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Research Article

ASSESSMENT OF THE GENETIC DIVERSITY OF *Dendrocalamus hamiltonii* ALONG ALTITUDINAL GRADIENT

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Abstract- *Dendrocalamus hamiltonii* is a commercially important bamboo species of India, experiencing population depletion due to heavy extraction from natural forests. The present study has been taken up to generate the basic knowledge of the genetic diversity of the natural stands of *D. hamiltonii* along altitudinal gradient. Seventeen polymorphic markers developed in other bamboo species were validated in *D. hamiltonii* and used for genotyping the populations of different altitudinal class (low = 100m, medium = 600m and high = 1200m). A total of 109 alleles generated in 88 individuals of three populations revealed higher genetic diversity at species (h = 0.209, I = 0.339) and population level (h = 0.169, I = 0.269) which is comparable to other bamboo species. Despite of large proportion of the genetic variation confined within the populations (75%), significant level of genetic differentiation (PhiPT= 0.250 and G_{ST} =0.199) was observed among the populations. Relatively higher genetic diversity was exhibited by the populations located at higher altitude than the populations of middle and low altitude. Large number of private bands recorded in the higher altitude populations showed less gene flow among the populations across wide altitudinal range. Mantel test showed significant correlation between genetic distance and altitudinal distance (r^2 = 0.315, p ≤ 0.010) and indicates that altitude also plays key role in influencing genetic differentiation of the populations of *D. hamiltonii*. The clustering pattern obtained in UPGMA dendrogram, PCoA plot and Structure analysis revealed that structuring among the populations is fairly good.

Key words- *Dendrocalamus hamiltonii*, Bamboo, Genetic diversity, Microsatellite markers, Altitudinal gradient

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Introduction

Bamboo plays a noteworthy role in improving the livelihoods of rural communities due to its multiple utility and accessibility to common man. They are multipurpose crops, with more than 1500 documented uses. Worldwide, more than 2.5 billion people depend on use or trade of bamboo [1]. India is endowed with vast biodiversity of bamboo represented by 125 species belonging to 23 genera and covers about 15.69 million ha area [2]. North East India supports about 50% of the total genetic resources, followed by peninsular India (Eastern and the Western Ghats) which accounts for about 23% of the bamboo genetic resources. North-western India, Indo-Gangetic plains and the Andaman & Nicobar Islands account for the remaining diversity. More than 50% of bamboo species occurring in India are endemic, and roughly 19 species are rare and threatened [3]. With the increasing population pressure and ban on green felling, natural stands of bamboo are being indiscriminately cut to fulfil the ever-increasing gap between the demand and the supply of wood. The common practice of 'jhum' (a form of shifting cultivation) in the north eastern states has resulted in genetic erosion of several bamboo species; overexploitation of some species for fuel wood and other purposes has endangered others. Since natural variation is the basic genetic resource required for selection and improvement, conservation of available genetic resource needs to be accorded the highest priority [4]. Forestry species are generally long lived and extremely diverse and evolved under several periods of climatic change. Genetic diversity provides the fundamental basis for evolution and enables forestry species to adapt to changing and adverse conditions for thousands of years. Understanding the genetic diversity is important in all types of forests for the conservation, management, breeding and genetic improvement. Intra-specific genetic diversity is influenced by environmental heterogeneity, breeding systems, the degree of biogeographic isolation and the species evolutionary history.

Its characterization and documentation is considered as a central component of conservation and management of individual species [5]. The number of evolutionary processes can impact the genetic diversity of natural populations like spontaneous mutations, gene flow, migration, inbreeding, natural selection and random genetic drift [6]. The occurrence of a population bottleneck causes a significant reduction in the effective population size and represents a major reason for the loss in allelic diversity [7]. Distance and discrete geographic barriers play a role in isolating populations due to limited seed and pollen dispersal [8-10]. The change pattern of genetic diversity along an altitudinal gradient is one of the important aspects in forestry research because altitudinal variations regulate the ecological conditions in a particular plant habitat [11]. Despite of the growing importance of bamboo and vast distribution range, only few studies have been undertaken to assess genetic diversity or understand population structure of individual bamboos species. Bamboo is a group of plants having wide range of diversity and distribution in India. Bamboo can grow in diverse environmental conditions tropical to temperate regions and from plains of central and south India to hill top in North east India. It can tolerate extreme temperature of about -200C and excessive precipitation ranged from 32 to 50 inch annual rainfall [12]. Therefore this is a suitable candidate plant group to study the impact of altitudinal or climatic gradient on genetic diversity. A study has been conducted to assess the genetic diversity of four subpopulations of *Chimonobambusa rivularis* along altitudinal gradient in Sichuan province of China [13]. *Dendrocalamus hamiltonii* is a large, evergreen or deciduous, ceasptiose, densely clumped bamboo, sometimes growing tall and erect, but more often sending out its stems at an angle or curved downwards. It is one of the priority bamboo species at national and international level [14, 15]. It is one of the most exploited species for its various uses like paper and pulp, house building, construction, making of basket, mats ropes and as container for water, milk and other eatable items.

