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Full Length Research Paper

Isolation and characterization of micro satellites in *Bambusa arundinacea* and cross species amplification in other bamboos

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Isolation and characterization of microsatellites was analyzed in *Bambusa arundinacea* and cross species amplification in other bamboos. Six microsatellites, three polymorphic and three monomorphic, were characterized in a bamboo species, *Bambusa aruninacea* belonging to the family Poaceae. The numbers of alleles per locus ranges form 2 to 6. Allelic diversity ranges from 0.128 to 0.789. Polymorphic Information Content (PIC) values for two loci were > 0.3, as 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. Utilization of the SSR loci in genetic diversity study of *B. arundinacea* and other cross amplified bamboo species is discussed.

Key words: *Bambusa arundinacea*, microsatellite marker, cross species amplification, population study, species identification.

INTRODUCTION

Bamboos, a group of arborescent grasses, have closely been associated with mankind since ancient time. They are used for a variety of purposes such as mat making, traditional instruments, furniture, musical instruments, flooring and construction materials, paper making, fencing, fodder, fuel wood, cooking utensils, floats for timber and rafts, sericulture industry, and waste water management. They belong to family Poaceae subfamily Bambusoideae. They have one of the widest habitats with more than 1500 species thriving in every continent but the poles. They are found mostly in tropical and subtropical regions. These are just grasses but vary in height from dwarf, one foot (30 cm) plant to giant timbers that can grow to 100 ft (40 meters). Bamboo flowers irregularly and usually at very long time interval 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/). Genetic/genotypic diversity may be more in the natural bamboo growing region due to occasional flowering. Out of the different type of DNA marker systems available for genetic diversity study microsatellites are proved to be the best one. Microsatellites also known as simple sequence repeats (SSR), are short tandemly repeated sequence motifs consisting of a repeat unit of 1 - 6 bp in length (Tautz and Schlötterer, 1994). They are highly polymorphic DNA markers with discrete loci and co-dominant alleles. Microsatellites are currently the marker of choice for genome mapping (Brondani et al., 2002; Sharopova et al., 2002), population genetic analysis, (Mariette et al., 2002, Dutech et al., 2002), genetic diversity study (Kisuki and Isagi, 2002), cultivar and variety identification (Cantini et al., 2001; Faria et al., 2000), and evolutionary genetics because of their ubiquitous and uniform distribution within both eukaryotic and prokaryotic (Field and Wills, 1996). Microsatellites

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are also characterized with high information content, ease of genotyping through PCR, high discrimination power and high degree of allelic variability which is presumably caused by a high mutation rate and replication strand slippage that changes microsatellite array length.

Microsatellites have been used in a number of plant species for construction of genetic linkage map (Brondani et al., 2001; Sharopova et al., 2002), quantitative trait loci mapping (Liu et al., 2002), and gene tagging (Kim et al., 2002, Xie et al., 2001). 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., 2001). Microsatellites have been identified in a number of economically important pant species such as maize, sunflower, avena, eucalyptus, barley, grape, rice, and chestnut (Sharopova et al., 2002; Paniego, 2002; Li et al., 2000; Glaubitz et al., 2001; Ramsay et al., 2000; Lefort et al., 2002; McCouch, 1997; Marinoni et al., 2003). In clonally propagated crops, microsatellites have been used for cultivar identification, population genetic structure and genetic diversity study. Here, we report the isolation and characterization of microsatellites in *Bambusa* species.

MATERIALS AND METHODS

Plant materials

Leaf samples of *Bambusa arundinacea* Willd were collected from 15 different clumps arbitrarily from Regional Plant Resource Center, Bhubaneswar forest area. Care was taken to collect samples from the clumps with at least 100 meters away from the nearby clump. Leaf samples of other bamboos including *Dendrocalamus giganteus* Munro, *D. strictus* Nees, *Dinochlea mclellandii* Kurtz, *Cephalostachyum pergracil* Munro, *Bambusa vulgaris* Schr, *B. vulgaris* var *striata* Schr, *B. nana* Roxb., *B. multiplex* Raeush, *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 other bamboo species such as *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* were collected from Sikkim, one of the treasure house 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 modification, as the leaves of bamboos contain silica materials. 2.5 g of leaf material was surface sterilized by wiping with 80% ethanol, then cut into small pieces and ground into fine powder using liquid nitrogen. 10 ml of preheated extraction buffer [100 mM Tris-HCl (pH 8.0), 2 mM EDTA, 3% CTAB (w/v), 0.2% β-mercaptoethanol (v/v), 1.4 M NaCl] was added to the powder 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 µl of TE (10 mM Tris pH 8.0, 1 mM EDTA). DNA quantification was made by visualizing under UV light against a λ DNA uncut marker after electrophoresis on 0.8% (w/v) agarose gel containing 0.5 µg ml^{-1} of ethidium bromide.

