Genetic diversity and structure of *Dendrocalamus hamiltonii* natural metapopulation: a commercially important bamboo species of northeast Himalayas

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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. Nuclear simple sequence repeats (nSSRs) were used to study the genetic diversity and population genetic structure of 19 natural stands of *D. hamiltonii* distributed across the northeast Himalayas. A total of 68 nSSR primer pairs of *D. latiflorus* and *Bambusa arundinacea* have been tested in *D. hamiltonii* for their transferability, out of which 17 primers showing positive and polymorphic amplification were used for genotyping. A total of 130 alleles were generated in 535 individuals of all the populations using selected primer pairs. The marker analysis indicated that *D. hamiltonii* populations have maintained a low level of genetic diversity ($h=0.175$, $I=0.291$) in northeastern region of India. Despite a large proportion of the genetic variation (83.47%) confined within the populations, a moderate level of genetic differentiation ($F_{ST}=0.165$) was observed among the populations. The clustering pattern obtained in UPGMA and STRUCTURE analysis revealed that most of the populations were clustered in accordance with their geographical distribution. Two populations (DH03 and DH13) exhibiting significant genetic admixture were identified and recommended for in situ conservation. In addition, six highly diverse populations were also suggested for conservation in different geographical area under study. The study has revealed useful nSSR markers for *D. hamiltonii*, which were lacking earlier and the information generated herein is of paramount importance in devising programs for species conservation and genetic improvement.

Keywords Bamboo · *Dendrocalamus hamiltonii* · Genetic diversity · Genetic differentiation · Genetic structure · nSSR

Introduction

Bamboo constitutes a large group of taxonomically related plants having vital economic and ecological importance. Approximately, 1650 bamboo species have been documented in World Atlas of Bamboos and Rattans (Vorontsova et al. 2016). India is the third after China and Brazil in bamboo genetic resources (Greco et al. 2015); however, there are varying reports on the number of bamboo species existing in the country, viz. 123 species under 23 genera (Seethalakshmi and Kumar 1998), 102 species (Ohrnberger 1999) and 115 species under 20 genera (Naithani 2008). But the recent assessment of Indian bamboo by Sharma and Nirmala (2015) revealed 148 species under 29 genera. Bamboo species are distributed across India covering about 15.69 million ha representing 22.15% of the forest area (FSI 2017). They grow from tropical to temperate regions with diverse climatic conditions and well known for its ecological services such as erosion control, protecting riverbanks,

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preventing landslides, soil moisture retention, land rehabilitation, biodiversity conservation, carbon sequestration, etc. (Ben-zhi et al. 2005).

Bamboo support livelihood for a large part of the Indian population due to its diverse uses and wide availability. Multifarious characteristics of bamboo such as fast growth, strength, light weight, flexibility and potential of technological advancement for value addition make them a viable alternative to timber and green economy. Unfortunately, in Indian context, there is a wide gap in its demand and supply. The total demand for bamboo products has been estimated at 26.9 million tonnes against a supply scenario of 13.47 million tonnes (Salam 2013). The demand for the bamboo resources is substantial and it is projected that in the years to come, the demand would soon outrun the stock. This is particularly true in countries such as India, where almost all the requirements of bamboo are met from their natural resources (Chaluvaraju et al. 2001; Seethalakshmi 2001). To meet the ever-increasing demand and reduce pressure on natural stock, there is a need to encourage more bamboos under cultivation with improved productivity (Tomar et al. 2009).

As natural variation is the key requirement for selection of superior genotypes for improved productivity and adaptation towards changing climate, conservation of bamboo genetic resource is of utmost importance (Ramanatha Rao and Hodgkin 2002). Genetic variability in metapopulation is vital for long-term survival by accommodating new selection pressure induced by environmental changes and serves as a resource for future breeding and improvement programs (Frankham 2008; Porth and El-Kassaby 2014). Northeastern states of India, home for more than 50% bamboo genetic resources, have undergone a significant decline of total forest cover recently in 2017 compared to assessment of 2015. The same declining trend has also been observed for bamboo resources in states of Mizoram, Arunachal Pradesh and Sikkim (FSI 2017). Degraded populations of bamboo species suffer significantly from long vegetative phase, increased inbreeding through adelphogamy (sib-pollination) and reduced reproductive output. Hence, baseline knowledge about the genetic diversity and population structure needs to be developed for their conservation, management and genetic improvement (Yeasmin et al. 2015; Potter et al. 2017).