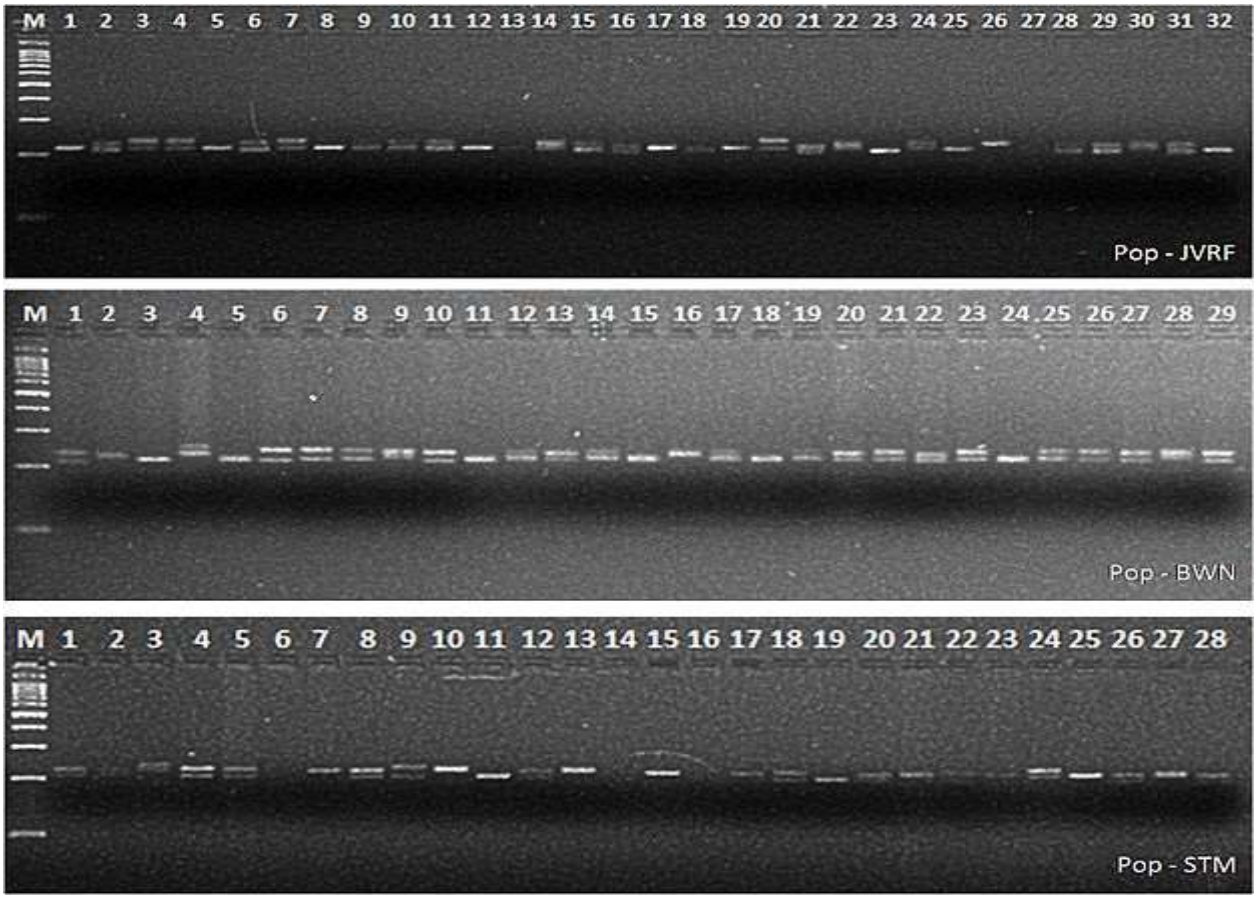

Fig-1 Banding pattern generated by SSR primer DLUGMS-52 in the populations of *D. hamiltonii*. Lane 'M' represents 100 bp DNA ladder; Lane '1' to 'n' represent n sample genotypes of corresponding population depicted in gel pictures.

