THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN-2319-2119

RESEARCH ARTICLE

Arnab Sen *et al*, The *Experiment*, *2014*., *Vol. 19(1), 1304-1315*

Molecular phylogeny of North Bengal bamboos inferred through PCR-RFLP based on the *trnL-trnF* region

ABSTRACT

Bamboo is a wonderful gift of nature to mankind which is associated with them since ages.This study presented the molecular diversity of 29 accessions of bamboos encountered in North Bengal through the PCR-RFLP technique based on the *trnL-trnF* region for the first time. The PCR amplification of the *trnL-trnF* region of chloroplast genome of bamboos using prime pairs Tab C & F, resulted in the production of single band of expected length i.e. 1029 bp compared to molecular marker. The PCR amplicons were subjected to restriction digestion using different restriction endonucleases, namely *Taq*I, *Alu*I, *Hinf*I, *Hae*III, *Hpa*I and *Msp*I. Only three of them *viz.* *Taq*I, *Alu*I, *Hinf*I were successful in digesting the PCR product which resulted in producing 19 bands of which 9 were polymorphic. The percentage of polymorphism was found to be only 47.37%. *Alu*I showed highest polymorphism of 70%. The band size ranged in between 33-1986bp. The PCR-RFLP study showed diversity among the 29 accessions. This was also confirmed by sequencing the PCR amplicons one from each genus. Thus, it can be inferred that PCR-RFLP can be used as a tool for screening the phylogenetics.

Keywords: Bamboo, genetic diversity, North Bengal, PCR-RFLP, *trnL-trnF* region

1. INTRODUCTION

Bamboo with altogether 1575 species belongs to the subfamily Bambusoideae of the true grass family Poaceae which include both woody and herbaceous bamboos [1]. Bamboo is popularly known as “Poor man’s timber”, “Green Gold of the forest” because of its innumerable applications [2]. Bamboo is naturally distributed round the globe. Major species of bamboo is found in Asia Pacific and South America but much less in Africa [3]. In India, there are about 136 indigenous and exotic species found to grow naturally and/or under cultivation [4]. North Bengal is houses some important genera of Bamboo like *Bambusa, Cephalostychum, Dendrocalamus, Drepanostachyum, Gigantocloa, Himalayacalamus, Melocanna, Pleioblastus, Phyllostachys, Pseudosasa, Sasaella, Shibataea, Yushnia* which include different species, subspecies and varieties [5,6].

Basic knowledge in the biology and genetics of bamboo is lacking due to its unusual life cycle with the vegetative phase ranging from one to 120 years [7]. The taxonomy of bamboo is in a state of flux. Moreover, since the morphological features are often influenced by the environment factors, molecular studies are required to help resolve systematic issues [8]. With the advent of molecular biology, the taxonomy of different plants has been revolutionized [9] including bamboo. The use of molecular markers has been increasing at an exponential state in all the fields of biology. The application of molecular marker in classifying bamboo where the basic biology due to long flowering time is so little understood can prove to be a landmark. In bamboo the use of molecular markers can be employed for dual function, firstly for proper identification of bamboo genotypes and secondly assessment of genetic variation within species irrespective of the geographic location or other factors responsible to phenotypic variability. Review of literature showed that different molecular markers have been widely used in the plant genetic diversity analysis either in the nuclear genome [1, 10-16] or nuclear r-RNA gene sequence [17-24] and bamboo is no exception. Some studies on bamboo based on *TrnL-trnF* region have been performed. Qiang and his group [25] and Yang et al. [23], used *TrnL-trnF* region to infer *Arundinaria* and *Schizostachyum* phylogeny respectively. In 2008, Yang and his co-workers [24] used this region to study the molecular phylogenetic of paleotropical woody bamboos. To resolve major phylogenetic groupings within Bambusoideae, Sungkaew and his team [26] performed multi-gene phylogenetic analysis including *trnL-trnF*. But however, PCR based RFLP analysis of the *TrnL-trnF* region of bamboo genome have not been attempted. Thus keeping this in mind in this study phylogenetic analysis of 29 accessions of bamboos encountered in North Bengal, India has been done amplifying the *TrnL-trnF* region of bamboo chloroplast genome and followed by digestion with restriction enzymes. This is perhaps the first study where PCR-RFLP analysis of the *trnL-trnF* region has been employed to study the phylogenetic relationship and genetic diversity among the 29 accessions of bamboos.

2. MATERIALS AND METHODS

2.1 The study area

The Northern half of West Bengal province of India i.e. North Bengal surrounded by International borders of Bhutan, Nepal and Bangladesh has wide and diverse forest cover that houses different type of bamboo. The inventory was conducted in the forests of North Bengal which comprises of six districts- Darjeeling, Jalpaiguri, Coochbehar, North Dinajpur, South Dinajpur and Malda, covering an area of about 21,540.66 sq. Km [6]. Exploration of different places like Kalimpong, Lataguri, Siliguri, Patharghata, Madarihat, Lava, Sukna, Kalijora, Pundibari, Gorubathan and Malda (Fig. 1) during different season of the year resulted in collection of several bamboo species.

THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN-2319-2119

RESEARCH ARTICLE

2.2 Collection of plant material

Twenty nine accessions of bamboo belonging to 13 different genera encountered in North Bengal were used in the present study (Table1) after proper authentication by the bamboo taxonomist Dr. Puniya P. Paudyal, Consultant, National Bamboo Mission, Sikkim. The germplasm is planted in the "Bambusetum' at Kuresong Research Range, Sukna, Darjeeling between 26º47'26.94'' N Latitude and 88º21'47.41'' E Longitude with an elevation of 532 feet [6]. Fresh leaf materials were used for DNA isolation.

