# Characterization of Microsatellites in *Bambusa arundinacea* and Cross Species Amplification in Other Bamboos

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Microsatellites, tandem repeats of short nucleotide (1–6 bp) sequences, are the DNA marker of choice because of their highly polymorphic, ubiquitous distribution within genome, ease of genotyping through polymerase chain reaction (PCR), selectively neutral, co-dominant and multi allelic nature. Six microsatellites, three polymorphic and three monomorphic, have been characterized for the first time in a bamboo species, *Bambusa arudinacea* belonging to the family Poaceae. The number of alleles per locus ranges form 2 to 13. Allelic diversity ranges from 0.041 to 0.870. Polymorphic information content (PIC) values for two loci were > 0.3, an indicator of polymorphic allele. Cross species amplification has been tested in other 18 bamboo species. Monomorphic simple sequence repeats (SSRs) have been found to be cross amplified in most of the tested species while polymorphic ones in only three to four species. The utility of the SSR loci in genetic diversity study of *B. arundinacea* and other cross amplified bamboo species have been discussed.

*Key words:* Microsatellite, *Bambusa arundinacea*, Cross Species Amplification

## Introduction

Bamboos, a group of arborescent grasses, have closely been associated with mankind since ancient time. They belong to the family Poaceae subfamily Bambusoideae. These are considered to be the strongest growing woody plants on earth with one of the widest ranging habitats with more than 1500 species thriving in every continent but the poles. They are found mostly in tropical and subtropical region. Bamboo flowers irregularly and usually at very long time intervals of 30 to 120 years and when it does, it takes so much of energy from the plant that it often dies. So, they are generally clonally propagated with occasional propagation from seeds (http://www.americanbamboo.org/). In a land without bamboo recruitment of clonally propagated plants the only option for plant introduction as a result of the clonal diversity/genetic diversity is very low in these areas. However, the genetic/genotypic diversity may be more in the natural bamboo growing region due to occasional flowering. Out of the different types of DNA marker systems available for genetic diversity study microsatellites are proved to be the best ones. Microsatellites, also known as simple sequence repeats (SSRs), are short tandemly repeated sequence motifs consisting of a repeat unit of 1–6 bp in length (Tautz and Schlötterer, 1994). Microsatellites have been used in a number of plant species for construction of genetic linkage map (Sharopova *et al*., 2002), QTL mapping (Liu *et al*., 2002), gene tagging (Kim *et al*., 2002), etc. These microsatellites need to be isolated *de novo* from the species or from the most related species that are being examined for the first time (Zane *et al*., 2002). Microsatellites have been isolated in a number of economically important plant species (Morgante *et al*., 2002). In the present study a set of hyper variable microsatellites have been isolated and characterized from a bamboo species of Indian origin, *i.e. Bambusa arundinacea* Willd., which will facilitate genetic diversity study and cross species amplification in other bamboos.

## Materials and Methods

### *Plant material*

Leaf samples of *Bambusa arundinacea* Willd. were collected from 15 different clumps arbitrarily from Regional Plant Resource Centre, Bhubaneswar forest area. Care was taken to collect samples from the clumps with at least 100 m away from the nearby clump. Leaf samples of other bamboos *viz*. *Dendrocalamus giganteus* Munro, *D. strictus* Nees, *Dinochlea m'clellandii* Kurtz,

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*Cephalostachyum pergracil* Munro, *Bambusa vulgaris* Schr., *B. vulgaris* var. *striata* Schr., *B. nana* Roxb., *B. multiplex* Raeusch, *B. balcooa* Roxb., *B. ventricosa* and *Sasa* sp. Makino and Shibata were collected from the Bambusetum of Regional Plant Resource Centre, Bhubaneswar. Leaf samples of the rest of the bamboo species, *i.e. Arundinaria mannii* Gamble, *Dendrocalamus sikkimensis* Gamble, *D. hookeri* Munro, *D. hamiltoni* Nees and Arn., *D. patellaris* Gamble, *Bambusa tulda* Roxb., *B. nutans* Wall. ex Munro, and *B. clarata*, have been collected from Sikkim, one of the treasure houses of Himalayan bamboos.