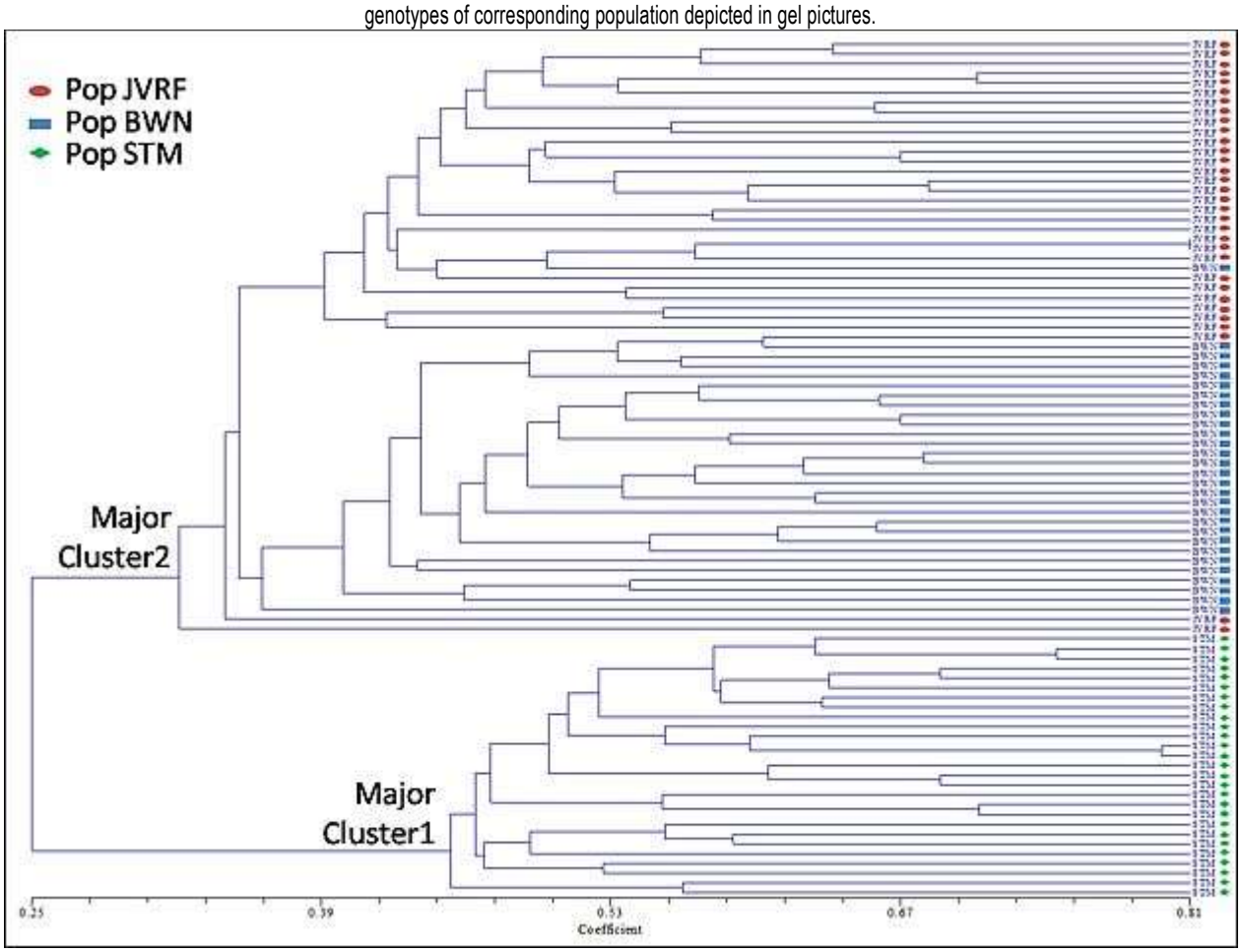

Fig-2 UPGMA dendrogram based on Jaccard similarity coefficient between genotypes.

