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Research Article

ASSESSMENT OF GENETIC DIVERSITY IN BAMBOO ACCESSIONS OF INDIA USING MOLECULAR MARKERS

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Abstract

Bamboo is an important grass with wide scale applications in paper industries, medicines, constructions industries. It is potential feedstock for advanced biofuel production due to its favourable characteristics, natural abundance, rapid growth, perennial nature and higher CO_2 sequestration. The objective of this study is to understand genetic diversity between the bamboo accessions with respect to geographical origin to correlate molecular information with feedstock characterization and adaptation to abiotic stress. In this study, genomic DNA was extracted from twenty bamboo accessions collected from different regions of India and genetic variations were assessed by inter simple sequence repeat (ISSR) based molecular marker approach using 8 primers. Maximum genetic distance was observed between *Bambusa wamin*-Itanagar & *B. ventricosa*-Durg (0.48221) & minimum genetic distance between *Bambusa balcooa*-Modasa & *Bambusa balcooa*-Tripura (0.00787). *Bambusa balcooa* and *Bambusa vulgaris* were genetically similar as compared to other accessions. The genetic distance is independent of geographical distance for the bamboo accessions considered in this study. The findings of this study will help to understand the degree of differences between bamboo accessions under the same environmental conditions and to identify the representative accessions that can be used for abiotic stress resistance studies. The information can be explored for screening of closely related bamboo accessions for abiotic stress resistance screening trials.

Keywords: Bamboo; Bioethanol production; CO_2 sequestration; Inter Simple Sequence Repeats markers; Lignocellulosic ethanol; Marginal land reclamation; Molecular markers; Salinity resistant screening.

Introduction

Bamboo is fast growing, resilient, perennial grasses comprising of more than 1400 species known to thrive in diverse climatic and soil conditions. Almost about 2.5 billion people use bamboo in routine life, mostly for fibre and food. India is the second richest country in bamboo genetic resources following China, ranking first in this aspect (Bystriakova *et al.,* 2003). More than 125 different bamboo species occur in India. Lately, worldwide interest in bamboo as a source of biofuel or bioenergy has rapidly increased (Scurlock, 2000) due to the concerns regarding energy security, oil price volatility and environmental pollution (International Energy Agency; 2011). Bamboo is considered a potential feedstock for the production of biofuels to meet the rising demands of fuel and to control the rising imports of oils because it shares a number of desirable fuel characteristics with certain other bioenergy feedstocks, such as low ash content and alkali index. The composition of bamboo is highly similar to other grasses utilised for biofuel purposes (e.g. switchgrass, Miscanthus) (Scurlock, 2000).

Literature on bamboo productivity and diversity is scarce with reference to its biofuel application and geographic widespread distribution. Identification and classification is necessary for collection and conservation of bamboo germplasms (Bahadur, 1979). The reproductive cycle of bamboo ranges from 3 to 120 years making floral identification process difficult (Janzen, 1976; Bhattacharya *et al..* 2009) while classifications based on vegetative traits are available but not reliable due to influence of ecological factors (Clayton. *et al.,* 2013; Ohrnberger, 2002).

Molecular data can provide useful information to deal with various aspects of taxonomic classification of plants (Das *et al.,* 2005). Application of molecular techniques for genetic diversity assessment of bamboo was limited till 2000. Several molecular marker approaches including random amplified polymorphic DNA (RAPD; Bhattacharya *et al.*2009) RFLP, isozyme analysis, chloroplast DNA phylogeny, analysis of rpl16 intron sequences and simple sequence repeats (SSRs; Sharma *et al.*, 2008) are available for Bamboo. ISSRs are widely used for population genetics studies (Zhang and Dai, 2010) that uses 16-25 bp primers in a polymerase chain reaction to generate multilocus markers.

ISSRs have high reproducibility, possibly due to the use of longer primers (16–25 bp) as compared to RAPD primers (10 bp). ISSR approach has been widely used to study the interspecies as well as intra species genetic variations in different plants however its use for assessment of genetic variation in bamboo accessions has not been reported yet for screening the potential drought and salt resistance bamboo accessions for biofuel application.

