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ORIGINAL PAPER

Evaluation of genetic fidelity of in vitro raised plants of *Dendrocalamus asper* (Schult. & Schult. F.) Backer ex K. Heyne using DNA-based markers

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Abstract *Dendrocalamus asper*, an edible bamboo is valued for its tender edible shoots in the food industry. However, overexploitation of natural stands of *D. asper* coupled with minimal conservation and reforestation efforts has led to its rapid depletion in nature. Therefore protocol for rapid multiplication of *D. asper* via direct regeneration using nodal segments from mature clumps was standardized and more than 25,000 plants were transferred to the field (Singh et al. 2012a). However, genetic fidelity of these in vitro raised plants needs to be authenticated for commercial scale application of the developed micropropagation protocol. PCR-based molecular markers have emerged as simple, fast, reliable and labor-effective tools for testing the genetic fidelity of in vitro raised plants. This study report the genetic fidelity analysis of in vitro raised plants of *D. asper* for the first time using arbitrary (Random Amplified Polymorphic DNA, RAPD), semi-arbitrary (Inter-Simple Sequence Repeat, ISSR; Amplified Fragment Length Polymorphism, AFLP), and sequence-based (Simple Sequence Repeat, SSR) markers. Bulked DNA samples of 20 in vitro raised shoots (collected after every three subculture cycles starting from 3rd to 30th passage) and field transferred plantlets were compared with the mother plant DNA using 90 primer combinations (25 each of RAPD, ISSR, SSR, and 15 AFLP) and scorable bands were produced by 78 (22 RAPD, 24 ISSR, 21 SSR, and 11 AFLP) primers. A total of 146 distinct and scorable bands were produced by 22 RAPD primers with an average of 6.6 bands per primer while the number of bands for ISSR primers varied from 3 (ISSR-4 and 9) to 13 (ISSR-17), with an average of 7.1 bands per primer. Similarly, SSR markers also showed wide variation in number of bands, ranging from 2 (RM 261) to 12 (RM 44, 140, and 224) with an average of 7.8 bands. AFLP primer combinations could generate 35–72 bands with an average of 48.7 bands per primer pair. Amplification of monomorphic bands with all primer combinations authenticated the true to type nature of the in vitro raised plants of *D. asper* which underwent up to 30 subculture passages over a period of approximately 2 years thereby supporting the commercial utilization of the developed micropropagation protocol.

Keywords Bamboo · Genetic fidelity testing · In vitro cultures · Micropropagation · Molecular markers · Nodal segments

Abbreviations

AdS	Adenine sulfate
AFLP	Amplified fragment length polymorphism
BAP	6-Benzyl-amino-purine

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CTAB	Cetyl trimethyl ammonium bromide
dNTPs	Deoxyribonucleotide triphosphates
FYM	Farm yard manure
IBA	Indole-3-butyric acid
ISSR	Inter-simple sequence repeat
MS	Murashige and Skoog's medium
NAA	α-Naphthalene acetic acid
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
SSR	Simple sequence repeat

Introduction

It is desirable to multiply only those trees and woody shrubs which have proved their potential in the field. However, vegetative propagation methods in these species are usually cumbersome, season-dependent, labor, and cost-intensive. Also, the regeneration potential of vegetative propagules declines with increase in age of the mother tree. Therefore, in vitro propagation has emerged as a powerful technique for large scale propagation of forest trees and other woody plants including bamboos with long flowering cycles of up to 120 years. However, culture method and environment, explant source, ploidy level and in vitro culture age are the primary factors controlling induction of somaclonal variation in vitro (Rani and Raina 2000). High concentrations of growth regulators usually administered in culture media for enhancing the rate of shoot multiplication has been reported to induce somaclonal variations in the micropropagated plantlets (Venkatachalam et al. 2007). Somaclonal variations may appear due to cell cycle disturbances caused by exogenously applied growth regulators (Peschke and Phillips 1992), increased mutation rate per cell-generation over time, and accumulation of mutations over a period of time (Rodrigues et al. 1998), alteration in DNA methylation patterns, DNA damage and mutation (Phillips et al. 1994), alteration of cell's ability to repair damaged and mutated DNA (Leroy et al. 2000). It is therefore extremely important to ascertain the suitability of a particular micropropagation protocol developed for a particular species, where commercial success in micropropagation depends solely on the maintenance of clonal uniformity (Heinz and Schmidt 1995). Dunstan and Thorpe (1986) rightly suggested that commercial application of tissue culture to perennial crops must await adequate quality checks and field testing with proper controls. Therefore, it is important to devise methods of quality control at all the tissue culture stages to minimize the potential danger of such defects at later stages.