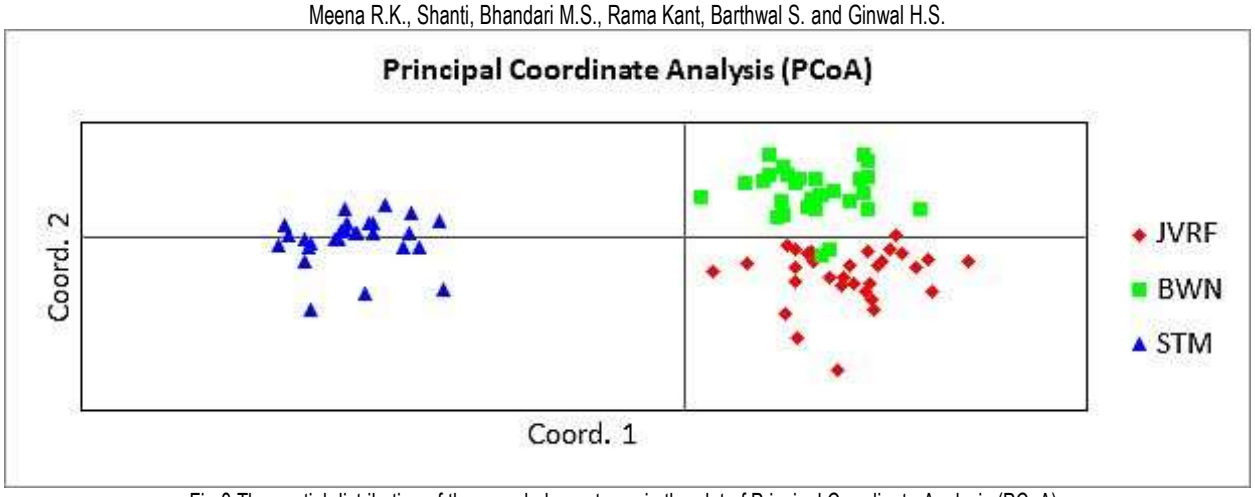

Fig-3 The spatial distribution of the sampled genotypes in the plot of Principal Coordinate Analysis (PCoA).