### *DNA extraction*

DNA was extracted from semi mature fresh/frozen/dried/semidried leaves following the *N*-cetyl-*N*,*N*,*N*-trimethyl ammonium bromide (CTAB) method of Doyle and Doyle (1990) with little modifications, as the leaves of bamboos contain silica materials. 2.5 g of leaf material were surface sterilized by wiping with 80% ethanol, cut into small pieces, ground into fine powder by liquid nitrogen, added to 10 ml of preheated extraction buffer [100 mM Tris-HCl (pH 8.0), 2 mM EDTA, 3% CTAB (w/v), 0.2% $\beta$-mercaptoethanol (v/v), 1.4 M NaCl] and incubated for at least 2 h at 60 °C with little mixing at every 15 min interval. After several standard steps, the pellet was washed with 70% ethanol and dissolved in 200–300 $\mu$l of TE (10 mM Tris, pH 8.0, 1 mM EDTA). DNA quantification was made by visualizing under UV light against a $\lambda$-uncut marker, after electrophoresis on 0.8% (w/v) agarose gel containing 0.5 $\mu$g ml$^{-1}$ of ethidium bromide.

20 mg of extracted DNA (only from one individual) was completely digested with M*bo*I (Life Technologies Inc., Rockville, USA). The digested DNA was concentrated (ethanol precipitation), dissolved in 30 $\mu$l of TE and electrophoresed on a 1% low melting agarose gel (Life Technologies Inc.) along with a molecular weight marker (MBI Fermentas Inc., New York, USA) as size standard. DNA fragments corresponding to 350–550 bp were excised from the gel and purified with QIAquick gel extraction kit (QIAGEN AG, Basel, Switzerland). The ligation reaction was performed with 50 ng of gel purified insert DNA, 100 ng of dephosphorylated pUC19/B*am*HI (Genei, Bangalore, India) plasmid DNA (vector:insert = 1:4) and 1 $\mu$l of T4 DNA ligase (Life Technologies Inc.) in a total volume of 20 $\mu$l at 16 °C for overnight. Ligated products were transformed into DH5$\alpha$ competent cells and transformants plated on 82 mm LB/ampicillin plates with X-gal and IPTG for blue white screening.

All the white colonies were transferred onto grided LB/ampicillin plates with the help of sterile tooth picks and incubated for another 12 h at 37 °C by putting nylon membranes (Hybond N+, Amersham Pharmacia Biotech. Inc., California, USA) over the plates. Then the membranes were lifted out of the plates, air-dried and processed by placing colonies side up in a petri dish containing sterile paper soaked with a) 10% SDS for 3 min; b) 1.5 M NaCl/0.5 N NaOH (fresh) for 5–10 min; c) 1.5 M NaCl/0.5 M Tris (pH 7.8) for 5–10 min; d) 2 × SSC for 1 min. Crosslinking of the DNA on nylon membrane was done in a UV cross linker (Hoefer, Amersham Pharmacia Biotech. Inc.). The probe (GT) 15, used to screen the library, was 3′ end labelled with the help of DIG oligonucleotide tailing kit (Roche, Mannheim, Germany) following the manufacturer's instruction. The oligonucleotide was enzymatically labelled at its 3′ end with terminal transferase by incorporation of digoxigenin-labelled deoxyuridine triphosphate (DIG-dUTP/dATP) tail. Pre-hybridization was done by incubating the blots with shaking at 65 °C in an appropriate amount of pre-hybridization buffer (5 × SSC, 0.5% SDS, 0.1 mg/ml, and 0.1% lactogen) in polythene bags for 1 h. Hybridization was done at 68 °C O/N in the same pre-hybridization solution with the labelled probe. Positive clones were detected colorimetrically, inoculated in 2 ml of LB/amp and grown overnight. Next day 1 ml of the above culture was stored at −80 °C with 15% glycerol for further use and plasmid DNA was isolated from 1 ml rest of the culture (Sambrook *et al.,* 1989). The above positive clones were reconfirmed through dot blot.