2.3 Isolation of genomic DNA

The genomic DNA was isolated using the standard protocol of Doyle and Doyle [27] with minor modifications. Tender leaves (approx. 5g) were ground into a fine powder with the help of liquid nitrogen. The pulverized material was mixed with 15 ml of prewarmed (at 65ºC) 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 hr at 65ºC with occasional mixing by gentle swirling. To it equal volume of chloroform:Isoamyl alcohol (24:1) and centrifuged for 15 minutes at 6,500 rpm (5,000 xg) at 24ºC 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 500µl of 1X 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 xg) for 15 minutes. 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 xg) for 15 minutes at room temperature.The upper aqueous phase was taken in a fresh tube and to it 0.1 volume of 3M sodium acetate (pH 5.2) and 2 volume of absolute ethyl alcohol was added and precipitated at 4ºC for 30 minutes in a cooling centrifuge at 12,000 rpm (13,500 xg). The pellet obtained was washed in 70% ethyl alcohol, dried and dissolved in 500µl of 1X 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 500µl of 1X TE buffer (pH 7.4) and it was incubated at 37ºC for 1 hr in a Dry bath. An equal volume of chloroform:Isoamyl alcohol (24:1) was added and mixed properly. Centrifuged at 10,000 rpm (10,000 xg) for 15 minutes at room temperature. The aqueous phase was then transferred to a fresh microcentrifuge tube. To the aqueous phase 0.1 volume of 3M 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 xg) for 30 minutes at 4ºC. The DNA pellet obtained was washed in 70% ethyl alcohol, air dried and finally dissolved in 100µl of 1XTE (pH 7.4) buffer. The quality and quantity of the isolated genomic DNA was estimated using two methods. Firstly gel analysis method i.e. where the DNA samples were run in 0.8% agarose gel using λ DNA/EcoRI/HindIII double digest as molecular weight marker and secondly spectrophotmetically recording the optical density at 260nm and 280 nm.

2.4 PCR amplifications

2.4.1 Primers used

Tab c-f in *trnL-trnF* [28] region of the bamboo genome was amplified. The primer sequence Tab c-5'-CGAAATCGGTAGACGCTACG-3' & Tab f -5'-ATTTGAACTGGTGACACGAG-3' was used on the basis of the known sequence from the Taberlet region of the other plant species.

2.4.2 PCR amplification of trnL-trnF region

The polymerase chain reaction contained 12.5µl of PCR master mix (GeNei™ Cat# 610602200031730 Pl. No. MME22), 1.25µl primer (each), DNA (25ng/µl) and Pyrogen free water to a final volume of 25µl. PCR amplification was performed on a Perkin-Elmer Thermocycler 2400 with the following specifications: initial denaturation 95ºC for 5min, followed by 34 cylces of denaturation at 95ºC for 45s, primer annealing at 37ºC 50ºC for 45s, primer extension at 72ºC for 2min with final extension at 72ºC for 7 min. The amplified products were resolved on 1.8% (w/v) agarose gel containing Ethidium bromide solution (0.5µg/ml) run in 0.5X 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 molecular weight marker.

2.4.3 PCR product restriction digestion

The PCR products were subjected to restriction digestion with 6 different restriction endonucleases, namely *Taq*I (GeNei™ Cat# 610100700041730 PI MBE7S), *Alu*I (GeNei™ Cat# 610101700041730 PI No. MBE17L), *Hinf*I (GeNei™, Cat# 610102100021730 PI No. MBE21S), *Hae*III (GeNei™, Cat# 610101000041730 PI No. MBE10L), *Hpa*I (GeNei™ Cat# 105753) and *Msp*I (GeNei™ Cat# 610103100021730 PI No. MBE31S) all 4-base cutters. The restriction digestion reaction mixture contained 2µl of restriction enzyme

THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN-2319-2119

RESEARCH ARTICLE

Arnab Sen *et al*, The *Experiment*, *2014*., *Vol. 19(1), 1304-1315*

buffer, enzyme (5U), PCR product (5µl) and pyrogen free water to a final volume of 20µl. In case of enzyme *Taq*I BSA was also added separately. The restriction digestion mix was spin for a moment. The mix was then incubated at temperature specific for enzyme for 1 hr in a Dry water bath. After 1 hr the restriction digestion mix products were resolved on 1.8% (w/v) agarose gel containing Ethidium bromide solution (0.5µg/ml) run in 0.5X 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 molecular weight marker.

2.4.4 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 [29]. 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) [30]. 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.

2.5 Sequencing of PCR-RFLP amplification products

To confirm the identity of the PCR bands generated by the primer pair, the corresponding amplification products of different species of bamboos (one from each genera) showing good amount of polymorphism in case of *trnL-trnF* region were directly sent for sequencing to Chromous Biotech Pvt. Ltd., #842, II, Floor, Shankar Bhawan, A Block, Shankar Nagar, Bangalore-5600092.

2.5.1 Sequence analysis

All the 26 (13 forward and 13 reverse) resulting sequences were individually compared with the equivalent sequences from a range of other bamboo species present in sequence banks using Basic Local Alignment Search Tool (BLAST) [31] obtained from National Centre for Biotechnology Information (NCBI) web site (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5.2 Sequence submission in public domain

The raw sequences of all the thirteen species (one from each genera) of bamboo were documented with the help of Sequin Application Version 12.30 Standard Release [Nov 13, 2012] for Database submission to GenBank providing necessary information's like, definition of the sequence (i.e. the specific region of the genome), source of the sequence (chloroplast DNA in this case; name of the plant species along with its taxonomic position, date and place of collection, tissue type etc.).