Attempts have been made in the past to judge the genetic fidelity of in vitro raised plants including bamboos using morphological and physiological traits (Agnihotri et al. 2009; Singh et al. 2012a, 2012b), however, these morphological traits require extensive observations until maturity. Further, the morphological and physiological differences may disappear over the growing seasons or the initially uniform looking plants may behave differently during flowering/fruiting stages due to genetic aberrations. Also, some changes induced during in vitro culture may not be apparent under ex vitro conditions (Palombi and Damiano 2002). Therefore, more efficient detection tools like DNA markers must be used to ascertain the genetic fidelity of in vitro raised plants as these markers are not influenced by the age or tissue of the plant, growth stage or the prevailing environmental conditions. Many PCR-, hybridization-, and sequence-based marker systems have come into existence (Kalia et al. 2011) which have specific advantages and limitations. Therefore, selection of a marker system and the technique used constitute two most important decisions in the experimental design (McGregor et al. 2000). Some primers target the genome randomly (RAPD, ISSR) while the sequence-based primers (SSR, RFLP) target specific regions of the genome. Many techniques combining the properties of two or more techniques are also available now (AFLP; PCR–RFLP). Combination of two or more primer types must be used for genetic fidelity testing of plants so as to target a larger part of the genome under study.

D. asper (Schult. & Schult. F.) Backer ex K. Heyne (Poaceae, Bambusoideae) native to China and commonly found in Thailand, Vietnam, Malaysia, Indonesia, and Philippines, is valued for its edible shoots. Conventionally *D. asper*, like other bamboos, is propagated by seeds, offsets, and culm cuttings which are beset with many problems (Singh et al. 2012a). For production of quality planting material at an accelerated pace within a short period of time, in vitro mass multiplication of *D. asper* was successfully attempted by Arya and Arya (1997), Arya et al. (1999, 2002, 2008), Ojha et al. (2009), Banerjee et al. (2011), and Singh et al. (2012a). However, no report authenticated the genetic fidelity of the in vitro raised plants of *D. asper* except Singh et al. (2012a) wherein morphological parameters were recorded for in vitro raised plants after field transfer. Although, some reports are available regarding assessment of genetic fidelity of other tissue culture raised bamboo species (Das and Pal 2005; Agnihotri et al. 2009; Negi and Saxena 2010, 2011; Mehta et al. 2011; Nadha et al. 2011), no report till date is available for fidelity testing of *D. asper* using molecular

markers. Therefore, the present study was undertaken to assess the probability of induction of somaclonal variation in *D. asper* cultures, maintained and propagated in vitro for over 2 years via enhanced axillary branching, using a combination of arbitrary (RAPD), semi-arbitrary (ISSR, AFLP), and sequence-based (SSR) markers.

Materials and methods

Plant material and culture conditions

The plant material consisted of in vitro raised shoots maintained in multiplication medium and in vitro raised

Table 1 Composition of media used at various stages of micropropagation of *D. asper* through axillary bud proliferation from nodal explants

Phases of micropropagation	In vitro and ex vitro media	Incubation period (weeks)	Remarks
Axillary bud proliferation	MS + 15 μM BAP	2–3	94–96 % buds break with 4–6 shoots of 1–1.5 cm length
Shoot multiplication	MS + 10 μM BAP + 75 μM AdS + 3 % table sugar	2–3	~4.0 fold shoot (2.5–3.5 cm long) multiplication rate was obtained by using a propagule of 8–10 shoots per subculture
Rooting of shoots	1/2x MS + 5 μM IBA + 5 μM NAA	2–3	100 % rooting with 10–16 roots (1.0–3.5 cm long) per clump of 3–4 shoots
Hardening and acclimatization of plantlets	Dune sand and vermi-compost (3:1); irrigated with 1/4x MS liquid medium without sucrose	4–6	90–95 % survival was obtained with good development of the plantlets

Data from Singh et al. (2012a)

Table 2 PCR conditions for different molecular markers assayed

A

Molecular marker	DNA	$MgCl_2$	Taq buffer	dNTPs	Primer	Taq polymerase	Total volume
RAPD	50 ng	1.0 mM	1x	0.2 mM	0.4 μM	0.6 U	25 μl
ISSR	70 ng	1.0 mM	1x	0.2 mM	0.4 μM	0.6 U	25 μl
SSR	140 ng	1.5 mM	1x	0.2 mM	0.2/0.2 μM	0.9 U	25 μl
AFLP							
Pre-selective amplification	5 μl R/L	1.6 mM	1.2x	–	20.0 μl	5.0 U	32 μl
Selective amplification	2.5 μl Preamp	0.62 mM	1x	0.1 mM	0.5 μM[a]/0.6 μM	0.3 U	10 μl

B

Molecular marker	PCR programme	Detection system
RAPD	5 min 94 °C, [1 min 94 °C/1 min 36–38 °C/1 min 72 °C] × 45 cycles, 7 min 72 °C	2 % agarose gel
ISSR	5 min 94 °C, [1 min 94 °C/1 min 45–53 °C/1 min 72 °C] × 38 cycles, 7 min 72 °C	2 % agarose gel
SSR	5 min 94 °C, [1 min 94 °C/1 min 50–66 °C/1 min 72 °C] × 38 cycles, 7 min 72 °C	4 % agarose gel
AFLP		
Pre-selective amplification	[30 s at 90 °C/60 s at 56 °C/60 s at 72 °C] × 20 cycles	1 % agarose gel
Selective amplification	[30 s at 94 °C/30 s at 65 °C/60 s at 72 °C] × 1 cycle; [30 s at 94 °C/30 s at 65 °C to 56 °C/60 s at 72 °C][b] × 11 cycles; [30 s at 94 °C/30 s at 56 °C/60 s at 72 °C] × 23 cycles	8 % polyacrylamide gel

