Grimm and Denk, 2008; Pilotti et al. 2009). The *ITS* regions can be amplified using universal primers because the ends are flanked by highly conserved sequences, and therefore have been used for various studies in Bamboos (Qiang et al. 2005; Peng et al. 2008; Song et al. 2012).

The entire *ITS* region, including *ITS*1, *ITS*2 spacers and the 5.8S subunit, of 18 species was analysed for cluster analysis by present investigator. The topology of Maximum-likelihood (ML) analysis was congruent with that of the Neighbor-joining (NJ) analysis. In present investigation *D. membranceous* and *D. strictus* were clustered together, similar observation was revealed by Yang et al. (2008) that *Dendrocalamus strictus* and D. *membranaceus* formed a subclade and were separated from the majority of the sampled *Dendrocalamus* taxa. However, *Dinochloa andamina* formed a separate clade, similarly Yang et al. (2008) studies also revealed that *Dinochloa* species formed a separate clade as a basal lineage.

Different workers have used *ITS* sequences for genetic variation and phylogenetic studies such as: Guo et al. (2001) studied twenty-three alpine species from three genera – *Fargesia, Thamnocalamus* and *Yushania*, *Phyllostachys* complex, Hodkinson et al. (2000) studied *Phyllostachys* complex, Sun et al. (2005) studied twenty-one species of *Bambusa, Dendrocalamus, Dendrocalamopsis, Guadua, Leleba* and *Lingnania*. Qiang et al. (2005) assessed the phylogenetic relationships of *Arundinaria* and related genera *(Pleioblastus, Pseudosasa, Oligostachyum, Bashania, Clavinodum,* etc.) by analyzing the sequences of the *ITS* and the cpDNA *trnL-F* intergenic spacer (IGS). Comparison with *trnL-F* IGS sequence, the *ITS* region provided the higher number of parsimony informative characters, and the interspecific variation of the *ITS* sequence was higher than that of the *trnL-F* IGS sequence.

In present investigation using *ITS* a distant relationship was established between *Guadua angustifolia, Melocanna bacifera* and *Dinochloa andamanica*. These three genera were clustered together in a separate clade represented as basal lineage with the species of other genera such as *Dendrocalamus, Bambusa* and *Gigantichloa.* Species of *Dendrocalamus* and *Bambusa* were clustered together into different clades. *Dendrocalamus longispathus* can be seen more closely related to the *Bambusa* species. Whereas, *Bambusa multiplex* can be seen more closely related to *Dendrocalamus* species.

Based on the whole analysis of molecular data very high resemblance was found between some species. *D. asper* and *D. giganteus* showed sister species relatedness using *ITS*, Similarly, *D.asper* and *D. hamiltonii* showed sister species relatedness using *ITS*. Earlier researchers have used various markers to analyze the Bamboo diversity and to find out their taxonomic relationship. Guo et al. (2001, 2002) used *ITS* for temperate woody Bamboos, Guo and Li, (2004) used *ITS* and *GSSI* for temperate woody Bamboos, Qiang et al. (2005) have used *ITS* and *trn*L-*trn*F to analyze species of Arundinaria and related genera,

Yang et al. (2008) worked on Pleotropical Woody Bamboos using *ITS*, *GBSSI*, *trnL-F*. Hodkinson et al. (2010) studied Arundinarieae using *trnL-F* and *ITS*, Ruiz-Sanchez and Sosa (2010) studied *Otatea* species using *ITS, atp*F-*atp*H, *psb*K*ps*I, *trn*L-*rpl*32. Zhou et al. (2010) studied 38 genera of woody bamoos using *Tyl*-copia rt, *ITS*. *ITS* based tree enlightened that *B. vulgaris* and *B. ventricosa* in two different clades shared a common node. Nayak et al. (2003) revealed closeness between *B. ventricosa* and *B. vulgaris* during his study and was supported by a previous finding that *B. ventricosa* is a cultivar originated variety of *B. vulgaris,* this would be a reason to bring these species in close relationship (Chua et al. 1996). A single species of *Gigantichloa* has been considerd in the present investigation, the *ITS* based sequence analysis exhibited a seperate clade with *Bambusa bambos* in tune with *Gigantichloa*.