### *Screening*

Positive clones were got sequenced through an automated sequencing facility. Oligo primers were designed from the flanking regions of identified repeats using a primer select module of DNASTAR software and synthesized by MWG-Biotech AG, Ebersberg, Germany. For PCR genotyping of microsatellites, amplification was carried out in a final volume of 25 $\mu$l containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.01% gelatin,

1.5 mM $MgCl_2$, 5 pmol of each primer, 200 $\mu$M dNTPs, 0.25 units of T*aq* DNA polymerase (Genei), and $\approx$ 20 ng genomic DNA templates. Amplifications were performed in a PTC 100 thermocycler (MJ Research Inc., Waltham, USA) programmed for initial denaturation of 4 min at 94 °C followed by 25 cycles of 1 min at 94 °C, 2 min at annealing temperature (Table I) and 2 min at 72 °C, a final extension of 7 min at 72 °C and indefinite halt at 4 °C. Amplified products were dried on a vacuum concentrator (DNA plus), mixed with 10 $\mu$l of formamide loading dye, heat-denatured and then separated on 8% denaturing polyacrylamide gel with 7 M urea and 0.5 × Tris-Borate-EDTA buffer. Separation was performed on vertical gel electrophoresis (Hoofer SE 600) at a constant current of 20 W for about 2 h. 100 bp ladder of MBI Fermentas Inc. was used as a size standard. Detection of the amplification products was done by silver staining (Riesner *et al.*, 1989). After initial fixation in a 10% ethanol/0.5% acetic acid solution for 10 min the gels were immersed for 15 min in a fresh 5.9 mM solution of silver nitrate, and developed for 10 min in 375 mM NaOH, 2.3 mM $NaBH_4$ and 0.125% formaldehyde (37% w/v).

For each locus, genotpypes, total number of alleles, allele frequencies and observed heterozygosity were determined from the gel. Expected heterozygosity, the proportion of expected heterozygotes under random mating condition, was calculated as per Nei (1987): $h_e = 2n\ (1 - \Sigma P_i^2)/(2n - 1)$ where $n$ is the number of samples and $P_i$ the allele frequency of the '$i$' th allele at a given locus. Inbreeding coefficient ($f_{is}$), a measure of heterozygosity deficit or excess, for a particular locus is calculated as per Wright (1978): $f_{is} = 1 - h_o/h_e$ where $h_o$ is the observed heterozygosity and $h_e$ the expected heterozygosity. The effective number of alleles ($A_E$) for a particular locus is calculated as per Hartl and Clark (1989): $A_E = 1/(1 - h_e)$. A polymorphic information content (PIC) value of a locus is calculated as per Anderson *et al.* (1992): $PIC = 1 - \Sigma^n j = 1\ P_{ij}^2$, where $P_{ij}$ is the frequency of the $j^{th}$ SSR pattern for locus $i$ and the summation covers $n$ patterns.

## Results and Discussion

Screening of four thousand recombinant colonies of the partial genomic library with (CA)15 oligo resulted in 15 positive clones. A second

Table I. Microsatellite loci identified from the genome of *B. arundinacea* with GenBank Accession number, primer sequence, annealing temperature and expected fragment size.