3. RESULTS AND DISCUSSION

PCR-RFLP being a simple and inexpensive method plays an important role in accessing the genetic diversity of different plant species [32-36] and was thus applied to study the fingerprinting of different species and varieties of bamboo found in North Bengal.

3.1 PCR amplification and agarose gel analysis

In the present study 29 species and varieties of bamboo were subjected to PCR amplification with locus specific primer pair developed based on the Tab c-f in "Taberlet" (*TrnL-trnF*) region of the chloroplast genome of bamboo for which the nucleotide information was available with respect to other plant species in the public domain. The primer pair successfully amplified the Tab c-f in "Taberlet" (*TrnL-trnF*) region of the chloroplast genome of bamboo. Using the template DNA, the primer pair generated a single band following PCR of expected length i.e. 1029 bp. The amplified product is shown in fig 2.

3.2 PCR product restriction digestion and agarose gel analysis

The PCR product obtained from the prime pair Tab c-f were subjected to restriction digestion using six different restriction enzymes like *Taq*I, *Alu*I, *Hinf*I, *Hae*III, *Hpa*I and *Msp*I to short out the degree of genetic variation among different species and varieties of bamboo. Three restriction enzymes (*Hae*III, *Hpa*I and *Msp*I) under study failed to digest the PCR products while the summary of the digestion by the other three restriction enzymes are depicted in table 2. Digestion with *Taq*I resulted in producing four bands in all except for *Bambusa multiplex* 'Alphanso-Karr', *B. multiplex* 'Rivierorum', *Cephalostachyum latifolium*, *Drepanostachyum khasianum* and Chinese bamboo (Unidentified) where five bands were produced (fig 3a). The polymorphism percentage was found to be 20% with band size between 354-740 bp. Restriction enzyme *Alu*I showed maximum polymorphism (70%) generating five bands in most of the accessions of bamboo, six bands were produced in *Bambusa multiplex* 'Alphanso-Karr', *B. multiplex* 'Rivierorum', *Cephalostachyum latifolium* and four bands in *Bambusa bambos*, *Drepanostachyum khasianum*, *Himalayacalamus hookerianus* and *Shibataea kumasaca* (fig 3b). When the PCR products were subjected to restriction digestion with *Hinf*I it produced four bands in almost all the bamboo species and cultivars under study with the exception of *Himalayacalamus hookerianus*, *Pseudosasa japonica* and *Shibataea kumasaca* where only three bands were visible (fig 3c), while the polymorphism percentage was found to be 25%.

3.3 PCR-RFLP data analysis

A total of 19 scorable bands were produced by the various restriction digestion enzymes ranging in between 200-1986 bp. Of the 19 cuts

9 were polymorphic. The number of polymorphic bands ranged from one in *Taq*I and *Hinf*I to seven in *Alu*I. These clear and distinct bands were scored and used for further analysis. The percentage of polymorphism ranged from 20% to 70%. Restriction enzyme *Taq*I revealed 20% polymorphism, while *Hinf*I showed 25% polymorphism and *Alu*I generated 70% polymorphism. The overall polymorphism was found to be 47.37% (table 2).

A dendrogram constructed on the basis of similarity estimates using the unweighted pair group method with arithmetic average (UPGMA) using NTSYSpc version (2.0) [30] is depicted in fig 4. The dendrogram was split into two clusters. *Phyllostachys nigra* was totally isolated and similarly *Melocanna baccifera* and *Shibataea kumasaca* were segregated from all other species of bamboo. The first cluster housed a total of 21 accessions of bamboo which was further divided into two groups. The first group consisted of 18 species, including 10 species from *Bambusa* of the total 12 under study, all the four species of *Dendrocalamus* along with *Gigantochloa* spp., *Pleioblastus argenteostriatus*, *Drepanostachyum intermedium* and *Sassaela ramosa*. The second group consisted of 2 species *Himalayacalamus hookerianus* and *Pseudosasa japonica* with a genetic similarity for *TrnL-trnF* region of 94.7%. The second cluster consisted of five species in which *Bambusa multiplex* 'Alphanso Karr', *B. multiplex* 'Rivierorum' and *Cephalostachym latifolium* clustered in the same sub-group. *Drepanostachyum khasianum* and Chinese bamboo formed the other part of the second cluster. Both the 2D and 3D plot (fig 5) of the correspondence analysis of the RFPL data corroborated the dendrogram. The above analysis showed no major and well supported incongruences in the *trnL-trnF* region of the chloroplast genome of different bamboo species. The probable reason for this is that the plastid DNA is generally non-recombining and maternally inherited in most angiosperms. However, from this study it is clear that the genus *Bambusa* is closely related to *Dendrocalamus* and *Gigantochloa* which is at par with the morphological taxonomy [37-39]. Similar observation was also made by Sungkaew and his co workers [26] while assessing the phylogenetic relationships among the bamboos taking into account five plastid DNA regions.