A PCR components. *B* PCR programmes used for each marker system

R/L restricted/ligated DNA

[a] Concentration of radio-isotope labeled *EcoR*I primer

[b] Touch down of 1 °C per cycle

plants transferred to the field after rooting, hardening, and acclimatization. These plants were regenerated clones obtained via enhanced axillary branching of nodal explants collected from mature clumps of *D. asper* (Singh et al. 2012a). The micropropagation procedure consisted of culturing the nodal explants on BAP supplemented medium, transfer of shoot clumps to multiplication medium, rooting of shoots on auxin supplemented media, and finally hardening and acclimatization of in vitro raised plants in the green house before field transfer. Details of media used at various steps, subculture duration, etc., are summarized in Table 1.

To determine the effect of number of subculture cycles/in vitro culture age on genetic fidelity, the shoot cultures were maintained continuously on multiplication medium for approximately 2 years (30 passages) and were transferred to fresh medium every 25 days. Shoots were randomly collected from 20 culture bottles after every three subcultures starting from 3rd to 30th subculture passage. Leaf samples of 20 randomly selected field transferred plants were bulked together for analysis, and three such bulks were used during the study—(1) plants at greenhouse/polyhouse stage, (2) plants after 1 year of field transfer, and (3) plants after 2 years of field transfer.

DNA extraction and quantification

Total genomic DNA of the mother plant, in vitro raised shoots and plants was extracted using the modified CTAB method as described by Lodhi et al. (1994). The yield and purity of isolated DNA was determined spectrophotometrically at the wavelength of 230 nm for the carbohydrates or polyphenols, 260 nm for the DNA and 280 nm for proteins, using Nanophotometer (NanoDrop ND 1,000 Spectrophotometer, NanoDrop Technologies, Wilmington, DE).

PCR amplification conditions

Four sets of primers including arbitrary (RAPD), semi-arbitrary (ISSR, AFLP), and sequence-based (SSR) markers were used in the present study to target a wider coverage of the genome. Twenty-five primers each of RAPD (Integrated DNA Technologies Inc.), ISSR and SSR (Life-Technologies, India Pvt. Ltd.) and 15 primer pair combinations of AFLP (Sigma-Aldrich) were used for genetic fidelity testing. Twenty-five microsatellite sequences which were developed to prepare the genetic map of rice (Gramene Cornell microsatellite RM markers) by Marulanda et al. (2007) were used for genetic fidelity analysis of in vitro raised plants of *D. asper*. DNA amplification was performed in an Eppendorf thermocycler (Mastercycler proS). Details of PCR mix and PCR thermal cycler conditions are provided in Table 2A, B, respectively. Concentration of various PCR components was standardized through experimentation. The agarose (Life Technologies, India) gels with 1x TAE buffer were stained with Nucleic Acid Stain (6 µl/100 ml gel; Life Technologies, India); run under a steady voltage of 100 V for 100 min and then photographed under gel documentation system (Alphaimager EP, Alphainnotech, California, USA). In all agarose gels GeneRuler 100 bp plus DNA ladder (Fermentas International Inc.) was used as size marker.

AFLP was performed according to the original protocol of Vos et al. (1995) with 15 primer pair combinations, in order to investigate the possibility of DNA changes more globally. Genomic DNA (400 ng) was subjected to enzymatic digestion with two restriction enzymes *Eco*RI and *Mse*I (2.5 U) in their corresponding restriction buffers in a final volume of 25 µl at 37 °C and then the restricted fragments were ligated with *Eco*RI and *Mse*I specific adapters. Restriction digestion and adaptor ligation was performed with AFLP core reagent kit (Invitrogen life technologies, California) as per the manufacturer's instructions. Ligation mixture (25 µl) was added to the restriction reaction and incubated for 2 h at 37 °C. Adapter-ligated DNA was diluted 10-fold and pre-amplified using *Eco*RI and *Mse*I primers having a single 3′ selective nucleotide, followed by electrophoretic verification. The pre-amplified DNA was diluted 50-folds and used as template for selective amplification using *Eco*RI and *Mse*I primers, each with three selective bases at the 3′ end. For detection, *Eco*RI primer was end labeled with γ-^{32}P-ATP (BRIT, Mumbai, India). The cycling parameters utilized for selective amplification are summarized in Table 2B. The AFLP reaction was terminated by adding equal volume of 98 % formamide dye. The amplified products were visualized in autoradiograms (Sambrook et al. 1989).

