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BRIEF COMMUNICATION

ISSR marker based analysis of micropropagated plantlets of *Nothapodytes foetida*

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Abstract

A DNA-based, inter simple sequence repeat (ISSR) markers were used to monitor genetic stability in micropropagated plantlets of *Nothapodytes foetida*. A total of 146 clear and distinct bands were produced using 26 primers resulting in 3 212 fragments. Out of 146, 135 bands (92.4 %) were monomorphic and 11 bands (7.53 %) were polymorphic which ranged from 200 to 21 226 bp in size. The number of bands per each primer varied from 1 to 11 with an average of 5.6 bands per primer. The banding pattern for each primer was uniform and comparable to mother plant from which the cultures had been established. The dendrogram based on the unweighted pair-group method with arithmetic averaging (UPGMA) depicted about 97 % homology between the mother plant and micropropagated plants. An attempt was made to reintroduce the micropropagated plants in the natural habitat and over 500 plants were successfully established.

Additional key words: genetic stability, medicinal tree, PCR, reintroduction, UPGMA.

Molecular techniques are at present powerful and valuable tools used in analysis of genetic fidelity of *in vitro* propagated plants. In comparison to amplified fragment length polymorphism (AFLP) and restriction fragment length polymorphism (RFLP), inter simple sequence repeat (ISSR) is cost efficient, overcomes hazards of radioactivity and requires lesser amounts of DNA (Zietkiewicz *et al.* 1994). The simple sequence repeats are abundant and scattered throughout the eukaryotic genomes. This prompts the use of ISSR based oligonucleotide primers for polymerase chain reaction (Gupta *et al.* 1994). The utility of ISSR markers have been found immensely useful in establishing genetic stability of several micropropagated plants such as cauliflower (Leroy *et al.* 2000), *Populus tremuloides* (Rahman and Rajora 2001), *Swertia chirayita* (Joshi and Dhawan 2007) and *Dictyospermum ovalifolium* (Chandrika *et al*. 2008). Screening the tissue culture-derived plants prior to reintroduction using molecular markers will assist in reintroducing true to type plants (Heinze and Schimdt 1995).

Nothapodytes foetida (Wight) Sleumer (*Icacinaceae*) is a threatened small medicinal tree, valued as a rich source of the potent cytotoxic quinoline alkaloids camptothecin and 9-methoxycamptothecin (Roja 2006). The tissue-culture propagation of *N. foetida* has been previously reported (Rai 2002). We report the assessment of clonal integrity in the regenerated plants along with the single mother plant using inter simple sequence repeat (ISSR) markers.

Fruits of *N. foetida* were collected from a single 20 - 25 year-old-elite tree in evergreen forest of Coorg, Karnataka State, India. The earlier standardized micropropagation protocol (Rai 2002) was followed for the surface sterilization and culture initiation. The surface-disinfected seeds were transferred to culture vessels, containing 35 cm^3 Murashige and Skoog (1962; MS) basal medium with 3.0 % sucrose and 0.8 % agar. The pH of the medium was adjusted to 5.8. The culture jars were incubated at temperature of 25 ± 1 °C, 16-h photoperiod with irradiance of 40 μmol m^{-2} s^{-1}. Hypocotyl segments from aseptically grown seedlings were cultured in MS medium fortified with different concentrations of thidiazuron (TDZ). For *in vitro* rooting, individual shoots were transferred to one-fourth MS medium containing indole-3-acetic acid (IAA) and

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Abbreviations: CTAB - cetyltrimethylammonium bromide, PCR - polymerase chain reaction, ISSR - inter-simple sequence repeat, UPGMA - unweighted pair group method with arithmetic averaging.

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indole-3-butryic acid (IBA) in different combinations. After 8 weeks, the well-rooted microshoots were transferred to plastic pots containing *Soilrite* (*Karnataka Explosives*, Bangalore, India) and the plantlets were hardened in greenhouse before transferred to field conditions.

Twenty one-tagged regenerated plants from hardening stage were randomly selected along with single mother plant for screening their genetic integrity. Total DNA was extracted from fresh young leaves of micropropagated plants and field grown mother plant using cetyltrimethylammonium bromide (CTAB) method described by Doyle and Doyle (1990) with minor modifications. Quantity of DNA was inspected by both gel electrophoresis and spectrometric assays using UV-visible double beam PC scanning spectrophotometer (*LABOMED*, Culver city, USA).