Therefore, the present study focuses on assessment of genetic variation using the ISSR approach to identify genetic and geographic diversity of bamboo accessions from different location. The molecular information could be correlated in future for bamboo cultivation and adaptation to any abiotic stress of marginally draught and saline land of India.

Methods and Materials

Germ Plasm Collection
A total of twenty widely founds bamboo germplasms growing in different region of India (Fig. 1) were collected from the Bambusetum Single khaanch-Ukai and other places of Gujarat with kind help and guidance Conservator of Forests and Additional Principal Chief Conservator of Forests, National Bamboo Mission, Gandhinagar.

All collected germplasms were established at a site maintained by Abellon Agrisciences at Rameshwar Kampa, Modasa in Aravalli district. The germplasms were named according to the site of collection as mentioned in Table-1.
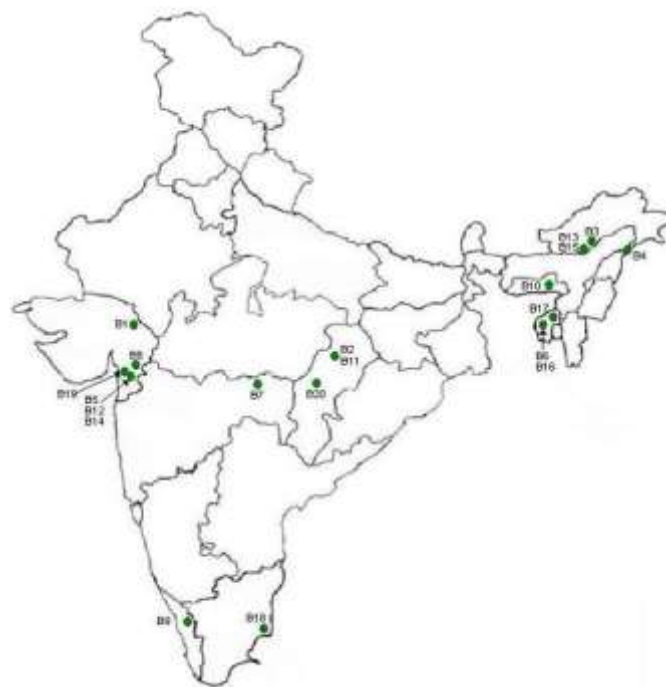

Fig. 1: Site of Collection of twenty Bamboo accessions

Table 1: Bamboo germplasm collection and site specific coding

Sr. No.	Samplecode	Bamboo variety	Collection site
1	B 1	*Bambusa balcooa*	Modasa-Gujarat
2	B 2	*Bambusa vulgaris*	Bilaspur- Chhattisgarh
3	B 3	*Bambusa vulgaris*	Tirap-Arunachal Pradesh
4	B 4	*Bambusa balcooa*	Papum Pare-Arunachal Pradesh
5	B 5	*Phyllostachys nigra*	Ukai-Gujarat
6	B 6	*Melocanna baccifera*	Gomati-Tripura
7	B 7	*Sasa fortuneii*	Amrawati-Maharashtra
8	B 8	*Dendrocalamus strictus*	Rajpipla-Gujarat
9	B 9	*Bambusa vulgaris*	Palakkad-Kerala
10	B 10	*Bambusa balcooa*	Nongpoh-Meghalaya
11	B 11	*Bambusa wamin*	Bilaspur-Chhattisgarh
12	B 12	*Bambusa vulgaris*	Ukai-Gujarat
13	B 13	*Bambusa wamin*	Itanagar-Arunachal Pradesh
14	B 14	*Bambusa multiplex*	Ukai-Gujarat
15	B 15	*Bambusa vulgaris*	Itanagar-Arunachal Pradesh
16	B 16	*Bambusa balcooa*	Agartala-Tripura
17	B 17	*Bambusa balcooa*	Karimganj-Assam
18	B 18	*Bambusa vulgaris*	Thiruvarur-Tamilnadu
19	B 19	*Sasa palmata*	Kakrapar-Gujarat
20	B 20	*Bambusa ventricosa*	Durg-Chhatisgarh

DNA Isolation

Fully expanded leaves from each of the twenty samples were collected and surface sterilized prior to DNA isolation. Genomic DNA was extracted using EZgene CP Plant Mini Kit (XcelGen, Xcelris Genomics) as per manufacturer's instructions. DNA concentration was measured using Nanodrop 8000 spectrophotometer (Thermo Scientific). The quality of DNA was determined using 0.8% agarose gel.