Data scoring and analysis

All reactions were performed at least twice, including internal controls (replicates of the same sample in each combination, and different DNA extractions of the same bulk) to ensure the consistency of results. Only consistently produced and well-resolved fragments obtained through amplification by RAPD, ISSR, SSR, and AFLP markers were considered and scored manually. The scoring of bands was done on the basis of their presence ('1') or absence ('0') in the gel and missing data was denoted by '9'. The genetic associations were evaluated by calculating the Jaccard's similarity coefficient for pair-wise comparisons based on the proportion of shared bands produced by the primers using NTSYS-pc version 2.1 software (Rohlf 2000).

Fig. 1 Micropropagation of *Dendrocalamus asper* through axillary bud proliferation, using nodal segments. **a** Axillary bud proliferation from nodal segments on MS + 15 μM BAP after 3 weeks of culture; **b** shoots multiplication on MS + 10 μM BAP + 75 μM AdS; **c** multiplied shoots before rooting on MS + 10 μM BAP + 75 μM AdS; **d** rooted plantlets; **e** acclimatized and hardened plantlets; **f**, **g** field transferred plant after 6 months and 3 years of plantation, respectively

Results and discussion

Micropropagation protocol for *D. asper* was standardized in our earlier report (Singh et al. 2012a) and was used for mass scale production of plants through axillary branching in vitro. More than 25,000 plants were successfully transferred to the natural field conditions. Neither morphological aberrations nor decrease in multiple-shoot forming capacity were observed even after long-term culturing for more than 2 years. However, Lakshmanan et al. (2007) documented morphological variation (e.g., hyperhydricity) in banana due to use of high levels of cytokinins especially benzyladenine combined with continuous availability of high levels of nutrients. The regenerated shoots of *D. asper* were rooted and transferred to the field as and when required (Fig. 1). The response of all the plants to acclimatization and hardening obtained from initial cultures as well as from long-term sustained cultures was found equal. The plants transferred to the field at successive subculture cycles up to 30 passages did not show any morphological variations. These results indicated true to type nature of the regenerated plants, however, the possibility of genetic variations arising during in vitro process cannot be ruled out because tissue culture techniques are known to induce somaclonal variations in regenerated or micropropagated plants due to stress induced by high concentration of growth regulators and fast rate of subculturing. Therefore, testing of clonal fidelity or true to type nature of regenerants remains one of the most important prerequisites (Lakshmanan et al. 2007) especially in trees and woody shrubs having long rotation cycles. DNA-based molecular markers have emerged as a powerful technique for the purpose and therefore are being used in many crops and trees (Cuesta et al. 2010; Negi and Saxena 2011; Pandey et al. 2012).

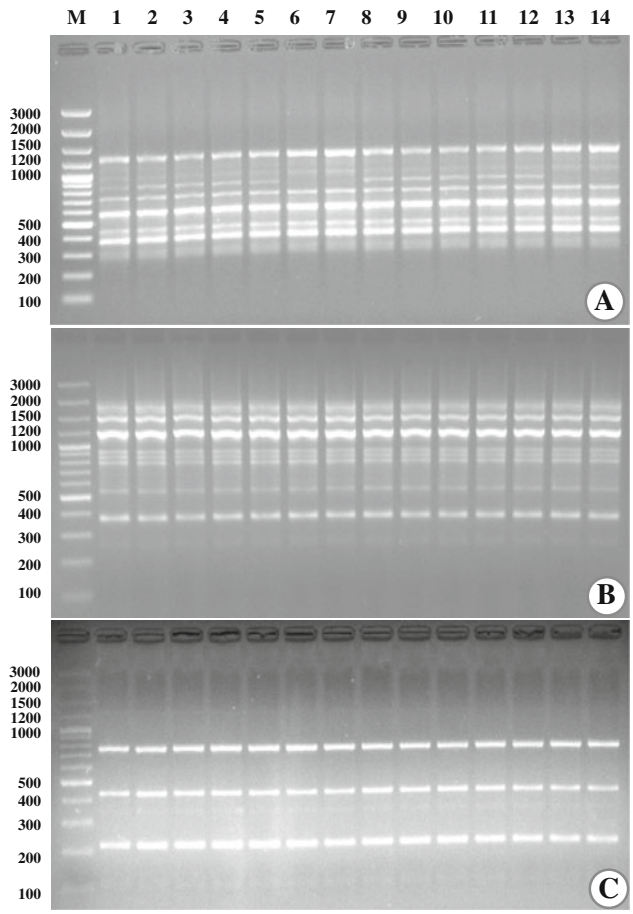

Fig. 2 DNA amplification obtained with primer: **a** RAPD (OPO-10), **b** ISSR (ISSR-14), and **c** SSR (RM-13). *Lanes M*-100 bp plus DNA ladder, *1* mother plant, *2–11* in vitro cultures from 3rd to 30th passage after every 3rd subculture cycle, *12* plant at greenhouse/polyhouse stage, *13* plant at field stage after 1 year and *14* plant at field stage after 2 years