Library construction

20 g of extracted DNA (only from one individual) was completely digested with *Mbo*I (GIBCO BRL, USA). The digested DNA was concentrated (ethanol precipitation), dissolved in 30 µl of TE and electrophoresed on a 1% low melting agarose gel (GIBCO BRL, USA) along with molecular weight markers (MBI fermentas, USA). DNA fragments corresponding to 350 to 550 bp were excised from the gel and purified with QIAquick gel extraction kit (QIAGEN). Ligation reaction was performed with 50 ng of gel purified insert DNA and 100 ng of dephosphorylated pUC19/*Bam*HI (Bangalore Genei, India) plasmid DNA (vector: insert =1:4) and 1 µl of T4 DNA ligase (GIBCO BRL, USA) in a total volume of 20 µl at 16°C for overnight. Ligated products were transformed into DH5α competent cells and transformants plated on 82 mm LB/ampicilin plates with X-gal and IPTG for blue white screening.

Screening for microsatellite repeat sequences

All the white colonies were transferred onto 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) 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/0.5N NaOH (Fresh) for 5-10 min; (c) 1.5 M NaCl/0.5MTris (pH 7.8) for 5-10 min; (d) 2x SSC for 1 minute. Crosslinking of the DNA on nylon membrane was done in a UV cross linker (Hoefer, Amersham Pharmacia). The probe, (GT) 15, used to screen the library, was 3'end labeled with the help of DIG oligonucleotide tailing kit (Roche) following manufacturer's instruction. Oligonucleotide was enzymatically labeled at their 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 appropriate amount of pre-hybridization buffer (5X SSC, 0.5% SDS, 0.1 mg/ml, and 0.1% lactogen) in polythene bags for 1 h. Hybrididization was done at 68°C overnight in the same prehybridization solution with labeled probe. Positive clones were detected colorimetrically, inoculated in 2 ml of BL/amp and grown overnight. Next day 1 ml of the above culture was stored in -80°C with 15% glycerol for further use and plasmid DNA was isolated from rest 1 ml of culture (Sambrook et al., 1989). The above positive clones were reconfirmed through dot blot.

SSR polymorphism

Positive clones were sequenced by an automated sequencing facility. Oligo primers were designed from the flanking regions of identified repeats using primer select module of DNASTAR software and synthesized by MWG-Biotech AG (Germany). For PCR genotyping of microsatellites, amplification was carried out in a final volume of 25 µl containing 10 mM Tris-HCl, pH 9.0; 50 mM KCl; 0.01% gelatin; 1.5 mM $MgCl_2$; 5 pmole of each primer; 200µM dNTPs; 0.25 units of *Taq* DNA polymerase (Bangalore Genei, India); and 20 ng genomic DNA. Amplifications were performed in a PTC 100 thermocycler (MJ research, 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 1) 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

Table 1. 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 temp. Tm	Amplification status
Ba10	(GT)12C(TA)10	AJ507486	F-GGTGGGGTCTAGCACCTAAG R-TACCAGCATGTAACGGTCGG	146	60°C	Amplified
Ba14	(CA)10	AJ507487	F-AGAGTTAAGGAAGCCAGGTC R-GTCTAGTAGCTGCTCAACTC	237	57°C	Amplified
Ba18a	(CT)5n(CT)5	AJ507488	F-TATCTCGACCTCCCCTTGCT R-GTCTAGAAGCAGGGAGGTAG	166	59°C	Amplified
Ba18b	(CA)13	AJ507488	F-CCAGGTCGTTTCACTGCTC R-ACAACGGTAGAGTTCACTCG	146	57°C	Amplified
Ba20	(AC)31(AT)7	AJ507489	F-TTGATTGCCCTACTCTGTCG R-TCAACGGTGGATGACCTAGG	169	57°C	Amplified
Ba25	(GACA)3(GATA)2 GAT(GATA)1	AJ507490	F-GTGAGATGGGCTGGGCAG R-GCTCCGATCTGTCAGTTTAC	242	57°C	Not amplified
Ba58	(CA)7	AJ507491	F-TCCGAAGCACACTCATGAAG R-TTCTACTATGCGCTAACTGC	187	55°C	Amplified
Ba202	(AC)3C(AC)2GC(AC)4	AJ507492	F-CAACTAGCAAACGCACAGTG R-CGAATTCGAGCTCGGTACC	261	57°C	Not amplified

plus), mixed with 10 µl of formamide loading dye, heat denatured and then separated on 8% denaturing polyacrylamide gel with 7 M urea and 0.5X TBE. Separation was performed on vertical gel electrophoresis (Hoofer SE 600) at a constant current of 20 watt for about 2 h. 100 bp ladder of MBI Fermentas 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 NaBH4 and 0.125% formaldehyde (37%, w/v).

Data analysis

For each locus, genotpypes, total number of allele, allele frequencies and observed heterozygosity (ho) were determined from the gel. Expected heterozygosity (he), the proportion of expected heterozygotes under random mating condition, was calculated according to Nei (1987). Inbreeding coefficient (fis), a measure of heterozygosity deficit or excess, for a particular locus was calculated according to Wright (1978). Effective number of allele (AE) for a particular locus was calculated according to Hartle and Clark (1989). A polymorphic information content (PIC) value of a locus was calculated according to Anderson et al. (1992).