ISSR PCR Amplification

Total eight ISSR primers were selected for this study based on the screening of twenty-five primers. Genomic DNA was PCR-amplified using the selected 8 primers. Primer details, sequences and codes are mentioned in Table-2.

Table-2: Primers description

Sr. No.	Primer code	Primer	Sequence
1	A	814	(CT)8 TG
2	B	844A	(CT)8 AC
3	C	17899A	(CA)6 AG
4	D	844B	(CT)8 GC
5	E	HB9	(GT)6 GG
6	F	HB14	(CTC)3 GC
7	G	ISSR 5	(AC)8 TG
8	H	17898B	(CA)6 GT

Amplification were performed using Veriti 96 well thermal cycler (Applied Biosystem). The 25 μl PCR reaction was set up using 50 ng of gDNA, 1 X reaction buffer with 2.0 mM MgCl₂, 10 pM primer, 200μM dNTPs, 1 unit of Taq polymerase (Fermentas) and nuclease free water (Ambion). PCR amplification conditions were Initial denaturation at 94°C for 5 mins, 35 cycles of denaturation at 94°C for 30 sec, annealing at 44°C for 45 sec, extension at 72°C for 90 sec and final extension at 72°C for 20 min. The PCR products were electrophoretically separated using 2.5 % agarose gel electrophoresis for 90 minutes at 110 Volt. A 2kb DNA ladder (Rovalab) was used as a size marker. Gel picture was captured using gel documentation system (Bio-rad-XR+ System). The number and intensity of monomorphic and polymorphic bands were recorded using QuantityOne software. Only clear and reproducible bands were considered for data analysis.

Data Analysis

Only clear and unambiguous bands were visually scored. The present bands were scored as (1) and absent bands were scored as (0). Each band was interpreted as one allele. Bands with the same mobility were assumed to be homologous. Each marker was treated as an independent unit character. The genetic distance and genetic similarity from ISSR data were calculated among species using Nei's coefficient. Cluster analysis were based on a similarity matrix obtained with the Unweighed Pair Group Method

using Arithmetic Averages (UPGMA) and relationships between species were visualized as a dendrogram. All data were scored in the form of a binary matrix. For each pair of species, the Nei's coefficient was calculated. The calculations were performed with the POPGENE 1.32 software for data analysis and MEGA 4.0 was used for dendrogram visualization.

Correlation between Genetic Diversity and Aerial Geographical Distance

A scatter plot was constructed for genetic diversity among the twenty bamboo accessions against the aerial geographical distance between the sites of collection of the bamboo accession. Pearsons correlation analysis of the genetic distance of the bamboo germplasms of different accessions (e.g. *B. balcooa* & *D. strictus*) as well as among the similar accession (e.g. *B. balcooa* & *B. balcooa* collected from different locations) against geographic distance between the sites of collection of bamboo germplasms was performed by using SPSS statistical software version-20 (IBM).