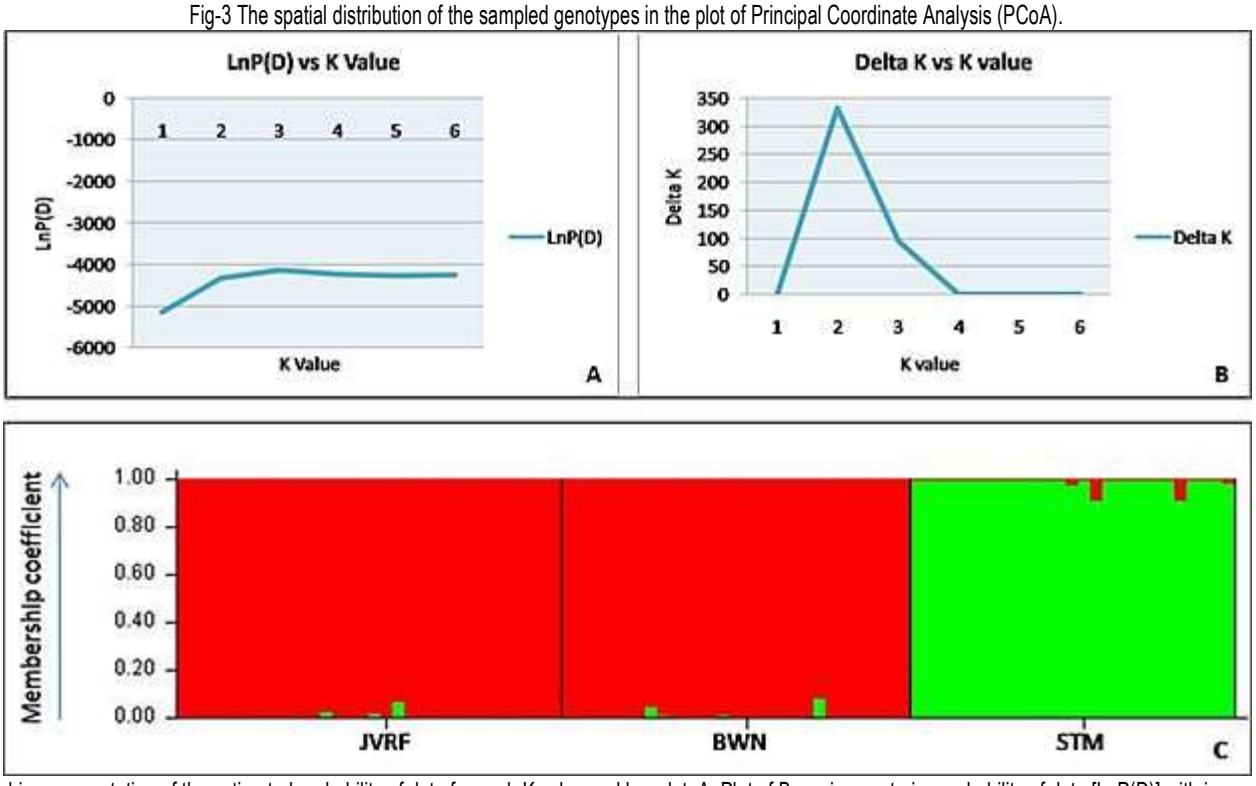

Fig-4 Graphic representation of the estimated probability of data for each K value and bar plot: A. Plot of Bayesian posterior probability of data [LnP(D)] with increasing K; B. Magnitude of ΔK as a function of K; C. Bar plot for estimated population Q-matrix at K=2 for the genotypes of *D. hamiltonii*.

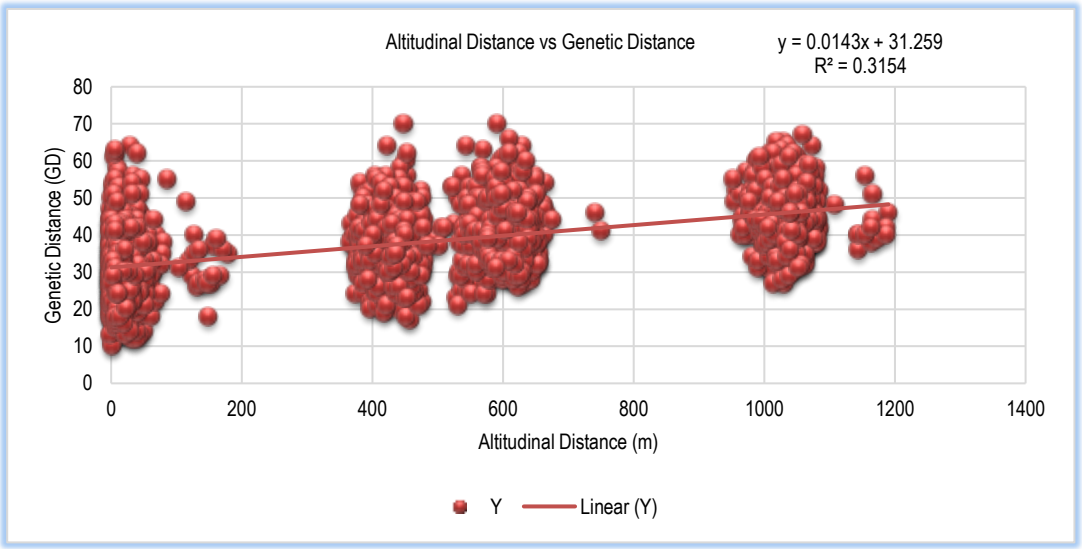

Fig-5 Relationship between genetic distance and altitude

Table-1 Location detail of sampled populations of *Dendrocalamus hamiltonii*

Population Id	Altitude (m)	Location	No. of samples	Latitude	Longitude
JVRF (Low)	100-150	Jatinga Valley Reserve Forest, Cachhar, Assam	32	N 24°59'27.31"	E 92°44'58.63"
BWN (Medium)	500-600	Bhandri, Wokha, Nagaland	29	N 26°12'23.0"	E 94°13'19.8"
STM (High)	1100-1200	Sialsuk, Thenzawl, Mizoram	27	N 23°25'01.49"	E 92°46'05.45"

Table-2 Details of 17 polymorphic SSR primers used for genotyping the samples of *D. hamiltonii*