3.4 Sequencing of PCR amplification product, performing BLAST and submission

A total of 13 samples (each representing one genera of bamboo) were sequenced from Chromous Biotech Pvt. Ltd, Bangalore for both the forward and reverse primers individually. The sequencing resulted in an average of 790bp for each reaction. In the present study the nucleotide BLAST was performed for each of the sequence obtained to find out the homology with the sequences already present in the GenBank. The nucleotide BLAST showed 95 to 100% identity with the bamboo sequence already available in the GenBank. After authentication of the sequences were submitted to the GenBank. The list of different species of bamboo along with their GenBank accession number is given in table 3.

4. CONCLUSION

Our results demonstrated that bamboo possess considerable polymorphism in the non-coding region of the chloroplast DNA. These results suggest the use of PCR-RFLP in elucidating the molecular phylogeny among the plant species in general and bamboos in particular. In summary, it is apparent from the above results that DNA sequencing can be used as a definitive means for identifying plant species including bamboos. However, additional molecular studies together with the morphological features are required to resolve the long-standing problem related to identification and systematic of bamboo.

THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN-2319-2119

RESEARCH ARTICLE

Table 1 List of bamboo species encountered in North Bengal

Sample ID	Scientific Name	Voucher No.
B1	*Bambusa vulgaris* Schrad 'Vittata'	SUK/KRR/001
B2	*B. multiplex* (Lour.) Raeusch. ex Schult. & Schult. f ' Alphanso-Karr'	SUK/KRR/002
B3	*B. bambos* (L.) Voss	SUK/KRR/003
B4	*B. multiplex* (Lour.) Raeusch. ex Schult. & Schult. f 'Rivierorum'	SUK/KRR/004
B5	*B. balcooa* Roxb	SUK/KRR/005
B6	*B. vulgaris* Schrad.'Wamin'	SUK/KRR/006
B7	*B. longispiculata* Gamble	SUK/KRR/007
B8	*B. atra* Lindl.	SUK/KRR/008
B9	*B. oliveriana* Gamble	SUK/KRR/009
B10	*B. sinospinosa* McClure	SUK/KRR/010
B11	*B. tulda* Roxb.	SUK/KRR/011
B12	*B. pallida* Munro	SUK/KRR/012
B13	*Cephalostachyum latifolium* Munro	SUK/KRR/013
B14	*Dendrocalamus hamiltonii* Munro	SUK/KRR/014
B15	*D. sikkimensis* Oliv.	SUK/KRR/015
B16	*D. asper* (Schult.)Backer	SUK/KRR/016
B17	*D. strictus* (Roxb.)Nees	SUK/KRR/017
B18	*Drepanostachyum khasianum* (Munro) Keng. F	SUK/KRR/018
B19	*D. intermedium* (Munro) Keng. F	SUK/KRR/019
B20	*Gigantochloa* Kurz ex Munro	SUK/KRR/020
B21	*Himalayacalamus hookerianus* (Munro)Stapleton	SUK/KRR/021
B22	*Melocanna baccifera* (Roxb.) Kurz	SUK/KRR/022
B23	*Phyllostachys nigra* (Lodd.) Munro	SUK/KRR/023
B24	*P. argenteostriatus* (Regel) Nakai	SUK/KRR/024
B25	*Pseudosasa japonica* (Steud.) Makino	SUK/KRR/025
B26	*Sasaella ramosa* (Makino) Makino	SUK/KRR/026
B27	*Shibataea kumasaca* Nakai	SUK/KRR/027
B28	*Yushania maling* (Gamble) R.B. Majumder	SUK/KRR/028
B29	Chinese bamboo (unidentified)	SUK/KRR/029

Table 2 Total fragments, number of monomorphic and polymorphic bands generated by using different restriction enzymes

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

THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY
ISSN-2319-2119
RESEARCH ARTICLE

Table 3 List of bamboo species with the GenBank accessions for *TrnL-trnF* region

Plant species	GenBank accession number
Pleioblastus argenteostriatus (TabC)	KC292011
Sasaella ramosa(TabF)	KC292012
Dendrocalamus hamiltonii(TabF)	KC292013
Pleioblastus argenteostriatus(TabF)	KC292014
Dendrocalamus hamiltonii (TabC)	KC292015
Pseudosasa japonica(TabC)	KC292016
Sasaella ramosa(TabC)	KC292017
Pseudosasa japonica(TabF)	KC292018
Yushania maling (TabF)	KC404798
Gigantochloa spp. (TabC)	KC404799
Shibataea kumasaca (TabF)	KC404801
Phyllostachys nigra (TabF)	KC404803
Shibataea kumasaca (TabC)	KC404807
Melocanna baccifera (TabC)	KC404808
Cephalostachyum latifolium (TabF)	KC404809
Bambusa vulgaris 'Vittata' (TabC)	KC404810
Drepanostachyum khasianum (TabC)	KC404811
Cephalostachyum latifolium (TabC)	KC404812
Drepanostachyum khasianum (TabF)	KC404814
Phyllostachys nigra (TabC)	KC404815
Melocanna baccifera (TabF)	KC404817
Himalayacalamus hookerianus (TabF)	KC404818
Himalayacalamus hookerianus (TabC)	KC404819
Yushania maling (TabC)	KC404821
Bambusa vulgaris 'Vittata' (TabF)	KC404822
Gigantochloa spp. (TabF)	KC404825

THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN-2319-2119

RESEARCH ARTICLE

Arnab Sen *et al*, The *Experiment, 2014., Vol. 19(1), 1304-1315*

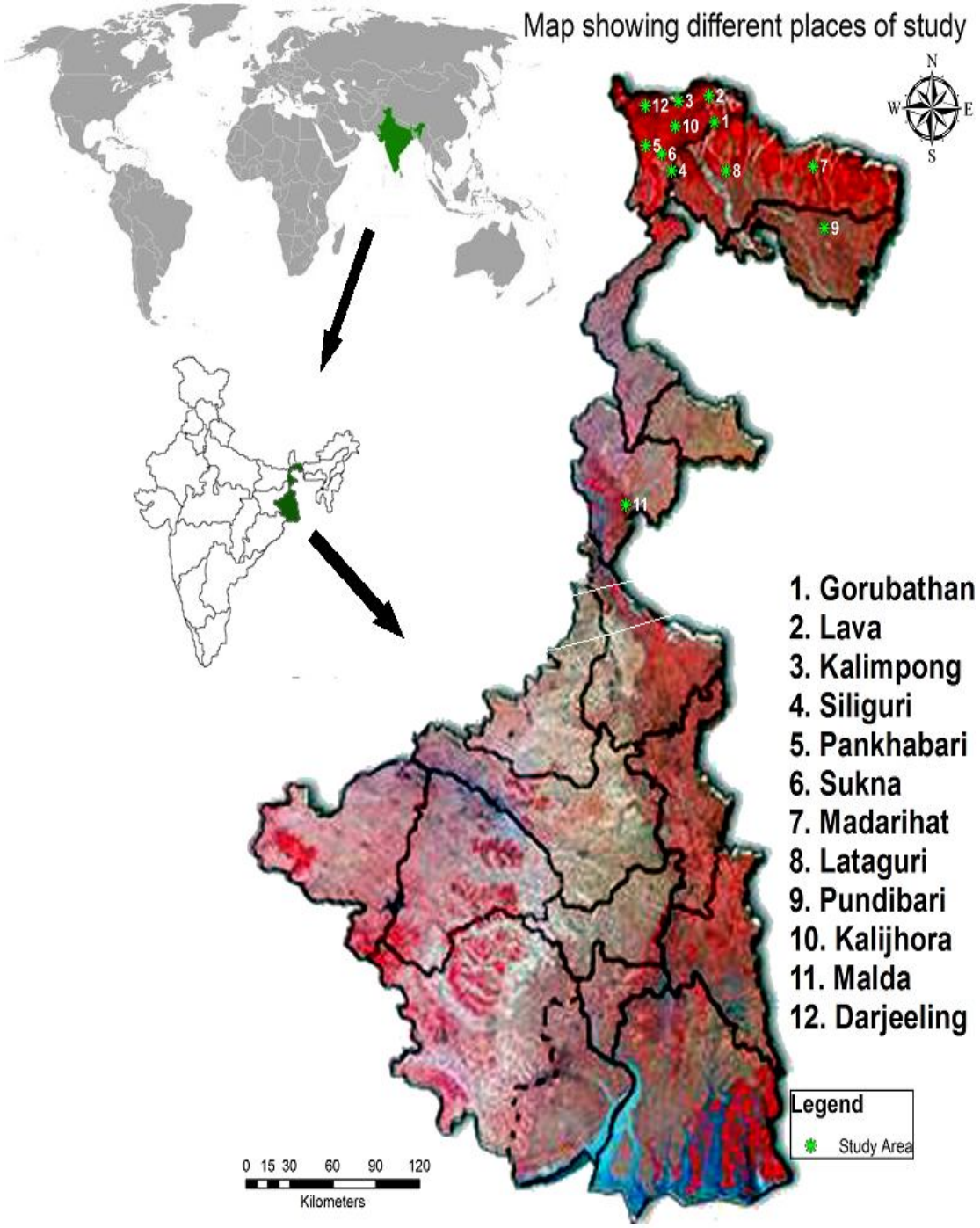


Figure 1

Map of the study area. The world map with map of India in green, which is further enlarged to show the map of West Bengal in green. The spots represent the places of bamboo collections in the North Bengal (consisting of six districts) part of the Indian state of West Bengal

ISSN-2319-2119

RESEARCH ARTICLE

Arnab Sen *et al*, The *Experiment*, *2014., Vol. 19(1), 1304-1315*

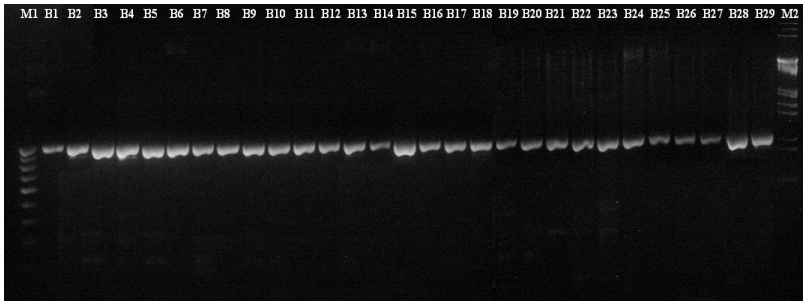


Figure 2

Amplification of bamboo accessions with primer Tab c-f (*TrnL-trnF*). Lane M1: 100bp DNA ladder; Lane B1-B29: Different species of bamboo as listed in table 1 and M2: λ DNA/EcoRI/HindIII double digest DNA ladder