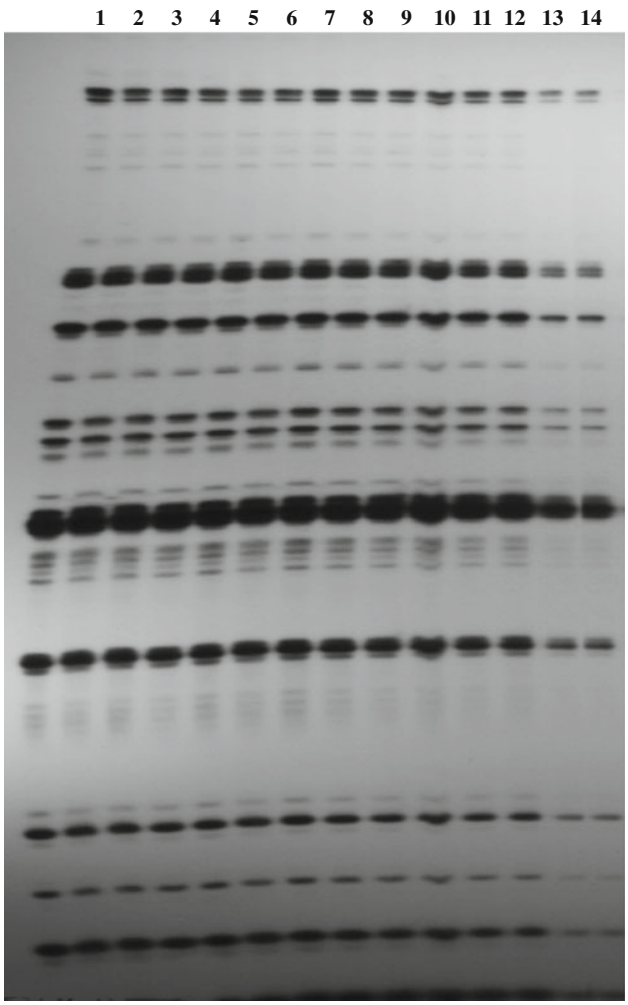

Fig. 3 AFLP fingerprint generated with primer combination E-AAG × M-CAC. *Lanes 1* mother plant, *2–11* in vitro cultures from 3rd to 30th passage after every 3rd subculture cycle, *12* plants at greenhouse/polyhouse stage, *13* plants after 1 year of field transfer and *14* after 2 years of field transfer

Use of more than one marker system has been suggested for better analysis of genetic stability of plants, as they will target different regions of the genome (Palombi and Damiano 2002; Lakshmanan et al. 2007). Therefore, four sets of primers (RAPD, ISSR, SSR, and AFLP) were used in the present study. The fact that RAPD (arbitrary, dominant) and ISSR (semi-arbitrary, medium to highly reproducible, dominant and more stringent) markers are capable to scan the whole genome randomly and quickly, SSR (highly polymorphic, highly reproducible) markers being sequence-based can detect variation at pre-determined sites such as repetitive regions of the genome, and AFLP markers (with high marker index, highly reproducible) on the other hand can check large portions of the genome, validated the selection of marker systems in the present study. This vide array of marker systems ensured screening for variations in the whole genome. Cuesta et al. (2010) used four marker systems (RAPD, ISSR, AFLP, and SAMPL) for ascertaining the genetic fidelity of micropropagated *Pinus pinea* L. plants. The fingerprinting profiles of the in vitro shoot cultures, regenerated plantlets and the mother plant generated using the above markers are shown in Figs. 2 and 3 and their scoring data is summarized in Tables 3, 4, 5, 6, and 7.

Twenty-five random decamer oligonucleotides were used as single primers for the amplification of RAPD fragments of which 22 primers amplified a total of 146 scorable bands (Table 4). All the bands obtained were monomorphic (Fig. 2a). Out of 25 ISSR primers used for the genetic fidelity testing, 24 primers produced amplification with 170 scorable bands (Table 5). The number of bands for each primer varied from 3 to 13, with an average of 7.1 bands per ISSR primer. Figure 2b gives a representative example of monomorphic bands obtained with ISSR primers. More fragments were amplified by ISSR

Table 3 Summary of results obtained with various molecular markers assayed for evaluating the genetic fidelity of micropropagated plants of *Dendrocalamus asper*

Molecular marker	Number of primers used	Number of combinations	Amplified combinations	Total number of bands	Average number of bands
RAPD	25	25	22	146	6.6
ISSR	25	25	24	170	7.1
SSR	25	25	21	164	7.8
AFLP (Mse +3/Eco + 3)	11	15	11	536	48.7

Table 4 Random amplified polymorphic DNA (RAPD) primers used for testing the genetic fidelity of *Dendrocalamus asper* shoots and plants produced through tissue culture