It brings to notice that the study of Loh et al. (2000) and Ramanayake et al. (2007), using RAPD and AFLP respectively, exhibited a close relationship between *Bambusa* and *Gigantochloa*. The joint indications from these earlier molecular studies and Song et al. (2012) suggested that taxa belonging to *Bambusa*, *Dendrocalamus* and *Gigantochloa* form a close complex but are relatively distant from *Dinochloa* and *Monocladus*. Similarly in present investigation *Dinochloa andamanica* was found to form a clade with the species other than *Bambusa* and Dendrocalamus.

Present study has shown a close relation between *Dendrocalamus* and *Bambusa*, a close relation of these two genera have also been shown by (Sun *et al.*, 2005). These results coincide with the investigation of Ghosh et al. (2012), who studied the AFLP marker and showed the polyphyletic origin of both genera. Due to the polyphyletic nature of genus *Bambusa* and *Dendrocalamus.* Some of the workers divided *Dendrocalamus* genus into two groups based on vegetative characters, inflorescence morphology and flowering behavior Wong (1995), whilst on the basis of biochemical parameters Chou and Hwang (1985) divided the same genus into four groups. Facing such taxonomic confusion, Wong (1995) concluded that *Dendrocalamus* genus needs more meticulous study using precise molecular biology methods.

Analysis of morphological and molecular data sets to present the synergies between Bamboo species. It was observed that *Gigantichloa albociliata* formed a separate clade with *Bambusa bambos* which was also supported by the vegetative character-based analysis where these two sequences formed a separate clade in a single cluster. However, Watanabe et al. (1994) the first to study phylogenetic relationships among Asian Bamboos using RFLP of chloroplast DNA recovered a clade representing subtribe Bambusinae Ohrnberger (1999) containing *Bambusa*, *Dendrocalamus*, and *Gigantochloa*, suggesting close relationships among these genera. Similarly, Loh et al. (2000) and Ramanayake et al. (2007), using AFLP and RAPD respectively, also indicated a close relationship between *Bambusa* and *Gigantochloa*. *B. Balcooa*, D. *hamiltonii* and *D. giganteous* formed a clade showing closeness which concides with the readings of vegetative character in one of the data sets and in other of *B. burmanica* and *D. asper* showed closeness. This supports the synapomorphies between two generas *Bambusa* and *Dendrocalamus*. Observations from molecular data sets suggests *Guadua angustifolia* a distinct species but using the morphological data sets revealed a close relationship of *Guadua angustifolia* with *B. multiplex* and *B. ventricosa.* No obvious relationship was found between *D.strictus* and *M. baccifera* from the analysis of molecular data whereas the two species formed a clade using morphological data.

Vegetatively B. *vulgaris* and B. *burmanica* showed closeness which corresponds the readings of *rbcL* and *ITS* data. Similarly, D. *giganteus* and *D. hamiltonii* showed closeness which conincides the readings of *psb*A and ITS. *B. multiplex* and *Polymorpha, D. strictus* and *M. baccifera* revealed closeness while analysis the observation from both the data sets.

5. Conclusion:

Morphological studies are essential at every mode for Bamboo identification and taxonomic classification. Erratic nature of flowering cycle has restricted the validity of morphological characterization to a certain extent. However, the other vegetative characters server the purpose by playing a curtail role. No doubt morphological information works as an important tool for the reorganization of Bamboo species but doesn't have an advocate quantity of information to fulfill the circumstantial clarifications to the changing nature of Bamboo morphotaxonomic characters. Molecular marker-based assessment may help in correlation between species in order to remove ambiguities of identification and taxonomic crunch which could be possibly because of the fact that the morphological characters can highly be influenced by environmental conditions.

Molecular phylogeny using ITS region was essentially used to resolve morpho-taxonomic ambiguity and taxonomic position of genera under Bambusoideae complex. Based on the sequence data the characterization of each species was obvious. However, molecular marker alone cannot be used for the characterization and identification of Bamboos. Utilization of morphological datasets is always needed due to their importance for field identification of Bamboos. Though, the cluster-based approach for taxonomic discrimination of species under Bambusoideae complex was found to be significant. However, for an accurate reconstruction of Bamboo evolutionary history needs to require more attention towards morphological and molecular combine data sets.

Declarations

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Conflict of interest- The authors declare that they have no conflict of interest.