Results

Analysis of Genetic Diversity using ISSR Primers

Out of 8 ISSR primer used, total 105 bands generated using twenty different bamboo accessions. The size of amplification products ranged from 100 bp to 2 kb (Figure-2).
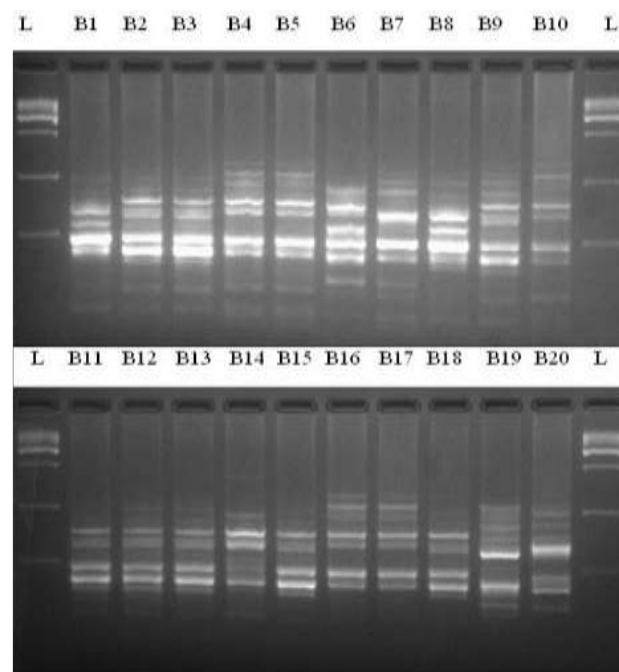
Fig. 2: Genetic profile of twenty bamboo vareities using ISSR primer HB-14.

The number of bands generated per primer were ranged from 11 (primer 844B and primer 17898B) to 16 (primers ISSR5) with an average of 13.125 bands per primer. Out of 105 bands generated, 76 bands (72.38%) were polymorphic and 29 bands (27.62%) were monomorphic (Table-3).

The percentage of polymorphism per primer ranged from 60% to 81%. Primer ISSR5 showed maximum number of polymorphic bands (13 bands out of 16) and primer 814 showed least number of polymorphic bands (6 bands out of 15).

Table 3: Amplification profiles of eight ISSR primers

Sr. No.	Primer code	No. of Amplified Bands	No. of Polymorphic Bands	% of Polymorphic Bands (PPB)
1	A	15	9	60
2	B	14	10	71.4
3	C	12	9	75
4	D	11	8	72.7
5	E	13	10	76.9
6	F	13	9	69.2
7	G	16	13	81
8	H	11	8	72.7
		Total=105	Total= 76	Average=72.38

Analysis of Genetic Distances

Genetic distances for all possible pairs based on Nei's coefficient using ISSR data were calculated. The population pair of *B. balcooa*-Modasa (B1) and *B. balcooa* (B16) showed minimum genetic distance (GD = 0.00787) and population pair *B. Wamin*-Itnanagar-Arunachal Pradesh (B13) and *B. ventricosa*-Durg-Chhattisgarh (B20) showed maximum genetic distance (GD = 0.48221).

Cluster Analysis

Phylogenetic tree for twenty bamboo accessions were constructed using Nei's coefficient (1972) and UPGMA tree, based on the values for the genetic distance (Fig. 3).

The phylogenetic tree was divided into 2 major groups. The first major group includes accessions of *Bambusa ventricosa*, 5 samples of *Bambusa balcooa* and 6 samples of *Bambusa vulgaris* collected from different locations. The second major group includes accessions of *Phyllostachys nigra*, *D. strictus*, *B. wamin* from two different locations, *B. multiplex* and *Sasa palmata*. The first major group was subdivided into two groups i.e. five germplasms of *Bambusa balcooa* collected from different locations in the first group and 6 germplasms of Bambusa vulgaris collected from different locations were placed in the another group.