Sr. no.	Primer code	Primer sequence (5′–3′)	Annealing temperature (°C)	Number of scorable/ monomorphic bands	Size range of amplification product (bp)
1	OPA-18	AGGTGACCGT	37	7/7	400–1,500
2	OPJ-02	CCCGTTGGGA	–	No amplification	–
3	OPJ-04	CCGAACACGG	37	10/10	400–3,000
4	OPJ-06	TCGTTCCGCA	37	8/8	300–1,200
5	OPJ-07	CCTCTCGACA	38	5/5	300–1,500
6	OPJ-09	TGAGCCTCAC	38	6/6	300–2,000
7	OPE-01	CCCAAGGTCC	38	7/7	600–2,000
8	OPE-02	GGTGCGGGAA	38	6/6	400–2,000
9	OPE-03	CCAGATGCAC	38	9/9	200–1,500
10	OPE-04	GTGACATGCC	38	7/7	400–2,000
11	OPE-05	TCAGGGAGGT	–	No amplification	–
12	OPE-06	AAGACCCCTC	38	6/6	600–2,000
13	OPE-07	AGATGCAGCC	–	No amplification	–
14	OPE-10	CACCAGGTGA	38	8/8	500–3,000
15	OPE-11	GAGTCTCAGG	37	6/6	500–2,000
16	OPE-16	GGTGACTGTG	38	5/5	600–1,500
17	OPO-1	GGCACGTAAG	38	6/6	500–3,000
18	OPO-04	AAGTCCGCTC	37	7/7	300–3,000
19	OPO-05	CCCAGTCACT	37	9/9	200–2,000
20	OPO-06	CCACGGGAAG	38	6/6	400–1,500
21	OPO-07	CAGCACTGAC	37	7/7	500–2,000
22	OPO-10	TCAGAGCGCC	37	9/9	300–1,500
23	OPO-11	GACAGGAGGT	38	11/11	300–2,000
24	OPM-11	GTCCACTGTG	38	6/6	600–3,000
25	OPF-02	GAGGATCCCT	36	6/6	600–2,000

primers compared to RAPD, which is contrary to the results of Cuesta et al. (2010) wherein ISSR markers amplified fewer bands compared to RAPD markers. Both RAPD and ISSR generated bands ranging in size from 200 to 3,000 bp, while Cuesta et al. (2010) reported a size range of 500–3,000 bp in *Pinus pinea* L. using RAPD and ISSR markers.

Amplification was obtained with 21 out of the 25 cross-species SSR markers used. A total of 164, all monomorphic, bands ranging in size from 100 to 2,000 bp were produced (Table 6; Fig. 2c). Genetic fidelity was also evaluated using 15 AFLP primer combinations generated from 5 *Eco*RI and 6 *Mse*I primers (Table 7). A total of 536 bands were generated with an average of 48.7 bands per primer combination and all the bands were monomorphic. A representative monomorphic AFLP fingerprint obtained with *E*-AAG × *M*-CAC is shown in Fig. 3. Various workers have already proved the suitability of SSR (Pandey et al. 2012) and AFLP markers (Mehta et al. 2011) for genetic fidelity studies.

Table 5 Inter-simple sequence repeat (ISSR) primers used for testing the genetic fidelity of *Dendrocalamus asper* shoots and plants produced through tissue culture

Sr. no.	Primer code	Primer sequence (5′–3′)	Annealing temperature (°C)	Number of scorable/ monomorphic bands	Size range of amplification product (bp)
1	ISSR-1	$(GAA)_6$	45.0	9/9	500–1,500
2	ISSR-2	GCAA + $(GACA)_3$	49.5	9/9	400–2,000
3	ISSR-3	GCTT + $(GACA)_3$	49.5	11/11	300–3,000
4	ISSR-4	$(GACA)_4$	49.5	3/3	800–1,500
5	ISSR-5	$(GGAT)_4$	47.5	6/6	600–2,000
6	ISSR-6	$(ACTG)_4$	47.5	8/8	800–3,000
7	ISSR-7	$(AGTC)_4$	52.5	4/4	700–1,500
8	ISSR-8	T + $(GA)_8$	–	No amplification	–
9	ISSR-9	T + $(GACA)_4$	49.0	3/3	400–1,200
10	ISSR-10	GC + $(AG)_7$	50.5	6/6	700–1,500
11	ISSR-11	GCGA + $(CA)_6$	51.5	5/5	1,000–3,000
12	ISSR-12	CCA + $(GTG)_4$	52.5	8/8	700–3,000
13	ISSR-13	$(CA)_8$ + AT	52.5	4/4	400–2,000
14	ISSR-14	$(AC)_8$ + TT	51.0	8/8	300–2,000
15	ISSR-15	$(AC)_8$ + TA	51.0	8/8	300–1,500
16	ISSR-16	$(AG)_8$ + AT	52.5	10/10	200–1,500
17	ISSR-17	$(AG)_8$ + AA	52.5	13/13	200–1,500
18	ISSR-18	$(AG)_8$ + TA	52.5	5/5	300–1,500
19	ISSR-19	$(AG)_8$ + TT	52.5	9/9	300–3,000
20	ISSR-20	GTG + $(GT)_7$	52.0	5/5	700–2,000
21	ISSR-21	$(AG)_8$ + C	51.0	8/8	600–2,000
22	ISSR-22	C + $(AG)_8$	51.0	9/9	500–2,000
23	ISSR-23	G + $(GACA)_4$	51.0	6/6	800–3,000
24	ISSR-24	$(AC)_8$ + CA	53.0	7/7	400–1,500
25	ISSR-25	$(AC)_8$ + TG	53.0	6/6	500–2,000