| Locus | Repeat sequence | GenBank accession number | Primer sequence (5'-3') | Expected PCR product [bp] | Annealing temperature [°C] | Amplification status |
|---|---|---|---|---|---|---|
| Ba10 | (GT)12C(TA)10 | AJ507486 | F-GGTGGGGTCTAGCACCTAAG<br>R-TACCAGCATGTAACGGTCGG | 146 | 60 | Amplified |
| Ba14 | (CA)10 | AJ507487 | F-AGAGTTAAGGAAGCCAGGTC<br>R-GTCTAGTAGCTGCTCAACTC | 237 | 57 | Amplified |
| Ba18a | (CT)5n(CT)5 | AJ507488 | F-TATCTCGACCTCCCCTTGCT<br>R-GTCTAGAAGCAGGGAGGTAG | 166 | 59 | Amplified |
| Ba18b | (CA)13 | AJ507488 | F-CCAGGTCGTTTCACTGCTC<br>R-ACAACGGTAGAGTTCACTCG | 146 | 57 | Amplified |
| Ba20 | (AC)31(AT)7 | AJ507489 | F-TTGATTGCCCTACTCTGTCG<br>R-TCAACGGTGGATGACCTAGG | 169 | 57 | Amplified |
| Ba25 | (GACA)3(GATA)2GAT(GATA)1 | AJ507490 | F-GTGAGATGGGCTGGGCAG<br>R-GCTCCGATCTGTCAGTTTAC | 242 | 57 | Not amplified |
| Ba58 | (CA)7 | AJ507491 | F-TCCGAAGCACACTCATGAAG<br>R-TTCTACTATGCGCTAACTGC | 187 | 55 | Amplified |
| Ba202 | (AC)3C(AC)2GC(AC)4 | AJ507492 | F-CAACTAGCAAACGCACAGTG<br>R-CGAATTCGAGCTCGGTACC | 261 | 57 | Not amplified |

round of screening through dot blot resulted in eleven positive clones. Out of the eleven clones sequenced seven were found to contain one or more repeat motifs of varying length. Oligo primers have been designed for eight loci of which six could be amplified (Table I). Half of the amplified loci (3) were found to be polymorphic and the rest three were monomorphic in a panel of 71 tested individuals. The number of alleles for all the polymorphic loci ranges from 2–13 with an average of 9.0. Locus B10 has 13 alleles, B20 has 12 alleles and B18a has only 2 alleles. Observed heterozygosity, expected heterozygosity, fixation index and PIC values for all the polymorphic loci are given in Table II. PIC value was highest, *i.e.* 0.864, for locus Ba10 and lowest, *i.e.* 0.041, for Ba18a.

The use of dephosphorylated pUC vectors helped in getting 100% positive white colonies in the first few LB/ampicillin plates with X-gal and IPTG. This eliminated the use of X-gal and IPTG for blue white screening of recombinants in the other LB/ampicillin plates. A second round of screening through dot blots helped in elimination of false positive which are often found through radioactive as well as non-radioactive detection systems. The chance of getting a microsatellite repeat motif after the first round of screening was less (46%) than that of the second round of screening (64%) in case of *B. arundinacea*. This might be true for all other bamboos.

About half of the loci amplified was found to be monomorphic and the rest polymorphic. This might be due to the nature of the plant itself. Being a highly clonally propagated and rarely sexually propagated (self/wind pollinated) plant there is less chance of replication slippage (Richards and Sutherland, 1994), a common method for formation of alleles in microsatellite loci. Further unequal crossing-over during homologous recombination in the site of microsatellite, another method of formation of allele in microsatellite loci (Jakupiak and Wells, 1999), may not be happening due to the very long and erratic flowering period. The presence of imperfect repeats in most of the identified loci suggests that mutation might have played a leading role in the formation or degradation of a microsatellite locus (Zhu *et al*., 2000). Loci containing longer repeat units (< 20) whether perfect or imperfect, *i.e.* Ba10 and Ba20, were found to be more polymorphic than loci containing shorter repeat units (Ba18a). This might be due to the rate of microsatellite mutations which is directly proportional to the repeat length (Schug *et al.,* 1997), thereby forming more alleles in a longer repeat motif. Further, the rate of contraction mutations increased exponentially over repeat length and that of expansion mutations was constant for all shorter and longer alleles (Xu *et al*., 2002).

