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Optimization of Protocol for Isolation of Genomic DNA from Leaves of *Selaginella* Species Suitable for RAPD Analysis and Study of their Genetic Variation

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ABSTRACT.—A simple and efficient protocol for isolating genomic DNA from leaves of *Selaginella* spp. (*S. delicatula, S. repanda, S. bryopteris, S. plana, S. monospora*) was developed, involving a modified CTAB protocol of Rogers and Benedich (1994). Increasing the incubation time with the precipitation buffer (1X CTAB) from 1–3 hours to 12–14 hours helped achieve higher quantity genomic DNA from the specimens, when compared with DNA extracted by protocols reported by Dellaporta *et al.* (1983), Murray and Thompson (1980) and Doyle and Doyle (1987). The DNA yield ranged from 846–1836 µg/ml from fresh and herbaria-preserved leaf samples. The DNA samples were found suitable for genetic diversity analysis with Random Amplified Polymorphic DNA (RAPD) markers. Nine random primers (OPA A17, OPB 4, OPB13, OPC 2, OPC 11, OPD 5, OPG 2, OPG 19 and OPK 10) were studied, of which two primers (OPD 5 and OPG 2) yielded reproducible amplification profile of polymorphic fragments.

KEY WORDS.—DNA extraction, RAPD, *Selaginella*, modified protocol

Selaginella (spike moss) is an enigma in the plant kingdom. At present only one genus is recognized in the Selaginellaceae, i.e., *Selaginella* (Family Selaginellaceae, Order Selaginellales, Class Lycopsida). The genus *Selaginella* is cosmopolitan in distribution and contains approximately 700 species that include temperate, tropical, frost-tolerant arctic, and drought-tolerant desert species. Such extremes are very rarely found in the same genus, and hence the family Selaginellaceae has been treated differently and sub-divided into myriad taxa by researchers (Spring, 1850; Braun, 1857; Baker, 1883; Hieronymus, 1901; Walton and Alston, 1938; Jermy, 1986).

Selaginella shows morphological variation within species and as such it is difficult to distinguish species depending on traditional morphology only. Thus, researchers have concentrated on molecular phylogenetic analysis to gain information about its evolutionary relationships. A recent molecular phylogenetic analysis of the genus has revealed that rates of molecular evolution among species are remarkably high, including when compared to the angiosperm families (Korall and Kenrick, 2004). Although many subtle morphological and developmental differences exist between species, few of these differences are phylogenetically useful markers for classifying the species in a way that is consistent with molecular data (Korall and Kenrick

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2002, 2004). In this perspective, studies involving isolation and characterization of DNA are very useful, since they open up the possibility for detection of an evolutionary pattern that implies both morphological and genetic changes. Molecular marker based phylogenetic studies (e.g., RAPD, ISSR, SNP, etc.) have been utilized in complementing and supplementing morpho-taxonomy in many cases. The success of these procedures relies on inexpensive, rapid and simple DNA extraction methods (Weishing *et al.*, 1995), as they require large amounts of high quality genomic DNA.

The main aim of the present study is to evaluate different protocols of DNA isolation and to standardize a protocol for obtaining better DNA yield and amplification quality for RAPD analysis from milligram amounts of living and herbarium *Selaginella* leaf specimens. The use of dried herbarium specimens is essential due to the unavailability of suitable quantity of required plant material from the living plant materials. The detriment is that satisfactory quantity and quality of DNA from herbarium specimens cannot be obtained due to the rapid degradation of plant material during preservation. DNA isolation from dried specimens usually requires some modifications to frequently used protocols (Rogers, 1994) to ensure quality DNA extraction from even very small amounts of dry herbarium tissues are available. Here we report a DNA isolation protocol from milligram amounts of both living and herbarium plant materials that has been standardized and proved to be suitable for RAPD analysis.

Materials and Methods

Sample collection.—*Selaginella* samples including *S. delicatula* (Desv. ex Poir) Alston, *S. repanda* (Desv. ex Poir) Spring, *S. bryopteris* (L.) Baker, *S. plana* (Desv. ex Poir) Hieron., *S. monospora* Spring were collected from the Darjeeling district (West Bengal) and Nainital (Uttarakhand) regions of India. Herbarium specimens were obtained from the Calcutta University Herbarium (CUH).