Molecular marker techniques such as random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR) and amplified fragment length polymorphism (AFLP) have been routinely used for characterization of bamboo germplasm (Tian et al. 2012; Yang et al. 2012; Ma et al. 2013; Nag et al. 2013; Nilkanta et al. 2017). Simple sequence repeats (SSRs) are preferred tool for plant genotyping because they are abundant in genome, informative, codominant and multi-allelic genetic markers; that are also

experimentally reproducible and transferable among related species (Mason 2015). In the last decade, several microsatellite markers have been identified in bamboo either by mining from the sequence information available in database (Barkley et al. 2005; Dong et al. 2011; Bhandawat et al. 2014) or development of novel markers using method based on microsatellite-enriched genomic library (Nayak and Rout 2005; Kaneko et al. 2008; Abreu et al. 2011; Ndiaye et al. 2013). Availability of draft genome sequence of *Phyllostachys heterocycla* var. *pubescens* (Peng et al. 2013) opens new horizons to use its SSR markers in other bamboo species.

Dendrocalamus hamiltonii Nees & Arn. ex Munro is one of the priority bamboo species recommended by National Bamboo Mission, Ministry of Agriculture and Farmers Welfare, Govt. of India, for plantations in India due to its commercial and economical viability (Salam and Pongen 2008). It is used in house building, scaffolding, making of baskets, mats, ropes and as containers for eatable items. It is also used in paper and pulp industries in large quantity. This is the primary species harvested for young shoots in the north-eastern states of India (Sood et al. 2013). In recent years, there has been an increasing concern over the conservation of bamboo genetic resources. However, for most species of bamboo, critical information on the extent of intra-specific genetic diversity of the populations is unavailable. The present work is aimed to characterize the metapopulation of *D. hamiltonii* across its range of distribution for their genetic diversity, differentiation and population structure using microsatellite markers. The knowledge generated for the metapopulation of *D. hamiltonii* would be vital for developing conservation approaches and to assess the resilient response of the species against future climate change and global warming.

Materials and methods

Plant materials

Young fully expanded foliage samples of *D. hamiltonii* were randomly collected from 535 clumps representing 19 populations distributed across the entire range in northeast Himalayas during February–October 2015 (Table 1). To avoid the possibility of sampling a clone twice or more, two sampled clumps were at least 100 m apart for each site. Fresh leaves were instantly desiccated with silica gel to prevent the DNA degradation and avoid any fungal contaminations during the long field tours. The desiccated plant material was brought to the laboratory of Genetics and Tree Improvement Division, Forest Research Institute Dehradun, and stored at -80°C till further use. The map of the sampled populations in the distribution range of *D. hamiltonii* was prepared using the program Arc-GIS ver 9.2 (Suppl. Fig. 1).

Table 1 Geo-spatial detail of sampled populations of *Dendrocalamus hamiltonii*

Population ID	Location	Number of samples	Latitude	Longitude	Altitude AMSL (m)
DH01	Sairang, Aizawl, Mizoram	25	N 23°47'08.45"	E 92°40'18.66"	405
DH02	Sialsuk, Thenzawl, Mizoram	27	N 23°25'01.49"	E 92°46'05.45"	1169
DH03	Buarpui, Thenzawl, Mizoram	28	N 23°17'57.59"	E 92°40'39.25"	442
DH04	Haulawng, Lunglei, Mizoram	32	N 23°01'17.40"	E 92°45'29.88"	762
DH05	Thenzawl, Mizoram	24	N 23°13'56.71"	E 92°44'33.11"	721
DH06	Vishnupur Reserve Forest, Cachar, Assam	33	N 24°31'55.67"	E 92°53'32.35"	76
DH07	Jatinga Valley Reserve Forest, Cachar, Assam	32	N 24°59'27.31"	E 92°44'58.63"	125
DH08	North Cachar Hill, Assam	13	N 25°25'45.66"	E 93°08'26.63"	283
DH09	Hatikali, NC Hill, Assam	31	N 25°31'59.63"	E 93°06'17.53"	159
DH10	Mariani, Jorhat, Assam	30	N 26°36'1.98"	E 94°20'39.48"	155
DH11	Medziphema, Nagaland	18	N 25°46'20.9"	E 93°54'42.7"	622
DH12	Bhandari, Wokha, Nagaland	29	N 26°12'23.0"	E 94°13'19.8"	549
DH13	Eaglenest Wildlife Sanctuary, Tippi, Arunachal Pradesh	32	N 27°05'38.0"	E 92°35'52.1"	203
DH14	Sinki View Wildlife range, Nahrlagun, Arunachal Pradesh	30	N 27°07'35.8"	E 92°41'01.2"	320
DH15	Kimin, Arunachal Pradesh	30	N 27°20'07.3"	E 93°50'29.3"	860
DH16	Likabali, Arunachal Pradesh	31	N 27°45'40.6"	E 94°42'39.4"	430
DH17	Ruksin, Paschigat, Arunachal Pradesh	30	N 27°57'55.7"	E 95°00'45.2"	578
DH18	Simpu, Itanagar, Arunachal Pradesh	30	N 26°59'00.1"	E 93°36'27.1"	170
DH19	Nongkhylllem Wildlife Sanctuary, Meghalaya	30	N 25°56'08.8"	E 91°46'33.0"	205