SN	Primer Name*	Primer sequence (5' - 3')	Motif
1	DLUGMS03	F: TGCCGGTGCTTTCTTACTCT , R:GGAGGAAGGGATGGGAGTAG	(CT)11
2	DLUGMS13	F: CCTTCCTCGTTTCCCTTTTC , R:TTCGCTTCGAGGGTTAAATG	(CGG)8
3	DLUGMS15	F: GGGGACCATTTGACAACTCA , R:CTCTTTGCGAGGAAGTCACA	(TC)10(CA)5
4	DLUGMS16	F: GGGAGATACAGTTCCGTTGG , R:TCCTTGATGGAGCGGACTAC	(GA)26
5	DLUGMS17	F: CGGTTGGCCTTCTATGAGAG , R:CCATCGATGATAGCACAGGA	(TC)24
6	DLUGMS23	F: AAGGAAAAAGGGCTGGGTTA , R: TCGTCGTCATCACTTTGCTC	(ATG)14
7	DLUGMS25	F: GAGGGACTTGATGGATTGGA , R: ATGTTATTGCGCTTGTGCTG	(GAA)8
8	DLUGMS45	F: CACCGTGTGGTTACCTTTCC , R: TGAGGAGGAGCTTGAAGAGG	(TC)16
9	DLUGMS47	F: GGGGACTCTCCTCTTCGTCT , R: GATCTGAGGCTTCTCCATCG	(TC)11
10	DLUGMS50	F: AGACTCTCCACTCGTGACTCG , RCCGCGAACTCCACAGACTAT	(CTCCG)6
11	DLUGMS51	F: CATTGGCCCATGTAACTTTTC, R: CGAGCAAGTGTTGTCCTGAA	(CAG)8
12	DLUGMS52	F: CCATCTCTCGTCTCCTCTCG , R: TTGCTCAGAAATGGCAAGAA	(CAT)9
13	DLUGMS54	F: CACAGGGAGCAACATCAAGA , R: CCGATCATAAAACCAACTGAAA	(TTTC)7
14	DLUGMS56	F: CAATCTCGGAGCCGAACTAC , R: ATACCACCAGGCACAAGAGC	(ACCTG)4
15	DLUGMS57	F: AGCCAGTCCACCATTACCAG , R: GGGAGAGTCGACTGAATTGG	(CTCCG)5
16	DLUGMS61	F: TTCCTCATCTTGCAGGCTTT , R: GCAAAATTTCCGTCGATTGT	(GGAGA)7
17	DLUGMS62	F: ATAGCCATGTACGCATGCAC , R: GCTTACAGGTTTCACACAACCA	(CGTG)6

*Primers were designed as per Bhandawat *et al*., 2014 [17]

Table-3 Gene diversity over loci for each sampled population of *D. hamiltonii*

Population ID	Percentage of Polymorphic Bands (*PPB*)	No. of Private Alleles (*PA*)	Nei's gene diversity (*h*)	Shannon's Information Index (*I*)
			Mean (SD)	Mean (SD)
JVRF	67.89	3	0.167 (0.172)	0.265 (0.247)
BWN	67.89	6	0.159 (0.163)	0.256 (0.236)
STM	69.72	19	0.182 (0.180)	0.285 (0.256)
Mean of Populations	68.50	-	0.169 (0.012)	0.269 (0.014)
Overall for Species	98.17	-	0.209 (0.156)	0.339 (0.209)

Table-4 Analysis of molecular variance (AMOVA) for sampled populations

SV	DF	SS	MSS	Est Var	% Var	GD	*P*(rand ≥ data)
Among populations	2	332.84	166.42	5.16	25%	*PhiPT* = 0.250	0.001
Within populations	85	1318.55	15.51	15.51	75%		
Total	87	1651.39		20.67	100%		

SV: Source of variation; DF: Degree of freedom; SS: Sum of Square; MSS: Mean Sum of Square; Est Var: Estimated variance; % Var: Percentage of variation; GD: Genetic differentiation

This is the primary species harvested for young shoots in the north-eastern states. It is naturally distributed from Shimla eastward extending to the upper Myanmar [15]. Huge distribution is found in almost all North-eastern states of India and also occasionally cultivated in the plains. Generally, the best growing stock is observed in foot hills of Assam, Nagaland, and Arunachal Pradesh but it can be found in small patches upto 2500m A.M.S.L. In the current study, populations of *D. hamiltonii* belonging to different altitudinal class were characterized for the genetic diversity using microsatellite markers.

Material and Methods

Plant Materials

Surveying and sampling of *D. hamiltonii* was carried out throughout the North East India during 2015-16. Natural populations were found to be distributed at various locations of Assam, Mizoram, Meghalaya, Nagaland and Arunachal Pradesh at the altitude ranges from 70 to 1200m A.M.S.L. Though the species distributed mostly at the foot hills but some populations were found to be growing at extremely low and high altitude and therefore the sampling area was divided into three classes *i.e.* Low, medium and high. Leaf samples of 88 genotypes belonging to three populations for three altitudinal classes were collected and desiccated with silica gel immediately [Table-1]. To avoid the possibility of sampling a clone twice or more, two sampled clumps were at least 100 m apart at each site. The desiccated plant material was brought to the laboratory and stored at -80ºC till further processing.