Correlation Analysis

The correlation analysis for genetic diversity of twenty bamboo accessions against the aerial geographical distance between the sites of collection of bamboo accessions showed that the bamboo plants of the same accession had low genetic diversity irrespective of geographical distance (Table 4).
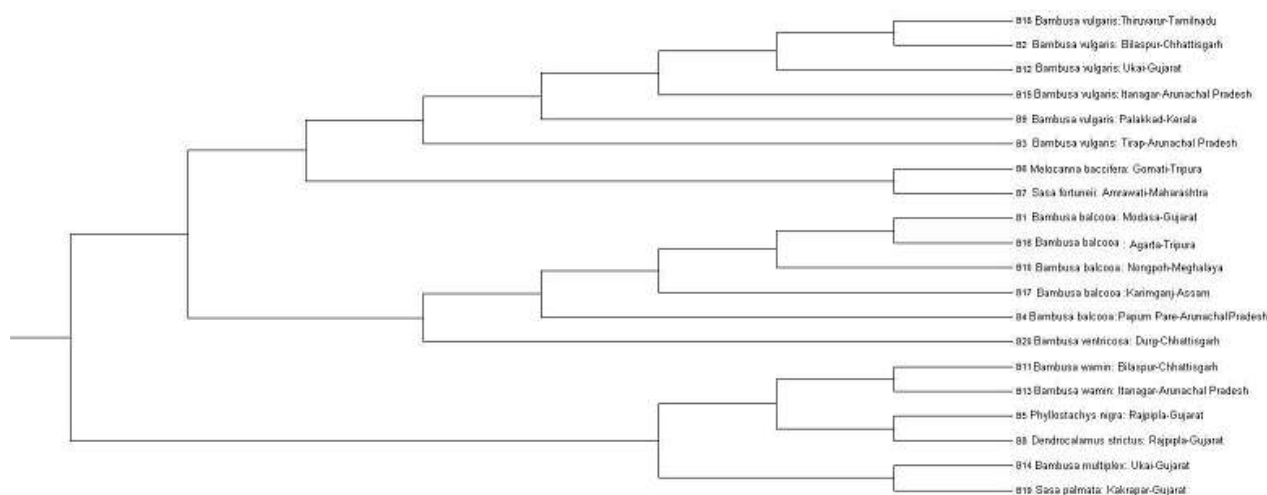
Fig. 3: UPGMA dendogram based on Nei's (1972) genetic distances among 20 different varieties of Bamboo.

Table 4: Correlation between genetic distance and geographical distance between the sites of collection

Correlation		Genetic Distance	Geographical Distance
Genetic Distance among different bamboo varieties v.s aerial geographical distance between the site of collection	Pearson Correlation	1	-.018
	Sig. (2-tailed)		.814
	N	164	164
Genetic Distance among different bamboo varieties collected from different locations v.s aerial geographical distance between the site of collection	Pearson Correlation	1	.710**
	Sig. (2-tailed)		0.000
	N	46	46

**. Correlation is significant at the 0.01 level (2-tailed).

B. balcooa germplasms collected from different locations had very less genetic diversity (less than 0.03) among themselves whereas different germplasms of *B. vulgaris* and *B. wamin* collected from different locations had higher genetic diversity (0.07 to 0.133) as compared to *B. balcooa*. The bamboo plants of different accessions s had the genetic diversity in the range of 0.2 to 0.5 with most of the correlation values concentrated in the range of 0.3 to 0.4 GD. The genetic distance among different bamboo accessions was in the range of 0.2 to 0.4 GD even among the accessions collected from the same location. Pearson's correlation analysis for different bamboo accessions against the geographical distance was not significantly correlated (p=0.814). Correlations analysis for similar bamboo accessions collected from different locations showed that genetic distance had statistically significant positive relationship (Two-tailed sigma=0.000) with the aerial geographical distance between the sites of collection of bamboo accessions, the strength was 0.286.

Discussion

Bamboo is a fast growing grass that has recently been identified as a promising feedstock for biofuel and bioenergy generation (Scurlock, 2000). In recent years, the need for development of sustainable liquid biofuels is realised due to the concerns regarding energy security, oil price volatility and environmental pollution (International Energy Agency; 2011). Several studies like Sathitsuksanoh *et al.* (2010) have reported efficient and cost effective pretreatment methods for second generation biofuel (cellulosic ethanol) generation from bamboo. In 2011, India's oil import bill in terms of value has increased from Rs 409,077 crore in 2009-10 to Rs 726,386 crore in 2011-12 (Press Information Bureau, Govt of India) and in 2013 India became the third biggest oil-importer in 2013 after USA and China (Reuters, 2014). Experts predict that by the year 2020, there will be 450 million vehicles plying on Indian roads (India Transport Portal, 2012). These issues contributed to the rise in worldwide interest in bamboo as a source of biofuel or bioenergy.