A total of thirty two ISSR primers were screened initially and 26 primers were selected in the present study. Each reactions were performed in a total volume of 0.02 cm^3 reaction containing, 2.0 mm^3 of 1× assay buffer [10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl and 0.01 % gelatin, pH 9], 200 µM primer, 250 µM of dNTPs, 1.5 unit (U) of *Taq* DNA polymerase (*Genei*, Bangalore, India) and 50 ng genomic DNA. Amplifications were performed in *UNO II* (*Biometra*, Goettingen, Germany) thermocycler, programmed for initial denaturation at 94 °C for 3 min, followed by 40 cycles for 60 s at 94 °C for denaturation, 1 min at annealing temperature, 2 min extension step at 72 °C and a final extension step at 72 °C for 10 min. The annealing temperature was adjusted according to the primers used in the PCR (Table 1).

Amplified products were analyzed by electrophoresis on 2 % (m/v) agarose gel (*Amersham*, Uppsala, Sweden) using 1× TBE buffer (Tris-Borate-EDTA buffer). The gels were stained with ethidium bromide solution. The amplified products were visualized and photographed under UV transilluminator and documented using *Bioprofile Image Analysis System* (Vilber Lourmat, France). The size of the amplification products was estimated from lambda DNA/*Eco*R I-*Hind* III Double digest (*Genei*, Bangalore, India) as molecular marker. All PCR reactions were repeated twice.

PCR amplified DNA bands were scored with the selected ISSR primers and only clear and reproducible bands were taken into consideration. The bands were transformed into a binary character matrix, "1" for presence and "0" for absence of band in each plant at a particular position. Data analysis was performed using the *NTSYS-pc* version *2.1* computer program package (Rohlf 2000). Genetic similarities between micropropagated and mother plants were measured by the Jaccard's similarity coefficient (Jaccard 1908) with

Table 1. ISSR primers used to screen twenty one micropropagated *Nothapodtyes foetida* plantlets.

Primer	Sequence (5' to 3')	Annealing temperature [°C]	Number of bands polymorphic	monomorphic
ISSR02	CTCTCTCTCTCTCTCTAC	40	2	4
ISSR03	CTCTCTCTCTCTCTCTGC	40	-	6
ISSR04	CACACACACACAAC	45	-	7
ISSR05	CACACACACACAGT	45	-	7
ISSR06	CACACACACACAAG	45	1	4
ISSR07	CACACACACACAGC	45	-	6
ISSR10	GAGAGAGAGAGACC	48	-	5
ISSR12	CACCACCACGC	45	2	9
ISSR13	GAGGAGGAGGC	45	-	7
ISSR15	GTGGTGGTGGC	48	2	6
ISSR16	GAGAGAGAGAGAGAGAGAT	48	1	5
ISSR21	GATAGATAGATAGATA	40	-	4
ISSR22	CCTACCTACCTACCTA	40	-	4
ISSR01/w3	ACACACACACACACACC	52	-	10
ISSR02/w4	ACACACACACACACACG	46	-	8
ISSR04/w7	GGGTGGGGTGGGGTG	50	-	4
ISSR05/w8	CTCTCTCTCTCTCTCTG	42	2	6
ISSR06/w9	CACACACACACACACAG	42	-	4
ISSR07/w11	TCTCTCTCTCTCTCTCA	40	-	3
ISSR09/w30	GGAGAGGAGAGGAGA	36	-	4
ISSR10/15	CCCGTGTGTGTGTGT	40	-	3
ISSR11/20	CCAGTGGTGGTGGTG	36	-	1
ISSR12/32	AGAGAGAGAGAGAGAGC	40	-	3
ISSR13/33	GAGAGAGAGAGAGAGAT	40	-	6
ISSR14/34	GAGAGAGAGAGAGAGAC	40	-	4
ISSR15/8082	CTCTCTCTCTCTCTCTCTG	45	1	5

SIM-QUAL module. The similarity coefficients thus generated were used for constructing dendrogram with the unweighted pair-group method with arithmetic average (UPGMA) option in *NTSYS-pc* software package.

The micropropagated plants were grown in polytunnel for a period of 2 - 3 months before moving to the greenhouse. One-year-old acclimatized plants were transplanted to field at University of Mysore Campus, Karnataka, India and Wynad forest range, Kerala, India. The growth conditions were 25 ± 2 °C with 65 % RH and 22 ± 2 °C with 75 % RH respectively, in normal daylight conditions.