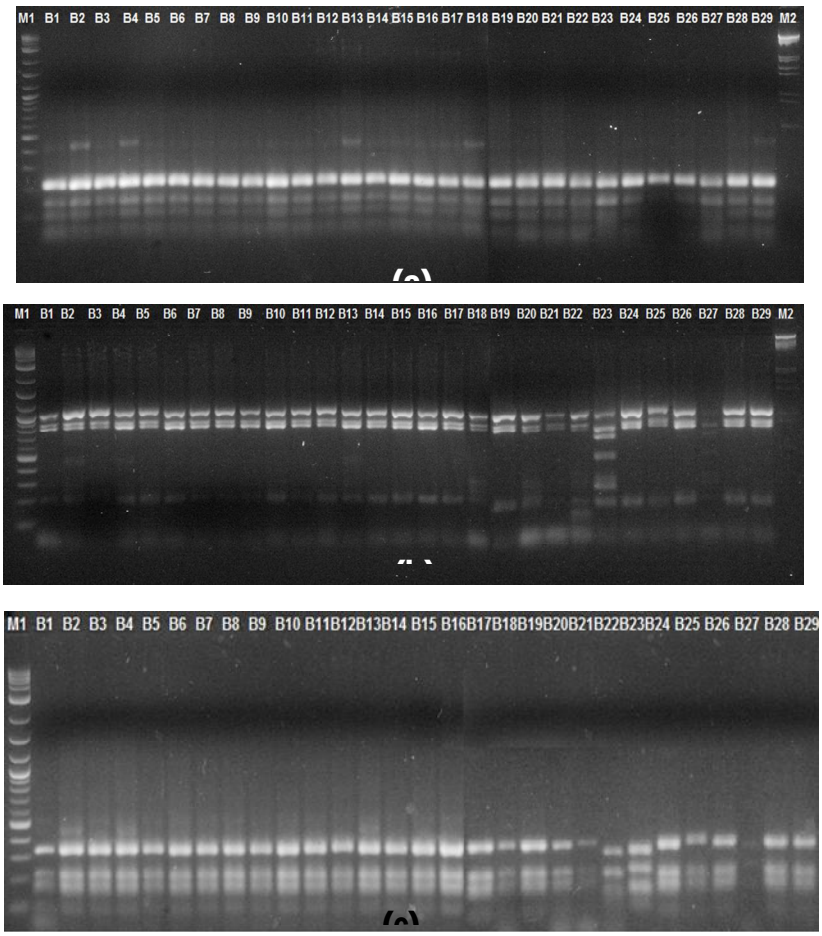


Figure 3

Restriction digestion products of *TrnL-trnF* region of chloroplast genome. (a) *Taq*I; (b) *Alu*I and (c) *Hinf*I. Lane M1: 0.1-10 kb DNA ladder; Lane B1-B29: Different species of bamboo as listed in table 1 and M2: λ DNA/*Eco*RI/*Hin*dIII double digest DNA ladder

THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN-2319-2119

RESEARCH ARTICLE

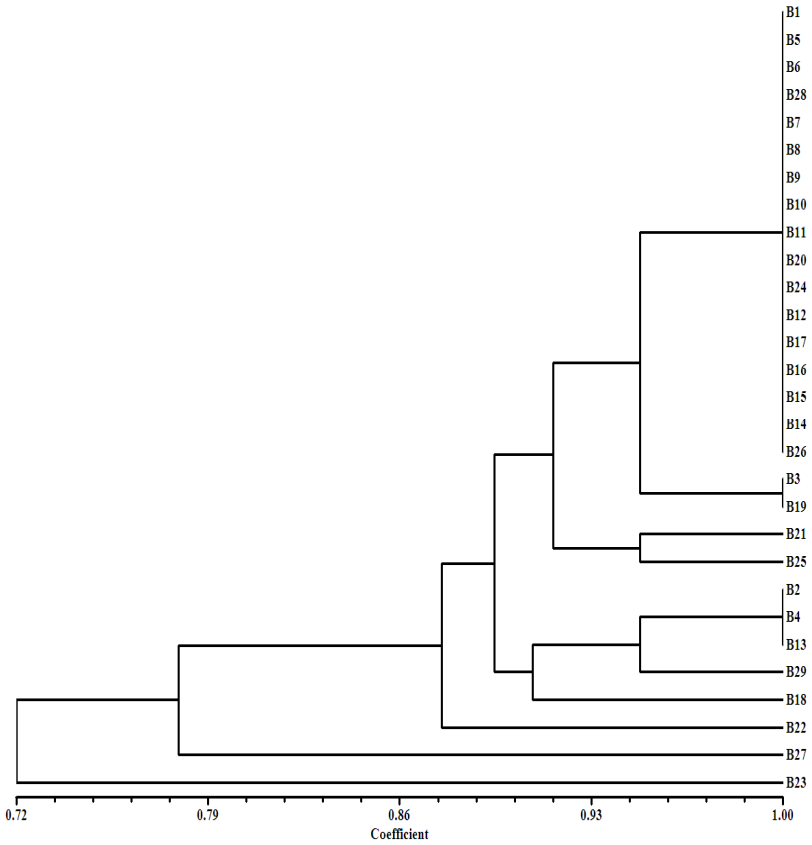
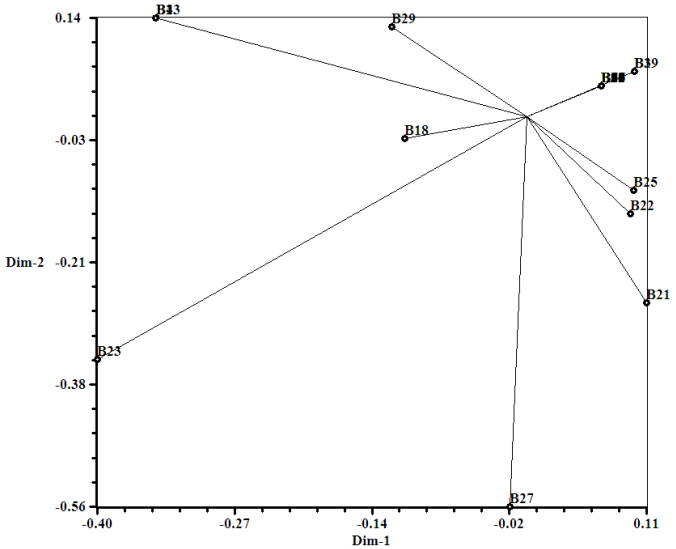