Genomic DNA extraction

Genomic DNA was extracted using standard protocols of Doyle and Doyle (1987) with minor modifications. Leaf tissues were ground in liquid nitrogen and incubated at 60 °C for 45 min 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). Qualitative and quantitative analyses of genomic DNA was carried out using 0.8% agarose gel and Biophotometer (Eppendorf), respectively. Final working solutions of the concentration 30 ng µl⁻¹ were prepared for setting of PCR reaction.

Cross-species amplification of SSR primers

Sixty-eight microsatellite markers were selected from the published literature for screening. Among these, 62 primers were developed in *Dendrocalamus latiflorus* (Bhandawat et al. 2014) and another six primers in *Bambusa arundinacea* (Nayak and Rout 2005). To obtain the amplification products of the appropriate size range, the annealing

temperature was optimized for each primer through gradient polymerase chain reaction (PCR) where a gradient range of ± 3 was set for primer-specific melting temperature (T_m). PCR amplification was carried out with the 15 µl PCR reaction mixture, containing 60 ng of template DNA, 1.5 µl of 10× PCR buffer, 1.75 mM MgCl₂, 0.2 mM dNTPs, 100 nM of each 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, primer-specific T_m range for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. The PCR products were electrophoretically separated using 2% agarose gel buffered with 1× TBE (Tris/borate/EDTA). Positively amplified primers were further tested for polymorphism by subjecting them to PCR amplification in about 20 random samples and resolved in 4% high-resolution agarose (Sigma-Aldrich).

Marker genotyping

The selected polymorphic markers were used for genotyping through PCR amplifications using a standard programme. Composition of reaction mixture and cycling programme were kept same as gradient PCR except the annealing which was done at fixed optimized temperature varied among primers from 51.2 to 61.7 °C. The PCR products were separated using 4% high-resolution agarose gel buffered with 1× TBE.

A 100-bp DNA ladder was used as a size marker. After staining with ethidium bromide ($0.5 \mu\text{g ml}^{-1}$), the DNA fragments were visualized under UV light and image captured using gel documentation system (UVP, USA). Each individual band was scored manually and marked with their respective size by comparing to DNA ladder. Since most of the bamboo species are polyploids (Triplett et al. 2014) including *D. hamiltonii* (Thakur et al. 2015), multiple bands were obtained at most of the loci in individual genotype and, therefore, it was difficult to assign the correct allele dosage (Sampson and Byrne 2012; Garcia-Verdugo et al. 2013). Although some methods based on electropherogram peak height have been successfully applied for estimating the allele dosage in tetraploids but these calculations are often unreliable in high-order polyploids (Obbard et al. 2006). Despite losing the advantage of co-dominance, SSR markers were proved as more powerful tool with higher discriminatory power for fingerprinting of polyploids due to the presence of a large number of reproducible alleles per locus (Pfeiffer et al. 2011). Therefore, each allele or band was scored as individual loci for each sample and the multi-locus data were transformed into a binary matrix of presence (1) and absence (0) as per the Teixeira et al. (2014).

Marker data analysis

Gene diversity was analyzed for all the loci in each individual population as well as at species level using software POPGENE ver 1.32 (Yeh et al. 1999). The parameters such as observed number of alleles (n_a), effective number of alleles (n_e), percentage of polymorphic band (PPB), Nei's gene diversity (h) and Shannon's Information Index (I) were calculated to describe the statistics of genic variation. Nei's analysis of gene diversity in subdivided populations was also carried out by calculating parameters such as total gene diversity (H_T), within population gene diversity (H_S), the coefficient of gene differentiation (G_{ST}) and gene flow (N_m). Banding patterns such as band frequency and number of private bands (bands unique to a single population) were calculated for each population using software GenAlEx ver 6.5 (Peakall and Smouse 2012).