The scoring data of well-resolved bands of RAPD, ISSR, SSR, and AFLP markers was subjected to calculation of similarity matrix based on Jaccard's similarity coefficient. The pair-wise value of the in vitro cultures, regenerated plantlets and the mother plant was 1, indicating 100 % similarity. This confirmed the true-to-type nature of the in vitro raised clones and authenticated that *D. asper* cultures can remain free from somaclonal variations over a culture period extending up to 2 years. Most of the organized (pre-existing meristems) cultures especially shoot tips and axillary buds maintain strict genotypic and phenotypic stability compared to de novo originating meristematic structures like adventitious buds differentiating from callus or directly from cultured tissues. Propagation by axillary buds circumvents the dedifferentiation or redifferentiation of cells or tissues, avoiding genomic aberrations and consequently maintaining the clonal fidelity of in vitro raised plantlets (Negi and Saxena 2010). Studies on the length of culture period have also been reported in *Bambusa balcooa* Roxb. (Negi and Saxena 2010), *Curcuma longa* L. (Panda et al. 2007), *Swertia chirayita* (Roxb. ex Fleming) H. Karst (Joshi and Dhawan 2007), and almonds (Martins et al. 2004) wherein the in vitro cultures were maintained for a period of >2 years, 26 months, 44 months, and 4 years, respectively. The retention of clonal uniformity for prolonged period under in vitro conditions has immense commercial significance because initiation of in vitro cultures is difficult in bamboo due to season specificity, persistent contamination, phenolic exudation, etc. (Saxena and Dhawan 1994; Ramanayake et al. 1995; Das and Pal 2005; Yadav et al. 2008; Bisht et al. 2010). Therefore, it is possible to maintain a continuous supply of genetically uniform plants over a prolonged duration without resorting to initiation of fresh cultures frequently, which in turn will reduce the overall cost of plant production at commercial scale.

The 100 % similarity index obtained between the mother plant, in vitro cultures and their regenerants from

Table 6 Simple sequence repeat (SSR) primers used for testing the genetic fidelity of *Dendrocalamus asper* shoots and plants produced through tissue culture

Sr. no.	Primer code	Primer sequences (5′–3′)	Annealing temperature (°C)	Number of scorable/ monomorphic bands	Size range of amplification product (bp)
1	RM 7	F: TTCGCCATGAAGTCTCTCG	52	4/4	100–1,500
		R: CCTCCCATCATTTCGTTGTT			
2	RM 13	F: TCCAACATGGCAAGAGAGAG	52	4/4	200–800
		R: GGTGGCATTCGATTCCAG			
3	RM 17	F: TGCCCTGTTATTTTCTTCTCTC	52	5/5	100–1,200
		R: GGTGATCCTTTCCCATTTCA			
4	RM 19	F: CAAAAACAGAGCAGATGAC	55	8/8	100–700
		R: CTCAAGATGGACGCCAAGA			
5	RM 27	F: TTTTCCTTCTCACCCACTTCA	52	5/5	200–1,500
		R: TCTTTGACAAGAGGAAAGAGGC			
6	RM 31	F: GATCACGATCCACTGGAGCT	51	4/4	100–400
		R: AAGTCCATTACTCTCCTCCC			
7	RM 44	F: ACGGGCAATCCGAACAACC	55	12/12	100–2,000
		R: TCGGGAAAACCTACCCTACC			
8	RM 135	F: CTCTGTCTCCTCCCCCGCGTCG	66	5/5	100–700
		R: TCAGCTTCTGGCCGGCCTCCTC			
9	RM 140	F: TGCCTCTTCCCTGGCTCCCCTG	52	12/12	200–2,000
		R: GGCATGCCGAATGAAATGCATG			
10	RM 142	F: CTCGCTATCGCCATCGCCATCG	52	9/9	100–800
		R: TCGAGCCATCGCTGGATGGAGG			
11	RM 154	F: ACCCTCTCCGCCTCGCCTCCTC	66	10/10	100–1,500
		R: CTCCTCCTCCTGCGACCGCTCC			
12	RM 167	F: GATCCAGCGTGAGGAACACGT	55	10/10	100–1,200
		R: AGTCCGACCACAAGGTGCGTTGTC			
13	RM 201	F: CTCGTTTATTACCTACAGTACC	–	No amplification	–
		R: CTACCTCCTTTCTAGACCGATA			
14	RM 206	F: CCCATGCGTTTAACTATTCT	–	No amplification	–
		R: CGTTCCATCGATCCGTATGG			
15	RM 224	F: ATCGATCGATCTTCACGAGG	50	12/12	100–2,000
		R: TGCTATAAAAGGCATTCGGG			
16	RM 234	F: ACAGTATCCAAGGCCCTGG	52	10/10	200–1,000
		R: CACGTGAGACAAAGACGGAG			
17	RM 240	F: CCTTAATGGGTAGTGTGCAC	51	6/6	100–1,200
		R: TGTAACCATTCCTTCCATCC			
18	RM 245	F: ATGCCGCCAGTGAATAGC	–	No amplification	–
		R: CTGAGAATCCAATTATCTGGGG			
19	RM 253	F: TCCTTCAAGAGTGCAAAACC	52	10/10	200–900
		R: GCATTGTCATGTCGAAGCC			
20	RM 254	F: AGCCCCGAATAAATCCACCT	52	11/11	100–1,500
		R: CTGGAGGAGCATTTGGTAGC			
21	RM 261	F: CTACTTCTCCCCTTGTGTCG	50	2/2	100–400
		R: TGTACCATCGCCAAATCTCC			
22	RM 290	F: ACCCTTATTCCTGCTCTCCTC	51	5/5	100–1,200
		R: GTGCTGTAGATGGAAGGGAG			
23	RM 309	F: GTAGATCACGCACCTTTCTGG	–	No amplification	–
		R: AGAAGGCCTCCGGTGAAG			