Cross species amplification of different bamboo species by taking primers of *B. arundinacea* microsatellite loci is presented in Table III. All the polymorphic/functional microsatellite loci could be amplified in few of the bamboo species. Locus Ba10 amplified in four other bamboo species, *i.e. B. clarata*, *B. vulgaris*, *B. vulgaris* var. *striata* and *B. ventricosa*. Locus Ba20 could be amplified in *D. strictus*, a species belonging to the genus *Dendrocalamus*. Nonspecific amplification of Ba20 could be observed in *B. nana* and *B. multiplex*. So, these loci may be useful for population genetic studies in the cross amplified species. However, interspecific differences in the repeat motif may be varied and influence the level of variability in the cross amplified taxon (Eustoup *et al.,* 1995). All the three monomorphic microsatellites could be amplified in almost all the tested species. These loci might be present in the transcribed region and cross amplify readily in related genera and species. Among plant species, the overall frequency of microsatellites is inversely related to the genome size and proportion of the repetitive DNA but remained constant in the transcribed portion of the genome (Morgante *et al*., 2002). Homology search of bamboo SSR loci in BLAST showed no concrete similarity with any of the transcribed or EST regions. Only 20 bp of locus Ba18a is similar to a) human L1 Heg repetitive element from the intergenic region of the epsilon and G-gamma globin gene and b) human 3′ end of the gene for protein tyrosine kinase phosphatase receptor type K.

Table II. Number of alleles, observed ($h_o$) and expected ($h_e$) heterozygosity, effective number of alleles ($A_E$), fixation index ($f_{is}$) and polymorphic information content (PIC) of the three polymorphic microsatellite loci of *B. arundinacea*.

| Locus | Sample size ($n$) | No. of alleles | $h_o$ | $h_e$ | $f_{is}$ | $A_E$ | PIC |
|---|---|---|---|---|---|---|---|
| Ba10 | 71 | 13 | 0.535 | 0.870 | 0.385 | 7.733 | 0.864 |
| Ba20 | 73 | 12 | 0.424 | 0.866 | 0.510 | 7.506 | 0.860 |
| Ba18a | 71 | 2 | 0.042 | 0.041 | –0.014 | 1.043 | 0.041 |

Table III. Cross species amplification of *B. arundinacea* microsatellite loci in different bamboo species.

| Species | Ba10 | Ba14 | Ba18a | Ba18b | Ba20 | Ba58 |
|---|---|---|---|---|---|---|
| *A. mannii* Gamble | – | – | – | – | – | +** |
| *B. balcooa* Roxb. | – | + | + | + | – | + |
| *B. nana* Roxb. | – | + | + | + | + | +* |
| *B. multiplex* Raeusch | – | + | + | + | + | +* |
| *B. clarata* | + | + | + | + | – | + |
| *B. nutans* Wall. ex Munro | – | + | + | + | – | + |
| *B. vulgaris* Schr. | + | + | + | + | – | + |
| *B. vulgaris* var. *striata* Schr. | + | + | + | + | – | + |
| *B. ventricosa* | + | + | + | + | – | + |
| *C. pergracil* Munro | – | + | + | + | – | + |
| *D. m'clellandii* Kurtz | – | + | + | – | – | + |
| *D. giganteus* Munro | – | + | + | + | – | + |
| *D. strictus* Nees | – | + | + | + | + | + |
| *D. hookeri* Munro | – | + | + | + | – | + |
| *D. hamiltoni* Nees and Arn. | – | – | + | – | – | + |
| *D. patellaris* Gamble | – | – | – | – | – | – |
| *D. sikkimensis* Gamble | – | + | + | + | – | + |
| *Sasa* sp. Makino and Shibata | – | + | – | – | – | + |

\+ Specific amplification.
– No amplification.
* Nonspecific amplification.
** Very less amplification product.

In conclusion, three polymorphic microsatellite loci in *B. arundinacea* have been identified and characterized for the first time in bamboo. These will help in population genetics study and genetic diversity study in the clonally propagated *B. arundinacea,* and other bamboos.