An unbiased estimate of Wright's fixation index (F_{ST}) which is an indicator of the degree of differentiation among populations was calculated according to Weir and Cockerham (1984) and partitioning of molecular variance among the genotypes within and among populations was studied through analysis of molecular variance (AMOVA) using the software Arlequin version 3.11 (Excoffier et al. 2005). Genetic relationship of the populations was studied by constructing a dendrogram based on Nei's genetic distances (Nei 1972) using unweighted pair group method with arithmetic mean (UPGMA) in POPGENE ver 1.32. Principal coordinate analysis (PCoA) was carried out to check the

consistency of clustering pattern obtained in UPGMA dendrogram, and the Mantel test (Mantel 1967) was performed to understand relationship of genetic distance of the sampled population with physical distance, using the software GenAlEx ver 6.5.

The Bayesian model-based clustering method was used to elucidate the genetic structure of the sampled populations using STRUCTURE software ver 2.2 (Pritchard et al. 2000). Ancestry model with admixture under the assumption of correlated allele frequencies was employed to determine the posterior probability [$\text{Pr}(K)$] or estimated Ln probability of data [$\text{Ln}P(D)$]. Simulations were run ten times for each value of K (2–10) with 300,000 Markov Chain Monte Carlo (MCMC) sampling runs after a burn-in period of 500,000 iterations. The results of the STRUCTURE output file were further utilized to determine the optimum k value manually using the graphical method of Evanno et al. (2005), which is based on the second-order rate of change of the likelihood function with respect to K .

Results

Gene diversity

In the initial screening of 68 nuclear microsatellite primer sets, 47 (74%) showed positive amplification in *D. hamiltonii* but only 21 (31%) were found polymorphic across the genotypes. Band profile generated by three representative primer sets is shown in Fig. 1. Out of the 21 primer sets, 17 showing reproducible banding pattern were further used for genotyping (Table 2). A total of 130 alleles were generated in 535 individuals when subjected to PCR amplification with selected primer pairs. All the loci were found to be highly polymorphic and displayed 2–14 alleles per marker loci across the genotypes and populations, with an average of 7.65 alleles. Out of the 130 loci, 129 (99.23%) were found to be polymorphic at the species level while an average percentage of polymorphic bands (PPB) at the population level was 53.72%, ranging from 40.77% (DH16) to 65.38% (DH01). The total numbers of alleles for all the loci were ranged from 62 in DH16 to 87 in DH01 and DH10. Additionally, eight private alleles were also detected, of which six were present in the populations of Mizoram (DH01, DH02 and DH04) while rest of the two present in one population of each from Assam (DH09) and Arunachal Pradesh (DH18). Assuming the Hardy–Weinberg equilibrium, the expected heterozygosity (Nei's gene diversity, h) was estimated to be 0.132 at population level and 0.175 at species level. The estimated mean genetic diversity (Shannon's Information Indices, I) was recorded as 0.211 for populations and 0.291 at species level (Table 3). Among the 19 populations, highest degree of variability was recorded for DH01 ($h=0.169$ and

Fig. 1 Primer polymorphic survey in random samples. Lane M represents 100-bp DNA ladder; Lanes 1–24 represent 24 random samples of *Dendrocalamus hamiltonii*. **a–c** Banding pattern generated by three different primer sets

$I=0.267$) and lowest for DH16 ($h=0.092$ and $I=0.149$). Among the different geographical area, the populations of Mizoram revealed highest diversity while lowest was recorded for the populations of Arunachal Pradesh.

Genetic differentiation among the populations

As per the Nei's analysis of gene diversity in subdivided populations, total genetic diversity (H_T) of the species was recorded as 0.167 and major part of this (82.51%) was found to be resided within the populations ($H_S=0.138$). The findings were consistent with the results of AMOVA which revealed 83.47% of the genetic variation existed among the individuals within populations and 16.53% among the populations (Table 4a). The hierarchical structuring was also done assuming three levels, i.e. among groups (as defined by UPGMA dendrogram), among populations within groups and within populations. The analysis revealed that 81.26%

of the total variation was attributable to differences among the individuals within populations, 12.45% was due to differences among populations within groups and only 6.28% was due to variation among groups (Table 4b). Variance estimates were based on 1000 permutations and the difference between the individuals within the populations was statistically significant ($P<0.001$). The calculated value of Wright's fixation index ($F_{ST}=0.165$) indicate the presence of moderate level of genetic differentiation among the populations. For comparisons with earlier findings another widely used measure of genetic differentiation (G_{ST}) was also calculated for the sampled populations and the value obtained is 0.247. Mantel test was performed to find out the relationship between genetic and physical distance among the populations. The test result supports the isolation by distance model showing a linear relationship with significant correlation ($r=0.498$; $P<0.001$) between genetic and physical distances (Suppl. Fig. 2).