Several high biomass yeilding varieties of bamboo are capable of growing under drought and saline affected land. Gujarat has 20108.06 sq. km. of wasteland, around 10.26% of total geographical land (Wasteland Atlas of India published in 2011). This land lays barren throughout the year due to high soil salinity and lack of irrigation water. Screening and identification of drought and saline resistant accessions of bamboo and its cultivation in this wasteland would contribute to feedstock for production of bioethanol along with wasteland reclamation, sequestering of carbon dioxide in the soil to reduce atmospheric CO_2 concentrations, thus mitigating the effects global warming.

Screening based on molecular markers provide precise information on genetic diversity and identification of variety-specific markers because of the independence of the confounding effects of environmental factors. In recent years, DNA profiling through ISSR technique has been used for the analysis of genetic diversity, phylogenetic relationship, and varietal identification but application of ISSR method for segregating the potential resistant accessions for drought and saline stress screening experiment is not yet reported elsewhere. This is the first reported study to employ ISSR markers for the identification of representative accessions for drought and saline resistance screening experiments.

In the present investigation, twenty bamboo accessions that are widely found in India were collected from eight ISSR primers were used to detect DNA polymorphism in twenty accessions of bamboo. ISSR primers produced 105 amplified fragments varying from size range of 100bp to 2000bp. ISSR marker system revealed high levels of polymorphism of about 72.38 % among the bamboo accessions.

Cluster analysis was performed using the unweighted pair group method with arithmetic averages (UPGMA). Dendrograms were constructed using the UPGMA algorithms in the MEGA 4.0 software (Tamura *et al.*, 2007) and showed that the phylogenetic tree was divided into two major clusters. The first major group included accessions of *B. balcooa*, *B. ventricosa* and *B. vulgaris*. The second major group included *P. nigra*, *D. strictus*, *B. wamin* , *B. multiplex* and *S. palmata*. Nayak *et al.*(2003) showed that *B. vulgaris*, *B. ventricosa*, *B. balcooa*, were placed in the same cluster and *D. strictus* and *B. multiplex* were placed in the different cluster based on genetic similarity. Sun *et al.*(2006) performed RAPD based genetic diversity assessment for *Bambusa* species and placed *B. multiplex* and *B. ventricosa* in one cluster and *Bambusa vulgaris* in other cluster. In the present study, *B. balcooa*, *B. vulgaris* and *B. ventricosa* were placed in one cluster, *B. multiplex* and *D. strictus* were placed in the second major group together and *B. vulgaris* and *B. ventricosa* were found to be more closely related to each other than *B. multiplex*. The results of the present study are similar to the results reported by Nayak *et al.*(2003) and this easy reproducibility of results obtained from inter-simple sequence repeat makes it most reliable molecular markers based method as it permits detection of polymorphisms in inter-microsatellite loci, using a primer designed from dinucleotide or trinucleotide simple sequence repeats. ISSR markers have been extensively used for DNA finger-printing (Moreno *et al.*, 1998), population genetics studies (Nebauer *et al.*, 1999) and phylogenetic studies (Hess *et al.*, 2000). ISSR analysis has also been applied in studies involving genetic identity, parentage, clone and strain identification, and taxonomic studies of closely related species (Godwin *et al.*, 1997; Gupta *et al.*, 1994).The difference in the results of the present study and the results reported by Sun *et al.* (2006) may be due to difference in the techniques used for analysis. In RAPD

analysis random primers may cause artifacts in the results due to their short length, whereas in ISSR technique primers have enough length (16–25 bp) that permits the subsequent use of high annealing temperature (45–60ºC) leading to higher stringency.

Pearson's correlation analysis was performed for the genetic distance among the bamboo accessions and aerial geographical distance of the site of collection of bamboo germplasms and the results show that there is no significant correlation between the genetic distance and aerial geographical distance for the different bamboo accessions but the genetic distance and aerial geographical distance are significantly positively correlated for the same bamboo accessions collected from different locations.

Conclusions

This study utilized ISSR based molecular study for screening out the potential bamboo accessions based on geographic and genetic distance. This results would be useful to select bamboo accessions from different genera for salt and drought resistance experiments based on their geographic and genetic distance and/or similarities.

Acknowledgement

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