RESULTS

Screening of four thousand recombinant colonies of the partial genomic library with (CA) 15 oligo resulted in 15 positive clones. Second 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 1). The use of dephosphorylated pUC vectors helped in getting 100% positive white colonies in first few LB/amp plates with X-gal and IPTG. This eliminated the use of X-gal and IPTG for blue white screening of recombinants in the subsequent LB/amp plates. 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 first round of screening was less (46%) than the second round of screening (64%) in case of *B.arundinacea*. About half of the loci amplified found to be monomorphic and rest were polymorphic. Half of the amplified loci (3) found to be polymorphic and rest three were monomorphic in a panel of 15 tested individuals. The number of allele for all the polymorphic loci ranged from 2 - 6 with an average of 4.6. Loci B10 and B20 both have 6 alleles each and locus B18a have only 2 alleles. Observed heterozygosity, expected heterozygosity, fixation index and PIC values for all the polymorphic loci were presented in Table 2. PIC value was highest (0.7627) for locus Ba20 and lowest (0.1244) for Ba18a. Loci containing longer repeat units (< 20) were found to be more polymorphic than locus containing shorter repeat unit (Ba18a). Cross species amplification of other bamboo species by taking primers of *B. arundinacea* microsatellite loci were presented in Table 3. All the polymorphic/functional microsatellite loci amplified in

Table 2. Number of allele, observed (ho) and expected (he) heterozygosity, effective number of allele (AE), fixation index (fis) and polymorphic information content (PIC) of the three polymorphic microsatellite loci of *B. arundinacea*.

Locus name	Sample size (n)	No. of allele	ho	He	fis	AE	PIC
Ba10	14	6	0.357	0.736	0.515	3.787	0.709
Ba20	15	6	0.200	0.789	0.746	4.739	0.762
Ba18a	15	2	0.000	0.128	1.000	1.4775	0.124

Table 3. Cross species amplification *B. arundinacea* microsatellite loci in different bamboo species.

Name of the bamboo species	Ba10	Ba14	Ba18a	Ba18b	Ba20	Ba58
A. mannii Gamble	-	-	-	-	-	+**
B. balcooa Roxb	-	+	+	+	-	+
B. nana Roxb	-	+	+	+	+	+*
B. multiplex Raeush	-	+	+	+	+	+*
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.

some of the bamboo species. Locus Ba10 amplified in four other bamboo species such as *B. clarata*, *B. vulgaris*, *B. vulgaris* var striata and *B. ventricosa*. Locus Ba20 was amplified in *D. strictus*, a species belongs to genus *Dendrocalamus*. Nonspecific amplification of Ba20 was observed in *B. nana* and *B. multiplex*. All the three momomrphic microsatellites were amplified in almost all the tested species.

DISCUSSION

The primary objective of this study was to isolate and characterize of microsatellites in in *B. arundinacea* and cross species amplification in other bamboos. Screening about four thousand recombinant colonies of the partial genomic library with (CA) 15 oligo resulted in 15 positive clones. Second 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. The chance of getting a microsatellite repeat motif after first round of screening was less (46%) than that of second round of screening (64%) in the case of *B.arundinacea*. This might be true for all other bamboos. About half of the loci amplified found to be monomorphic and rest were polymorphic. This might be due to the nature of the plant itself. Being highly clonally propagated and rarely sexually propagated (self/wind pollinated) plant, there is less chance of replication slippage (Schlötterer and

Tautz, 1992; Richards and Sutherland, 1994), a common methods for formation of alleles in microsatellite loci. Further unequal crossing over during homologous recombination in the site of microsatellite, other method of formation of allele in microsatellite loci (Jakupiak and Wells, 1999), may not be happening due to very long and erratic flowering period. 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 such as Ba10 and Ba20 were found to be more polymorphic than locus containing shorter repeat unit (Ba18a). This might be due to the rate of microsatellite mutation which is directly proportional to the repeat length (Goldstein and Clark 1995; Schug et al., 1998), there by forming more alleles in a longer repeat motif. Furthermore, the rate of contraction mutations increase exponentially over repeat length and that of expansion mutations constant for all shorter and longer alleles (Xin et al., 2002). Cross species amplification of different bamboo species occured in few species. Locus Ba10 amplified in four other bamboo species (*B. clarata*, *B.vulgaris* , *B. vulgaris* var striata and *B. ventricosa*). Locus Ba20 was amplified in *D. strictus*, a species belongs to genus *Dendrocalamus*. Nonspecific amplification of Ba20 was observed in *B. nana* and *B. multiplex*. So, these loci may be useful for population genetic studies in the cross amplified species. However, inter specific differences in the repeat motif may be varied and influence the level of variability in the cross amplified taxon (Eustop et al., 1995).

All the three monomorphic microsatellites were 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. Cordeiro et al. (2001) observed that the *Saccharum* EST (Expressed Sequence Tag) - SSRs could cross amplify readily in related genera such as *Erianthu*s and *Sorghum sp* but not the *Saccharum* genomic region SSRs. Frequency of microsatellites in plants is higher in the transcribed region especially in the untranslated region (Morganate et al., 2002) than in the repetitive fraction (Ramsay et al., 1999). 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 (Morganate 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 phospatase receptor type K.

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

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