Genomic DNA Extraction

Genomic DNA was extracted using standard protocols of Doyle and Doyle (1987) with minor modifications [16]. Frozen tissues were grounded in liquid nitrogen and incubated at 60ºC for 45 minutes in pre-heated CTAB extraction buffer followed by mixing with chloroform-isoamyl alcohol (24:1). The supernatant was pipetted out after centrifugation and precipitated with equal volume of chilled isopropanol overnight. The precipitated DNA was first washed with a solution containing 96% ethanol and 0.3 M Sodium acetate followed by washing with 70% ethanol. After vacuum drying, DNA pellet was re-suspended in 100 µl of 10 mM Tris-EDTA buffer (pH 8.0). Quantitative analysis of genomic DNA was carried out using Biophotometer and quality was checked by agarose gel electrophoresis. Genomic DNA was diluted to obtain final working concentration of 30 ngµl^{-1} for PCR amplification.

Genotyping

Microsatellite primers developed in *Dendrocalamus latiflorus* were cross-amplified and validated by PCR amplification in *D. hamiltonii* [Table-2] [17]. The PCR amplification was carried out with the 15µL PCR reaction mixture, containing 60ng of template DNA, 1.5µl of 10x PCR buffer, 1.75mM $MgCl_2$, 0.2mM dNTPs, 100nM of each forward and reverse primer, 0.6 units of *Taq* DNA polymerase and nuclease free sterile water. The cycling conditions included an initial denaturation at 95°C for 5 min; then 35 cycles of 95°C for 1 min, 51.2-61.7°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. The PCR products were resolved in 4% high resolution agarose gel with 1x TBE buffer.

A 100-bp DNA ladder was used as a size marker. After staining with ethidium bromide (0.5 µgml-1), the DNA fragments were visualized and captured using gel documentation system. Each individual band was scored manually but multiple bands were obtained at most of the loci in individual genotype and therefore it was difficult to assign the correct allele dosage. Therefore, each allele was treated as individual loci and the multi-locus data was transformed to a binary matrix of presence (1) and absence (0) as per Teixeira *et al*. (2014) [18].

Marker Data Analysis

To obtain the various measures of genetic diversity like observed number of alleles (Na), effective number of alleles (Ne), percentage of polymorphic bands (PPB), allele frequency, number of private bands (PB), Nei's gene diversity (h) and Shannon's Information index (I), the binary data was subjected to analysis using software POPGENE ver 1.32 [19] and GenAlex ver 6.5 [20]. Inter and intra-population differentiation was determined by AMOVA analysis using the software GenAlEx. For interpretation, another measure of genetic differentiation (GST) was also calculated through POPGENE. The amount of gene flow (Nm) among populations is a measure of the effective number of migrants per population per generation. It was estimated indirectly from the GST values at each locus and from the average values over all loci by applying McDermott and McDonald's (1993) formula [Nm = 0.5(1-GST)/GST] for the studied populations using software POPGENE [21]. Genetic relationship among the genotypes was studied by constructing a dendrogram based on pair-wise genetic distances among the individual genotypes calculated through Jaccard similarity coefficient using Unweighted Pair-Group Method with Arithmetic averages (UPGMA) in NTSYSpc ver 2.02 [22]. To check the consistency of the clusters obtained in UPGMA dendrogram, Principal coordinate analysis (PCoA) was performed using GenALEx. The Bayesian model-based clustering method was used to elucidate the genetic structure of the populations using Structure software ver 2.2 [23]. Ancestry model with admixture under the assumption of correlated allele frequencies was used to determine the posterior probability. Simulations were run 10 times for each value of K (1 to 4) with 50,000 Markov Chain Monte Carlo (MCMC) sampling runs after a burn-in period of 50,000 iterations.To study the relationship of genetic distance with horizontal and vertical physical distance the Mantel test was performed with GenAlEx.

Results

Genetic diversity and differentiation

PCR amplification with 17 SSR primers generated 109 reproducible bands in 88 individuals representing 3 different populations of different altitudinal class. [Fig-1] presents the representative picture of the banding pattern generated by SSR primer DLUGMS-52 in the populations of *D. hamiltonii*. Of the 109 bands, 107 were polymorphic (98.17%) at the species level while the average percentage of polymorphic bands (PPB) at the population level was 68.50%. Mean genetic diversity was estimated by Shannon's Information indices (I) and was recorded as 0.269 at population and 0.339 at species level. Among 3 populations, highest degree of variability was exhibited by the high altitude population of Mizoram, STM (h = 0.182 and I = 0.285) and lowest for population of medium attitude from Nagaland, BWN (h = 0.159 and I = 0.256) [Table-3]. Total genetic diversity (HT) of the species was recorded as 0.211, of which 80% (0.169) remained to be restricted within the populations (HS). Analysis of molecular variance (AMOVA) also revealed that most of the genetic variation (75%) exists within the populations and only 25% among the populations [Table-4]. Variance estimates were based on 999 permutations and the difference between the individuals within populations was statistically significant with P value <0.001. High values obtained for the estimated measures of genetic differentiation (GST = 0.199 and PhiPT = 0.250) indicates the moderate level of genetic differentiation among the populations, that was further supported by the presence of large number of private bands (bands unique to a single population). A total of 28 private bands were detected, of which 19 (67.85%) were present in the population STM which suggest its genetic distinctness from others.