Figure 4

Dendrogram based on the restriction digestion products data of the *TrnL-trnF* region of different bamboo genome. For details on sample ID (B1– B29) please refer table 1.



THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN-2319-2119

RESEARCH ARTICLE

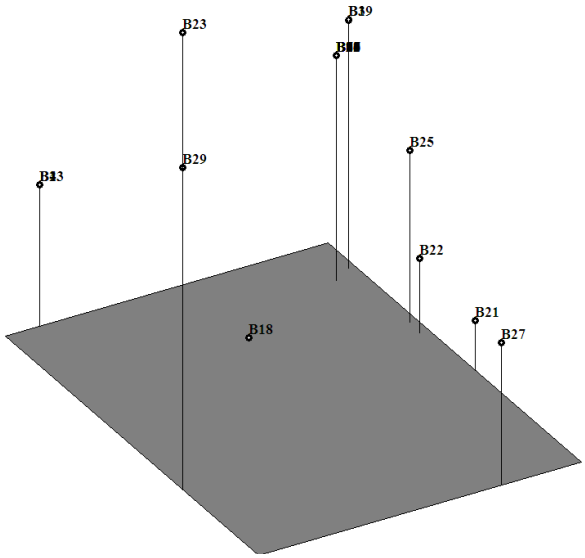


Figure 5

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

5. ACKNOWLEDGEMENTS

The authors are thankful to Mr. Prasanta Kr. Ghosh, Range Officer, Kurseong Research Range, Sukna, Darjeeling for providing necessary help, support and information. The authors are also obliged to the bamboo taxonomist, Mr. P.P. Paudyal, Consultant, Bamboo Mission, Sikkim for his help in identifying the species of bamboo.

6. REFERENCES

1. Goyal AK, Kar P, Sen A. Advancement of bamboo taxonomy in the era of molecular biology: a review. In: Sen A, editor. Biology of useful plants and microbes. Narosa publication house, New Delhi; 2013. p. 197-208.
2. Hadique P, Rethy P, Dutta BK, Das A, Doley B. Role of bamboo resources in socio economic development of the Tribal people of Arunachal Pradesh with special reference to Nyishi tribe of Papum Pare District. J Biosci Res 2010; 1: 216-226.
3. Bystriakova N, Kapos V. Bamboo diversity: the need for a Red List review. Biodiversity 2006; 6(4): 12-16.
4. Sharma Y M L. Inventory and resources of bamboos. In: Rao AN, G Dhanarajan G, Sastry CB, editors. Recent Research on Bamboos. CAF China and IDRC Canada; 1987. p. 14-17.
5. Goyal AK, Middha SK, Usha T, Chatterjee S, Bothra AK, Nagaveni MB et al. Bamboo-infoline: A database for North Bengal bamboo's. Bioinformation 2010; 5(4): 184-185.
6. Goyal AK, Ghosh PK, Dubey AK, Sen A. Inventorying bamboo biodiversity of North Bengal: a case study. Int J Fundam Appl Sci 2012; 1(1): 5-8.
7. Janzen DH. Why bamboos wait so long to flower. Annu Rev Ecol Evol Syst 1976; 7: 347-391.
8. Wu MCY. Classification of *Bambuseae* based on leaf anatomy. Bot Bull Acad Sin 1962; 3: 83-107.
9. Cameron KM. Bar coding for botany: A system modeled on commercial bar codes may soon enable anyone to identify any plant from a small fragment of its DNA. Natural History 2007; 116(2): 52.
10. Friar E, Kochert G. Bamboo germplasm screening with nuclear restriction fragment length polymorphisms. Theor Appl Genet 1991; 82: 697-703.
11. Friar E, Kochert G. study of genetic variation and evolution of *Phyllostachys* (Bambusoideae: Poaceae) using nuclear restriction