The adventitious shoot buds started forming within 20 - 25 d in thidiazuron (TDZ) containing MS medium. Best response for shoot induction was observed in medium with 0.5 mg dm^{-3} TDZ. Since, multiple shoots failed to elongate in TDZ, they were transferred to MS medium with 6-benzyladenine (BA; 0.5 mg dm^{-3}) for shoot elongation. *In vitro* rooting was obtained on one-fourth MS medium with auxins indole-3-acetic acid (IAA; 1.0 mg dm^{-3}) and indole-3-butyric acid (IBA; 0.5 mg dm^{-3}) after 30 - 35 d of culture. *In vitro* rooted plantlets were acclimatized in growth chamber (*Sanyo*, Moriguchi-City, Osaka, Japan) under temperature of 25 ± 2 °C, 80 % relative humidity, 16-h photoperiod with irradiance of 50 μmol m^{-2} s^{-1}. Then plantlets were hardened in greenhouse for a period of 3 - 4 months before moving to its original habitat.

True to type clonal fidelity is one of the most important prerequisites in the micropropagation of any plant species. A major problem encountered with the *in vitro* culture is the presence of somaclonal variation. Various factors, such as the *in vitro* process and its duration, auxin-to-cytokinin concentration and their ratio, nutritional conditions and *in vitro* stress, are all known to induce somaclonal variation (Devarumath *et al.* 2002). There are many studies available which regard axillary multiplication as the safest mode of micropropagation (Carvalho *et al.* 2004, Martins *et al.* 2004, Joshi and Dhawan 2007). The result of our study shows that adventitious multiplication is also the safe mode of micropropagation producing true to type progeny. Similarly, genetic fidelity of *in vitro* derived plants through adventitious multiplication was reported in *Lilium* bulblets (Varshney *et al.* 2001), *Drosera* plantlets (Kawiak and Lojkowska 2004), *Chlorophytum arundinaceum* (Lattoo *et al.* 2006) and *Hagenia abyssinica* (Feyissa *et al.* 2007). Whereas genetic variation among 11 regenerated clones from adventitious origin and the mother clone was reported in *Pyrus pyraster* (Palombi *et al.* 2007).

Hence it becomes imperative to regularly check the genetic integrity of the *in vitro* regenerated plants in order to produce clonally uniform progeny. We screened 32 ISSR primers for this analysis but only 26 primers were useful in reproducing the banding patterns. Therefore, we discarded remaining 6 primers as they were producing ambiguous and non reproducible amplification profiles (Table 1). Other 26 primers produced a total of 146 scorable, clear and reproducible

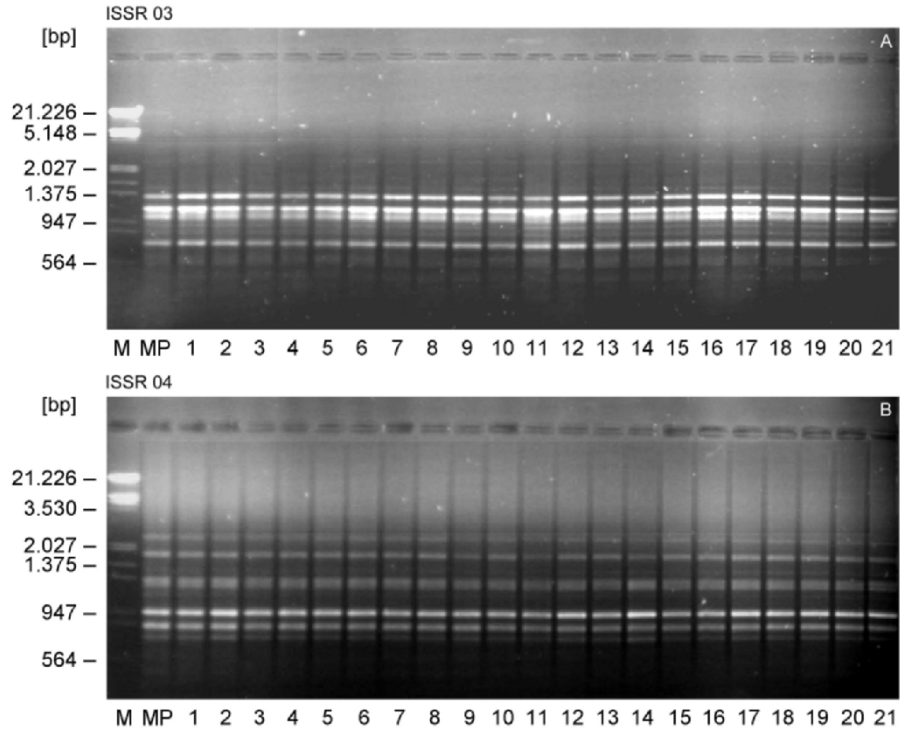

Fig. 1. ISSR products generated from 21 *in vitro* regenerated plants and mother plants of *N. foetida* amplified with primer ISSR-03 (*A*) and ISSR-04 (*B*) *Lanes*: M - molecular size marker (λDNA / *Eco*RI- *Hind*III Double digest DNA ladder), MP - donor plant, 1 to 21 - micropropagated plants of *N. foetida*.