Genetic structure and relationship among the populations

The UPGMA tree based on pair-wise genetic distance among genotypes revealed that all the genotypes belonging to their respective populations were clustered together with similarity coefficient ranging from 0.25 to 0.81 [Fig-2]. The genotypes were grouped in two major clusters and first major cluster constituted the genotypes of high altitude population (STM). Second major cluster was formed by the rest of the two populations of low and medium altitude *i.e*. JVRF and BWN. Clustering pattern resulted from UPGMA dendrogram was also supported by the results of Structure and Principal Coordinate Analysis (PCoA). The spatial distribution of the genotypes represented in PCoA plot showed that the first principal coordinate, accounting for 15.50% of total variation, separates population STM from other two. While other two populations *i.e*. JVRF and BWN could not be separated clearly by any of the coordinates [Fig-3]. Bayesian clustering method was used to elucidate the genetic structure of the sampled populations. A posterior probability [Estimated Ln Probability of Data, LnP(D)] was calculated for each K value. Optimum K value was determined using the graphical method suggested by Evanno *et al*. (2005) [24]. The highest value of ΔK with a clear peak was obtained for K = 2 and hence it was considered that two ancestral groups captured the entire variability among the sampled genotypes similar to as revealed by UPGMA dendrogram [Fig-4]. An analysis based on the Mantel test showed that there is a positive linear relationship between genetic distance and altitude (r^2 = 0.315, p≤ 0.07), suggesting that altitude is the principal factor influencing genetic differentiation in the populations of *D. hamiltonii* [Fig-5].

Discussion

In this study, efforts were made to assess the genetic diversity exist in the natural populations of *Dendrocalamus hamiltonii* along altitudinal gradient. Relatively higher genetic diversity has been recorded in *D. hamiltonii*. Moderate level of genetic differentiation has also been observed among the populations as indicated by estimated measures of genetic differentiation. The results were also supported by analysis of molecular variance (AMOVA) which revealed that 25% of the total genetic variation attributable to the differences among populations. Parameters of genetic diversity and genetic differentiation recorded in our species were comparable to that of another bamboo species like *Dendrocalamus membranaceus* (h = 0.219, I = 0.349 and GST = 0.252) and *Melocanna baccifera* (h = 0.1939, I = 0.321 and GST = 0.194) [25, 26]. Measure of genetic differentiation in our populations were also in accordance with the mean value of other out-crossing species (GST = 0.23) [27]. Relatively higher genetic diversity was exhibited by the populations located at higher altitude than the populations of middle and low altitude. This is not in accordance to the earlier study in which low genetic diversity has been recorded in high altitude populations of *Chimonobambusa rivularis* [13]. Large number of private bands recorded in the higher altitude populations showed less gene flow among the populations across wide altitudinal range. Mantel test showed that genetic distance was significantly correlated with the altitude, suggesting that altitude also plays important role in genetic differentiation of the bamboo. The Cluster and Structure analysis showed that the genotypes of low and medium altitude populations were clustered together while separate group was formed by higher altitude population, indicating that the structuring between subpopulations is fairly good. There is a significant variation in the distribution patterns of genetic diversity along altitudinal gradients as reported in different species [10]. Some study revealed peaks on higher slopes [28] while others have found greater diversity at lower or intermediate elevations [11, 29, 30]. In addition to these three patterns, there are also reports showing constant values of genetic variability all over the altitudinal range, suggesting a free gene flow among populations from different altitudes [8, 10, 31].

Application of research: The data generated here in will serve as basic knowledge to understand the status of genetic diversity of Indian bamboo species. The data is of paramount importance in devising programs for species conservation and genetic improvement.

Research Category: Population Genetics

Abbreviations:
A.M.S.L.: Above Mean Sea Level
EDTA: Ethylenediaminetetraacetic acid
TBE: Tris/Borate/EDTA
UPGMA: Unweighted Pair-Group Method with Arithmetic averages

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