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

Sl. no.	Primer name*	Primer sequence (5'–3')*	Motif*	Expected product range (bp)*	Obtained product range (bp)	Annealing temp (°C)	No. of alleles per loci
1	DLUGMS03	F: TGCCGGTGCTTTCTTACTCT R: GGAGGAAGGGATGGGAGTAG	(CT)11	150–1000	175–440	52	8
2	DLUGMS13	F: CCTTCCTCGTTTCCCTTTTC R: TTCGCTTCGAGGGTTAAATG	(CGG)8	150–500	220–270	51.2	9
3	DLUGMS15	F: GGGGACCATTGACAACCTCA R: CTCTTTGCGAGGAAGTCACA	(TC)10(CA)5	100–1000	180–200	56.9	2
4	DLUGMS16	F: GGGAGATACAGTTCGGTTGG R: TCCTTGATGGAGCGGACTAC	(GA)26	125–600	130–230	51.8	10
5	DLUGMS17	F: CGGTTGGCCTTCTATGAGAG R: CCATCGATGATAGCACAGGA	(TC)24	150–400	195–530	56.9	12
6	DLUGMS23	F: AAGGAAAAAGGGCTGGGTTA R: TCGTCGTCATCACTTTGCTC	(ATG)14	175–800	195–260	52.6	4
7	DLUGMS25	F: GAGGGACTTGATGGATTGGA R: ATGTTATTGCGCTTGTGCTG	(GAA)8	150–1000	240–530	53.3	14
8	DLUGMS45	F: CACCGTGTGGTTACCTTTCC R: TGAGGAGGAGCTTGAAGAGG	(TC)16	150–300	220–300	58.6	9
9	DLUGMS47	F: GGGGACTCTCCTCTTCGTCT R: GATCTGAGGCTTCTCCATCG	(TC)11	220–310	195–265	54.7	10
10	DLUGMS50	F: AGACTCTCCACTCGTGACTCG R: RCCGCGAACTCCACAGACTAT	(CTCCG)6	180–350	200–260	56.7	7
11	DLUGMS51	F: CATTGGCCCATGTAAGTTTTC R: CGAGCAAGTGTGTCCTGAA	(CAG)8	210–300	200–230	56.7	3
12	DLUGMS52	F: CCATCTCTCGTCTCCTCTCG R: TTGCTCAGAAATGGCAAGAA	(CAT)9	210–360	210–240	56.7	5
13	DLUGMS54	F: CACAGGGAGCAACATCAAGA R: CCGATCATAAAACCAACTGAAA	(TTTC)7	225–450	200–260	56.7	7
14	DLUGMS56	F: CAATCTCGGAGCCGAACTAC R: ATACCACCAGGCACAAGAGC	(ACCTG)4	230–260	150–230	56.7	7
15	DLUGMS57	F: AGCCAGTCCACCATTACCAG R: GGGAGAGTCGACTGAATTGG	(CTCCG)5	400–600	145–400	57.7	12
16	DLUGMS61	F: TTCCTCATCTTGCAGGCTTT R: GCAAAATTTCCGTCGATTGT	(GGAGA)7	170–320	170–220	55.7	4
17	DLUGMS62	F: ATAGCCATGTACGCATGCAC R: GCTTACAGGTTTCACACAACCA	(CGTG)6	175–380	180–250	61.7	7

*As per the Bhandawat et al. (2014)

Genetic relationship among the populations

Genetic relationship of the populations was studied by constructing a UPGMA dendrogram based on Nei's genetic distances. UPGMA dendrogram revealed the clustering pattern in accordance with their geographical distribution (Fig. 2). Exceptionally, one population of Assam (DH06) was separated from other Assamese populations and clustered with the population of Meghalaya (DH19) indicating their common origin despite spatial separation. Further, one population of Mizoram (DH03) was unexpectedly not found to be clustered with other populations of this region and showed a significant genetic admixture in structure analysis. The clustering pattern obtained in UPGMA dendrogram was also supported by principal coordinate analysis. The first

principal coordinate of two-dimensional PCoA plot accounting for 18.58% of total variation separates populations of group 4 (Assam, Nagaland and Arunachal Pradesh) from other populations (groups 1, 2 and 3) and a second principal coordinate accounting for 11.61% of the variation is able to separate two populations of group 2 from groups 1 and 3 (Suppl. Fig. 3).