bands, of which 135 bands were monomorphic (92.4 %) and remaining 11 bands were polymorphic (7.53 %). Indeed, all these 26 primers were found to generate identical banding patterns in two independent amplifications that were performed for all the samples. A total of 3212 fragments (numbers of plantlets analyzed × number of bands in all the primers) were generated showing homogeneous ISSR banding patterns. Twenty of the 26 primers produced monomorphic patterns, while each of the rest 6 primers generated polymorphic bands at least in one of 21 individuals relative to the donor plant. The number of bands per each primer varied from 1 to 11 with an average of 5.6 bands per primer. Samples of monomorphic gels obtained from ISSR primers (ISSR-03 and ISSR-04) were shown in Fig. 1.

The ISSR data was used to calculate genetic similarity among the 22 individuals. The similarity coefficient among the plants ranged from 0.92 to 0.99 with a mean of 0.97. A dendrogram, generated by cluster analysis using UPGMA method based on Jaccard's coefficient indicated the genetic similarity of 97 %. The plants were divided into three distinct groups with the donor plant in the second group (Fig. 2). However, NF-3 plant did not fall into any of the clusters.

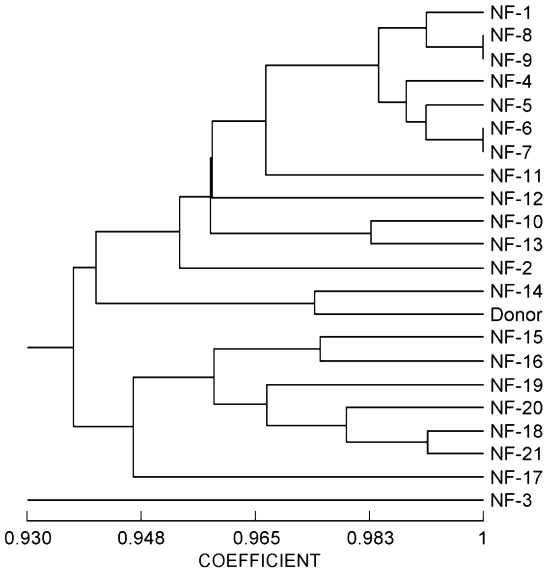

Fig. 2. Dendrogram illustrating similarities among 21 regenerated plants (NF-1 to NF-21) and the single donor plant of *N. foetida* by the UPGMA cluster analysis calculated from 146 ISSR bands generated with 26 selected primers.

The ISSR analysis of *N. foetida* revealed a low variation among regenerants, similar to *Tectona grandis* (Gangopadhyay *et al.* 2003) and *Gyposophila paniculate in vitro* regenerated plants (Rady 2007). ISSR analysis produced low percentage of 3.92 % polymorphism in 21 individual plants of *in vitro* grown *Dictyospermum ovalifolium* (Chandrika *et al.* 2008) and 10.62 % of polymorphism in *Robinia ambigua* among the 42 individual plants (Guo *et al.* 2006b). Further there was 15.72 % polymorphism reported among the 64 individual plants of medicinal plant *Codonopsis lanceolata* (Guo *et al.* 2006a) and 21.42 % seen among the three cultivars of banana (Ray *et al.* 2006).

Fig. 3. *Nothapodydes foetida* plantlet was reintroduced after one year growth to areas on Wynad forest range in Kerala, India.

Plants acclimatized in greenhouse were transplanted into the field condition where they exhibited normal growth. After one year the plants showed good establishment in areas of Wynad forest range in Kerala, India and University of Mysore campus, Karnataka, India with 68 % of survival rate (Fig. 3). Considering that ISSR markers detect the highest level of discrimination between any pair of genotypes (Powell *et al.* 1996), we can conclude that the *in vitro* plants developed in the present study detected low level of polymorphism. Since *N. foetida* is threatened medicinal plant, production of genetically true to type plants is reliable for the germplasm conservation and therapeutic purposes.

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