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Anderson J. A., Churchilll G. A., Austrique J. E., Tanskley S. D., and Sorells M. E. (1992), Optimizing parental selection for genetic linkage map. Genome **36**, 181–186.

Doyle J. J. and Doyle J. L. (1990), Isolation of plant DNA from fresh tissue. Focus **12**, 13–15.

Eustoup A., Taillieez C., Cornuet J. M., and Solignac M. (1995), Size homoplasy and mutational processes of interrupted microsatellites in two bee species, *Apies mellifera* and *Bombus terrestris* (Apidae). Mol. Biol. Evol. **12**, 1074–1084.

Hartl D. L. and Clark A. G. (1989), Principles of Population Genetics, 2nd Ed. Sinauer Association, California, MA, USA.

Jakupiak J. P. and Wells R. D. (1999), Genetic instabilities in (CTG.CAG) repeats occur by recombination. J. Biol. Chem. **274**, 23468–23479.

Kim N. S., Armstrong K. C., Fedak G., Ho K., and Park N. I. (2002), A microsatellite sequence from the rice blast fungus (*Magnaporthe grisea*) distinguishes between the centromeres of *Hordeum vulgare* and *H. bulbosum* in hybrid plants. Genome **4**, 165–174.

Liu D. C., Gao M. Q., Guan R. X., Li R. Z., Cao S. H., Guo X. L., and Zhang A. M. (2002), Mapping quantitative trait loci for plant height in wheat (*Triticum aestivum* L.) using a F2:3 population. Yi Chuan Xue Bao. **29**, 706–711.

Morgante M., Hanafey M., and Powell W. (2002), Microsatellites are preferentially associated with non-repetitive DNA in plant genomes. Nature Genet. **30**, 194–200.

Nei M. (1987), Molecular Evolutionary Genetics. Columbia University Press, New York.

Richards R. I. and Sutherland G. R. (1994), Simple repeat DNA is not replicated simply. Nature Genet. **6**, 114–116.

Riesner D. G., Steger R. Z., Owens R. A., Wagenhofer M., Hillen W., Vollbach S., and Henko K. (1989), Temperature-gradient gel electrophoresis of nucleic acids: analysis of conformational transition, sequence variations and protein nucleic acid interactions. Electrophoresis **10**, 377–389.

Sambrook J., Fritsch E. F., and Maniatis T. (1989), Molecular Cloning: a Laboratory Manual, 2nd Ed. Cold Spring Harbor Laboratory Press, New York.

Schug M. D., Mackay T. F. C., and Aquadro C. F. (1997), Low mutation rates of microsatellite loci in *Drosophila melanogaster*. Nature Genet. **15**, 99–102.

Sharopova N., McMullen M. D., Schultz L., Schroeder S., Sanchez-Villeda H., Gardiner J., Bergstorm D., Houchins K., Melia Hancock S., Musket T., Duru N., Polacco M., Edwards K., Ruff T., Register J. C., Brouwer C., Thompson R., Velasco R., Chin E., Lee M., Woodman-Clikeman W., Long M. J., Liscum E., Cone K., Davis G., and Coe E. H. Jr. (2002), Development and mapping of SSR markers for maize. Plant Mol. Biol. **48**, 463–481.

Tautz D. and Schlötterer C. (1994), Simple sequences. Curr. Opinion Genet. and Dev. **4**, 832–837.

Wright S. (1978), Variability within and among Natural Populations, Vol. 4. The University of Chicago Press, Chicago.

Xu X., Peng M., Fang Z., and Xu X. (2002), The direction of microsatellite mutation is dependent upon repeat length. Nature Genet. **24**, 396–399.

Zane L., Bargelloni L., and Patarnello T. (2002), Strategies for microsatellite isolation – a review. Mol. Ecol. **11**, 1–16.

Zhu Y., Queller D. C., and Strassman J. E. (2000), A phylogenetic perspective on sequence evolution in microsatellite loci. J. Mol. Evol. **50**, 324–338.