Genetic structure of the populations

In each simulation of the model, $\text{Ln}P(D)$ was calculated for all the K values. The K value with maximum $\text{Ln}P(D)$ is generally considered as the optimal number of subdivisions but no clear peak was obtained in distribution of $\text{Ln}P(D)$ values (Suppl. Fig. 4a). To overcome the difficulty

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

Population ID	Number of polymorphic loci	Percentage of polymorphic bands (PPB)	No. of private bands (PB)	Nei's gene diversity (<i>h</i>)	Shannon's information index (<i>I</i>)
				Mean (SD)	Mean (SD)
DH01	85	65.38	2	0.169 (0.174)	0.267 (0.250)
DH02	76	58.46	3	0.153 (0.178)	0.239 (0.257)
DH03	65	50.00	0	0.134 (0.174)	0.209 (0.254)
DH04	74	56.92	1	0.141 (0.165)	0.225 (0.244)
DH05	66	50.77	0	0.143 (0.178)	0.222 (0.259)
DH06	61	46.92	0	0.114 (0.153)	0.184 (0.230)
DH07	74	56.92	0	0.140 (0.169)	0.222 (0.246)
DH08	59	45.38	0	0.122 (0.168)	0.192 (0.245)
DH09	80	61.54	1	0.144 (0.162)	0.231 (0.238)
DH10	83	63.85	0	0.155 (0.167)	0.248 (0.242)
DH11	70	53.85	0	0.138 (0.172)	0.219 (0.250)
DH12	74	56.92	0	0.133 (0.161)	0.214 (0.236)
DH13	70	53.85	0	0.137 (0.166)	0.217 (0.244)
DH14	67	51.54	0	0.121 (0.165)	0.193 (0.241)
DH15	63	48.46	0	0.100 (0.143)	0.166 (0.215)
DH16	53	40.77	0	0.092 (0.148)	0.149 (0.220)
DH17	64	49.23	0	0.118 (0.162)	0.189 (0.238)
DH18	69	53.08	1	0.123 (0.156)	0.200 (0.231)
DH19	74	56.92	0	0.141 (0.162)	0.225 (0.240)
Mean of populations	69.84	53.72	–	0.132 (0.019)	0.211 (0.028)
Overall for species	129	99.23	–	0.175 (0.152)	0.291 (0.209)

Table 4 Analysis of molecular variance (AMOVA) for 19 populations of *D. hamiltonii*

Source of variation	Degree of freedom	Sum of square	Estimated variance	Percentage of variation	Genetic differentiation
(a) Partitioning of the variance assuming no hierarchical structure					
Among populations	18	649.65	1.09	16.53	$F_{ST}=0.165$
Within populations	516	2835.51	5.49	83.47	
Total	534	3485.16	6.58	100	
Source of variation	Degree of freedom	Sum of square	Estimated variance	Percentage of variation	Fixation indices
(b) Partitioning of the variance assuming hierarchical structure as defined by UPGMA clustering					
Among group	3	214.44	0.42	6.28	$F_{CT}=0.062$
Among populations within group	15	435.22	0.84	12.45	$F_{SC}=0.132$
Within populations	516	2835.51	5.49	81.26	$F_{ST}=0.187$
Total	534	3485.16	6.76	100	

for interpreting the real K value, another ad hoc quantity (ΔK) was calculated manually using the method described in methodology section. The highest value of ΔK with a clear peak was obtained for $K=4$ (Suppl. Fig. 4b), and hence it was considered that four ancestral groups captured the entire variability existing in the sampled genotypes. Clustering was found in accordance with the UPGMA

dendrogram and PCoA plot. The populations localized to a specific geographical area were clustered together and share the same ancestral gene pool with few exceptions (Fig. 3). As per the inferred ancestries (Q -matrix) of population, 17 populations have been clearly defined by four clusters with the proportional membership coefficient of more than 0.7 while two populations, namely DH03 and

Fig. 2 UPGMA dendrogram based on Nei's (1972) unbiased measures of genetic distance among 19 populations of *Dendrocalamus hamiltonii*. Genetic distance is indicated by branch length for each population

DH13, were not defined by any of the single clusters, indicating their admixed ancestry (Fig. 4).

Discussion

Genetic diversity

Germplasm characterization is an important link between its conservation and utilization (Stapleton and Rao 1995; Nayak et al. 2003). The levels of genetic diversity reflect the availability of genetic resources necessary for short-term ecological adaptations and long-term evolutionary

changes (Templeton et al. 1995). The development of SSR markers is still inefficient, laborious and costly, principally in organisms with large and complex genomes (Rai and Ginwal 2018). The availability of microsatellite markers in bamboo is limited and few genomic SSR markers have been developed for bamboo in recent years (Nayak and Rout 2005; Kaneko et al. 2007; Kitamura et al. 2009; Dong et al. 2012; Dong and Yang 2014). However, a number of researches have highlighted the ability of cross amplification of SSRs in various bamboo species (Sharma et al. 2009; Abreu et al. 2011; Dong et al. 2011; Ndiaye et al. 2013; Bhandawat et al. 2014; Lin et al. 2014; Attigala et al. 2017). Cross transferability is the rapid and cost-effective approach