fragment length polymorphisms. Theor Appl Genet 1994; 89: 265-270.
12. Nayak S, Rout GR, Das P. Evaluation of the genetic variability in bamboo using RAPD markers. Plant Soil Environ 2003; 49: 24-28.
13. Sun Y, Xia N, Stapleton CMA. Relationships between *Bambusa* species (Poaceae Bambusoideae) revealed by random amplified polymorphic DNA. Biochem Syst Ecol 2006; 34: 417-423.
14. Das M, Bhattacharya S, Basak J, Pal A. Phylogenetic relationships among the bamboo species as revealed by morphological characters and polymorphism analyses. Biol Plantarum 2007; 51: 667-672.
15. Mukherjee AK, Ratha S, Dhar S, Debata AK, Acharya PK, Mandal S et al. Genetic relationships among 22 taxa of bamboo revealed by ISSR and EST-based random primers. Biochem Genet 2010; 48: 1015-1025.
16. Zhang HK, Yang YM, Liu XZ. Bamboo species relations revealed by random amplified polymorphism chloroplast DNA. Afr J Agricult Res 2011; 6(5):1241-1245.
17. Hodkinson TR, Renvoize SA, Chonghaile GN, Stapleton CMA, Chase MWA. Comparison of ITS Nuclear rDNA Sequence Data and AFLP Markers for Phylogenetic Studies in *Plyllostachys* (Barn busoideae Poaceae). J Plant Res 2000; 113: 259-269.
18. Guo ZH, Chen YY, Li DZ, Yang JB. Genetic variation and evolution of the alpine bamboos (Poaceae: Bambusoideae) using DNA sequence data. J Plant Res 2004; 114: 315–322.
19. Guo ZH, Chen YY, Li DZ. Phylogenetic studies on Thamnocalamus group and its allies (Bambusoideae: Poaceae) based on ITS sequence data. Mol Phylogenet Evol 2002; 22: 20–30.
20. Guo ZH, Li DZ. Phylogenetics of the Thamnocalamus group and its allies (Gramineae: Bambusoideae) inference from the sequences of GBSSI gene and ITS spacer. Mol Phylogenet Evol 2004; 30: 1–12.
21. Albach DC, Chase MW. Incongruence in veroniceae (Plantaginaceae): evidence from two plastid and a nuclear ribosomal DNA region. Mol Phylogenet Evol 2004; 32: 183–197.
22. Wang YJ, Liu JQ, Miehe G. Phylogenetic origins of the Himalayan endemic *Dolomiaea, Diplazoptilon* and *Xanthopappus* (Asteraceae: Cardueae) based on three DNA regions. Ann Bot 2007; 99 311–322.
23. Yang HQ, Peng S, Li DZ. Generic delimitations of *Schizostachyum* and its allies (Gramineae: Bambusoideae) inferred from GBSSI and trnL-F sequence phylogenies. Taxon 2007; 56: 45–54.
24. Yang HQ, Yang JB, Peng ZH, Gao J, Yang YM, Peng S et al. A molecular phylogenetic and fruit evolutionary analysis of the major groups of the paleotropical woody bamboos (Gramineae: Bambusoideae) based on nuclear ITS, GBSSI gene and plastid trnL-F DNA sequences. Mol Phylogenet Evol 2008; 48: 809-824.
25. Qiang Z, Yu-long D, Chen X, Hui-yu Z, Min-ren H, Ming-xiu W. A preliminary analysis of phylogenetic relationships of Arundinaria and related genera based on nucleotide sequences of nrDNA (ITS region) and cpDNA (trn L-F intergenic spacer). J Forest Res 2005; 16: 5–8.
26. Sungkaew S, Stapleton CMA, Salamin N, Hodkinson TR. Non-monophyly of the woody bamboos (Bambuseae; Poaceae): a multi-gene region phylogenetic analysis of Bambusoideae ss. J Plant Res 2009; 122(1): 95-108.
27. Doyle JJ, Doyle JL. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 1987; 19: 11-15.
28. Taberlet P, Gielly L, Pautou G, Bouvet J. Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol Biol 1991; 17(5): 1105-1109.
29. Nei M, Li WH. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci 1979; 76: 5269-5273 .
30. Rohlf FJ. NTSYS-pc Numerical Taxonomy and Multivariate Analysis version 2.0 (Exeter Software, Setauket, New York) 1998.
31. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990; 125(3): 403-410.
32. B. Demesure B, Comps B, Petit RJ. Chloroplast DNA phylogeography of the common beech (Fagus sylvatica L.) in Europe. Evolution 1996; 50: 2515–2520.
33. King RA, Ferris C. Chloroplast DNA phylogeography of Alnus glutinosa (L.) Gaertn. Mol Ecol 1998; 7: 1151–1161.
34. A. C. Newton AC, Allnutt TR, Gillies ACM, Lowe AJ, Ennos RA. Molecular phylogeography, intraspecific variation and the conservation of tree species. Trends Ecol Evol 1999; 14: 140–145.
35. Fineschi S, Taurchini D, Villani F, Vendramin GG. Chloroplast DNA polymorphism reveals little geographical structure in Castanea sativa Mill. (Fagaceae) throughout southern European countries. Mol Ecol 2000; 9: 1495–1503.
36. Mohanty A, Martin JP, Aguinagalde I. A population genetic analysis of chloroplast DNA in wild populations of Prunus avium L. in Europe. Heredity 2001; 87: 421–427.

37. Li DZ. Taxonomy and biogeography of the Bambuseae (Gramineae: Bambusoideae). In Rao AN, Rao VN, editors. Bamboo—Conservation, diversity, ecogeography, germplasm, resource utilization and taxonomy. Proceedings of training course cum workshop, Kunming and Xishuanbanna, Yunnan, China, IPGRI-APO, Serdang, Malaysia; 1998. p. 14-23.
38. Li DZ, Xue JR. The biodiversity and conservation of bamboos in Yunnan, China. In Chapman GP, editor. The bamboos. 19th ed. Linnean Society Symposium Series; 1997. p. 83-94.
39. D. Z. Li DZ, Stapleton CMA, editors. Melocalamus Bentham. Flora of China, 22nd ed; 2006. p. 48–49.

Arvind Kumar Goyal[1,2] and Arnab Sen[1]*

[1]Molecular Cytogenetics Laboratory, Department of Botany, University of North Bengal, Siliguri- 734013, WB, India,

[2]Bamboo Technology, Department of Biotechnology, Bodoland University, Kokrajhar- 783370, BTAD, Assam, India