Data archiving statement

The data has been submitted to National *Center for Biotechnology Information from which the accession numbers have been obtained which has been presented in table III.*

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Figures

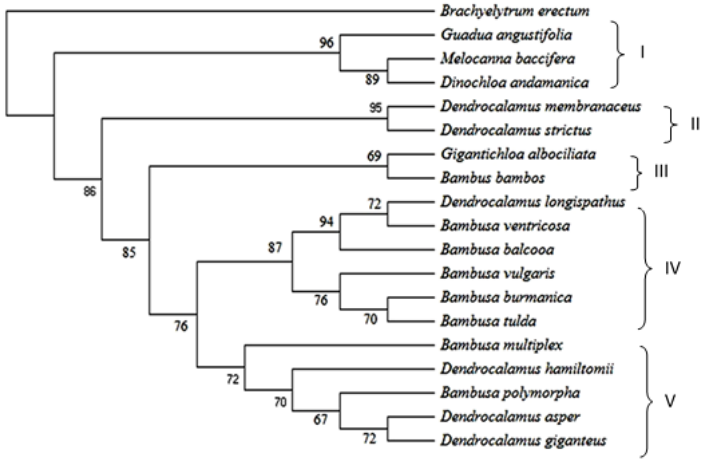

Figure 1

Phylogenetic tree constructed using maximum-likelihood algorithm with 1000 bootstrap replications following Kimura 2-parameter model bootstrap values are shown at the nodal positions of each branch of the respective tree.

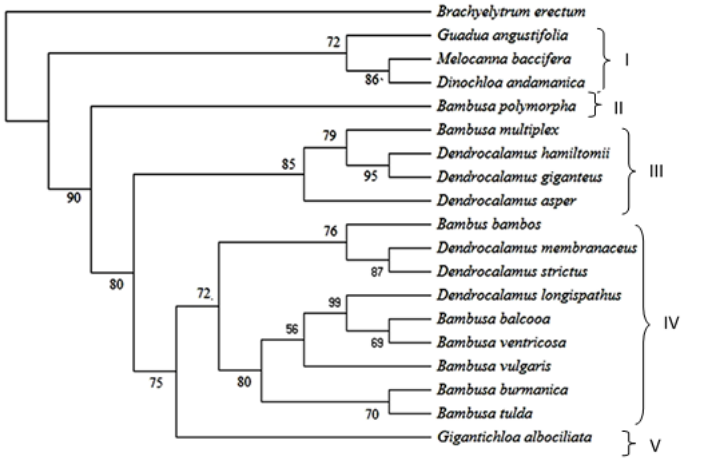

Figure 2

Phylogenetic tree constructed using Neighbor-joining algorithm with 1000 bootstrap replications following Jukes-Cantor model, bootstrap values are shown at the nodal positions of each branching of the respective trees.

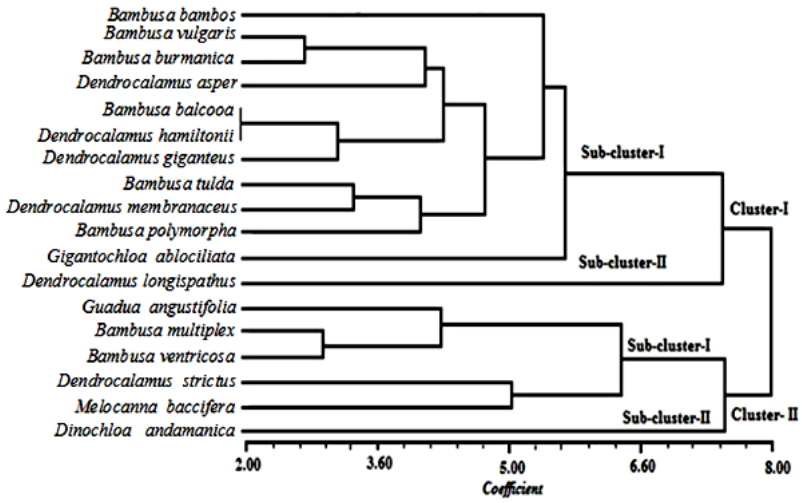

Figure 3

Dendrogram based on UPGMA for Eighteen Bamboo Species Khanday et al. 2015.