Fig. 3 Geo-spatial representation of the pattern of genetic admixture and clustering depicted by STRUCTURE analysis. Clusters defined by STRUCTURE shown by dotted lines

Fig. 4 Bar plot for estimated population Q -matrix at $K=4$ for all the genotypes of *Dendrocalamus hamiltonii*. Each population is separated by vertical line and individual samples are represented by coloured bars

for developing locus-specific markers in related species. In the present study, 17 robust nuclear microsatellite markers have been identified for *D. hamiltonii* through cross-specific amplification. These primers have been successfully utilized in genetic analysis of 19 populations of *D. hamiltonii* distributed across the northeast Himalayas. The study confirms that the nSSR primer pairs of other bamboo species can be

successfully transferred in *D. hamiltonii* and these markers could provide a valuable resource for future genetic research in this species.

A low level of genetic diversity ($h=0.175$, $I=0.291$) was observed in *D. hamiltonii* compared to other bamboo species such as *D. membranaceus* ($h=0.219$, $I=0.349$) and *Melocanna baccifera* ($h=0.194$, $I=0.321$) which were studied

using ISSR markers by Yang et al. (2012) and Nilkanta et al. (2017), respectively. Lower level of gene diversity has also been reported earlier for the populations of *D. strictus* ($h=0.197$) and *B. bambos* ($h=0.114$) in the central Western Ghats, India, using isozymes (Ravikanth et al. 2008). Lesser genetic diversity detected here for *D. hamiltonii* might be attributed to its long vegetative phase and widely separated episodes of flowering/seed set over a life cycle. This indicates that the population may have been mainly derived vegetatively, during the process of evolution. The other reason could be increased anthropogenic pressure, changing environmental conditions and selective removal of clumps by the local villagers/tribals causing fragmentation and shrinkage of the populations; hence, the small patches are at the risk of losing their genetic diversity. Large-scale destruction of habitats and over-harvesting of many economically important species of bamboo has led to irreversible loss of its genetic diversity in India (Ravikanth et al. 2008). While assessing sandal (*Santalum album*) genetic diversity in peninsular India, Nageswara Rao et al. (2007) also highlighted such strong association of loss in genetic diversity due to increased anthropogenic pressure and reduced population size. Earlier studies have shown that the demographic status of a species could be used as an appropriate indicator of the status of the genetic diversity of the populations (Lande 1988; Quattro and Vrijenhoek 1989); larger the population size, the better would be the status of the genetic resources (Gilpin and Soule 1986; Prober and Brown 1994).

Genetic differentiation

As expected, owing to the greater degree of geographical isolation, higher population divergence or differentiation is observed for *D. hamiltonii* ($F_{ST}=0.165$). For the interpretation of F_{ST} , it has been suggested that a value lying below 0.05 indicates little genetic differentiation; a value between 0.05 and 0.15, moderate differentiation; a value between 0.15 and 0.25, great differentiation and values above 0.25, very great genetic differentiation (Wright 1978; De Vicente et al. 2004). However, for interpretation through comparisons with earlier findings, another widely used measure of genetic differentiation such as G_{ST} was also considered along with F_{ST} . The measure of genetic differentiation ($G_{ST}=0.247$) recorded in *D. hamiltonii* was comparable to those of another bamboo species such as *D. membranaceus* ($G_{ST}=0.252$) and *Melocanna baccifera* ($G_{ST}=0.194$). But the parameters are quite different when compared to another bamboo species, i.e. *Dendrocalamus giganteus*, investigated in Yunnan Province of China (Tian et al. 2012). In this study, high genetic diversity ($I=0.4329$) was recorded at the species level but very low for the populations ($I=0.0624$), indicating high genetic differentiation among populations ($G_{ST}=0.847$). The reason of very high differentiation was

the use of cultured populations established by vegetatively multiplied planting material.