Table 6 continued

Sr. no.	Primer code	Primer sequences (5′–3′)	Annealing temperature (°C)	Number of scorable/ monomorphic bands	Size range of amplification product (bp)
24	RM 315	F: GAGGTACTTCCTCCGTTTCAC R: AGTCAGCTCACTGTGCAGTG	57	9/9	200–2,000
25	RM 325	F: GACGATGAATCAGGAGAACG R: GGCATGCATCTGAGTAATGG	51	11/11	100–1,500

Table 7 Amplified fragment length polymorphism (AFLP) primer pairs used for testing the genetic fidelity of *Dendrocalamus asper* shoots and plants produced through tissue culture

Sr. no.	Primer pair	Status	Number of bands scored
1	E-AAG × M-CAC	Amplified	44
2	E-AAG × M-CTG	Amplified	53
3	E-AAG × M-CTC	Amplified	35
4	E-AAG × M-CAT	Not Amplified	–
5	E-AAG × M-CTA	Not Amplified	–
6	E-ACA × M-CTG	Not Amplified	–
7	E-ACA × M-CTA	Not Amplified	–
8	E-ACA × M-CAC	Amplified	49
9	E-ACT × M-CTG	Amplified	72
10	E-ACT × M-CTA	Amplified	46
11	E-ACT × M-CTC	Amplified	47
12	E-AAC × M-CTG	Amplified	68
13	E-AAC × M-CTA	Amplified	42
14	E-AAC × M-CAC	Amplified	35
15	E-ACG × M-CTT	Amplified	45

all the DNA markers used revealed that cost-effective, quick to use, easy to apply and highly polymorphic RAPD and ISSR markers can be effectively used for genetic fidelity testing of in vitro raised clones in bamboos. In some of the earlier studies also these two markers (RAPD and ISSR) were successfully used to ascertain the clonal fidelity of the in vitro-generated plantlets of bambusoideae representatives namely *B. balcooa* Roxb. and *B. tulda* Roxb. (Das and Pal 2005), *D. hamiltonii* Nees et Arn ex Munro (Agnihotri et al. 2009), *B. balcooa* Roxb. (Negi and Saxena 2010), *B. nutans* Wall. ex Munro (Negi and Saxena 2011), and *Guadua angustifolia* Kunth (Nadha et al. 2011). AFLP markers have also been used for evaluating the genetic fidelity of some plants (Cuesta et al. 2010; Mehta et al. 2011), however; they require highly skilled manpower, costly equipments, and expensive reagents as compared to RAPD and ISSR methods. Similarly, SSR primers are sequence-based species-specific markers and their development cost is high compared to RAPD and ISSR. Thus, RAPD and ISSR markers provide a simple and cost-effective method to test the clonal fidelity of tissue culture raised plants. Cuesta et al. (2010) also recommended the use of RAPD primers after comparison of RAPD, ISSR, AFLP, and SAMPL markers for assessment of somaclonal variation in micropropagated plants of stone pine.

Conclusions

Assessment of genetic stability of in vitro raised plants at an early stage can help in fine tuning of the micropropagation protocol and also in getting rid of genetically instable plants thus reducing the cost of their maintenance in the field till maturity. Production of monomorphic bands by in vitro cultures, their regenerants, and mother plant of *D. asper* against various DNA-based markers confirmed the genetic stability of the plants produced and also approved the commercial scale utilization of the developed protocol. Therefore, it can be concluded that the micropropagation

protocol reported earlier (Singh et al. 2012a) based on axillary shoot proliferation can be used for large scale micropropagation of *D. asper* without any risk of genetic instability appearing for at least 30 passages of 25 days each or up to 2 years of continuous subculturing. The developed micropropagation protocol will continue to contribute significantly to fill the gap of demand and supply of true to type planting material of *D. asper* as has already been demonstrated by planting more than 25,000 in vitro raised plants in the forest land of Haryana state.

Author contribution Sharbati R. Singh and Rohtas Singh conducted the research work, Sunita Dalal, A. K. Dhawan and Rajwant K. Kalia designed the experiments and analyzed the data, A. K. Dhawan and Rajwant K. Kalia arranged the funds, and Rohtas Singh and Rajwant K. Kalia prepared the manuscript under the guidance of A. K. Dhawan.

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