The measures of genetic differentiation in our populations of *D. hamiltonii* were also in accordance with the mean value of other out-crossing species ($G_{ST}=0.23$) (Nyblom and Bartish 2000). Generally, long-lived out-crossing species with wide geographical distribution tends to maintain a high level of genetic diversity with lower genetic differentiation (Hamrick and Godt 1990, 1996). However, despite out-crossing and wide distribution range of bamboo, populations could be genetically differentiated over the period of time because of several factors such as flowering and breeding behavior, population size, habitat fragmentation and gene flow (George et al. 2009). The genetic differentiation detected here among the populations might be attributed to prolonged vegetative phase and widely separated unsynchronized flowering events over the life span. These are typical features of several woody bamboo species (Janzen 1976) including *D. hamiltonii* with the estimated flowering cycle of about 45 years (Banik 2016). Populations flowered during a year may not be likely to mate with populations flowered another year or later, and this temporal reproductive isolation acts as barriers to gene flow among the population and promote sib-pollination, i.e. among the individuals of same provenance. This may likely to have direct genetic consequences such as increase of the genetic distance between populations and subsequently differentiating into sub-populations (Thakur et al. 2015). This is also evident by the presence of private alleles in some population and low observed value of gene flow ($N_m=1.52$). The results from the Mantel test also supported the above observation as it showed a significant positive correlation between the genetic and geographic distances between different populations. Mantel test further suggests that genetic admixture between the population decreases with increase in distance as expected under isolation by distance model.

Population genetic structure

STRUCTURE analysis revealed that the populations localized to a particular geographical area were clustered together and shares the same ancestral gene pool that may be attributed to the physical barriers and geographical attributes of the sampling area. Cluster 1 defines two distant populations one from Assam (DH06) and another from Meghalaya (DH19). Both the populations were old and belong to either reserve forest or sanctuary area. Population DH06 was very small with a countable number of clumps located in Vishnupur Reserve Forest of the Assam and isolated from other populations of same species, while another population DH19 located in Nongkhyllam wildlife sanctuary, Meghalaya, was very large and old. Therefore, it can be inferred that population DH06 might have originated from the population DH19

of Meghalaya. Moreover, less diversity exhibited by population DH06 ($I=0.184$) may be due to smaller size and random genetic drift. Also, the reserve forest is located near the settlements which may face the problem of encroachment and over-extraction. Therefore, conservation efforts need to be done to protect the population. Diversity level and longevity of the population can be further enhanced by infusing the diverse germplasm and increasing the population size. Cluster 2 defines all the populations of Assam (except DH06) and adjoining parts of Nagaland. These populations were distributed from plain of the Assam to foot hills of Nagaland and well separated from other geographical area. Cluster 3 comprises the populations located at mid-high hills of Mizoram and gene flow might be restricted due to hilly terrain of the state, while cluster 4 comprises the populations of Arunachal Pradesh which were separated from other populations through the river Brahmaputra, one of the major river systems of India.

Population Q -matrix obtained in STRUCTURE analysis revealed that the population DH03 has admixed ancestry with the major component from populations of Mizoram and Arunachal Pradesh. This population seemed more like plantation rather than natural population, as seen during sample collection, while population DH13 of Arunachal Pradesh belongs to wildlife sanctuary which also showed admixed ancestry with major component from populations of Assam and Arunachal Pradesh. The perpetual propagation through vegetative means and sharing of planting material appears to be responsible for distribution of few *D. hamiltonii* cohorts, resulting in admixing of genomes across the locations of natural range.

Low level of genetic diversity and significant value of differentiation depicted in natural populations of the Himalayan landscape indicate that there is hindrance in the gene flow among the populations. Also heterogeneous genetic structures obtained in STRUCTURE analysis reflect the considerable genetic changes adopted by populations in response to habitat fragmentation, geographical isolation and environmental changes.

Conservation implications

The ultimate goal of conservation is to ensure the continuous survival of population and to maintain their evolutionary potential by preserving natural levels of genetic diversity (Desai et al. 2015). Considering the low level of genetic diversity of metapopulation and the degree of threats confronted by the species, great efforts are needed for conservation of existing populations. The population size is required to be enhanced up to standard effective size required for maintaining higher genetic diversity. Genetic drift in very small population might also cause rapid genetic erosion and increased the risk of extinction of the bamboo species

(Nilkanta et al. 2017). To make the best possible use of highly diverse populations, it will be important to conserve them in situ as well as ex situ and to use their available seeds for infusing diversity in less diverse populations. Other ex situ germplasm resources might include vegetatively propagated materials taken from multiple populations that represent the four major clusters. Considering the high degree of differentiation, conservation of any one population alone would not serve the purpose. As per the STRUCTURE analysis, two populations, DH03 (Mizoram) and DH13 (Arunachal Pradesh), have significant genetic admixture and encompassing diverse genotypes at a location, and may be treated as natural gene banks and recommended for in situ conservation. In addition, the populations comprising of higher genetic diversity and private alleles could also be prioritized for each geographical area, viz. populations DH01 and DH02 for Mizoram; DH09 and DH10 for Assam and Nagaland; DH18 for Arunachal Pradesh and DH19 for Meghalaya.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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