Genomic DNA isolation.—Genomic DNA was extracted from fresh and dried leaf samples using several reported protocols, including those of Dellaporta *et al.* (1983) (Protocol 1); Doyle and Doyle (1987) (Protocol 2); Murray and Thompson (1980) (Protocol 3); Rogers and Benedich (1994) (Protocol 4). A modified Rogers and Benedich (1994) protocol was devised to increase DNA yield. The essential modification is an increase in the incubation time which involves incubation of supernatant containing DNA after a second chloroform-isoamyl alcohol extraction with 1X CTAB overnight, instead of 1–3 hours as reported in the original protocol, and an additional step of RNase treatment was added, when required.

The modified protocol of Rogers and Benedich, 1994 (Protocol 5) that was standardized is as follows: fresh leaves (0.5 g) frozen at −20°C for 7–15 days or herbarium or dried leaves of *Selaginella* (0.2 g) were freeze dried in liquid nitrogen and crushed in a mortar and pestle. The crushed powder was transferred to a 50 ml tube and mixed with 2X CTAB extraction buffer (2% CTAB, 100 mM Tris, 20 mM EDTA, 1.4 M NaCl, 1% PVP) with

ß-mercaptoethanol, warmed at 55°C before use. The mixture was incubated while shaking at 66°C for 45 minutes. Then an equal volume of chloroform: isoamyl alcohol (24:1) was mixed and gently shaken for 10 minutes at room temperature. The mixture was centrifuged for 20 minutes at 10,000 rpm and the supernatant was recovered to which 1/10 volume of 10% CTAB warmed at 55°C was added. Equal volume of chloroform:isoamyl alcohol (24:1) was added again and mixed gently. The mixture was centrifuged at 10,000 rpm for 20 minutes. The supernatant was pipetted into a new tube and 2 volumes of 1X CTAB were added to it; after mixing, it was incubated overnight at room temperature. The mixture was centrifuged for 20 minutes at 10,000 rpm. The pellet thus obtained was dissolved in high salt TE to which 2 volumes of ice-cold ethanol were added and the mixture was incubated overnight at −20°C, and later centrifuged at 10,000 rpm for 10 minutes. The pellet was washed with cold 80% ethanol before centrifugation for 5 minutes at 10,000 rpm. The pellet containing nucleic acids thus obtained was dried and redissolved in 30 µl TE and stored.

In the case of DNA extraction from fresh *Selaginella* specimens, an additional step of RNase treatment was required. The RNase treatment was not an essential step for the herbarium specimens as RNA interference is absent in the case of preserved specimens.

The extracted genomic DNA was tested for purity index (A_{260}/A_{280} absorbance ratio) on UV-VIS Spectrophotometer and for size, purity and integrity on 1% agarose gel at 80V for 40 minutes.

Polymerase Chain Reaction.—PCR reactions for RAPD analysis were performed in a 25 µl volume containing 100 ng genomic template DNA, 2.5 µl of reaction buffer, 100 mM dNTP mix, 2.5 ng primer (random primer, Operon Technologies), and 3 U/µl *Taq* polymerase. Amplification was performed in a Gradient Thermal Cycler (Eppendorf). The reaction mixtures were amplified in an initial step of 94°C for 3 min and then subjected to 35 cycles of the following program: 94°C for 1 min, 37°C for 1 min, 72°C for 1 min. After the last cycle, the temperature was maintained at 72°C for 8 min. Amplified DNA was electrophoresed in a 1.2% agarose gel containing ethidium bromide and photographed on a UV transilluminator. Amplification products generated by a few decamer primers from OP series (Operon Technologies) are presented in the current study.

Results

When a modified version of the original CTAB protocol (Rogers and Benedich, 1994) was used for the extraction of DNA from the dried herbarium and living leaves of different species of *Selaginella,* DNA yield and quality was significantly increased. The extraction protocol of Dellaporta *et al.* (1983) and Doyle and Doyle (1987) did not yield quantifiable amounts of genomic DNA, while the extraction protocol of Murray and Thompson (1980) yielded comparatively less quantifiable DNA, which was not suitable for RAPD analysis. The spectrophotometric results for the five different species of

TABLE 1. Yield of genomic DNA measured by quantity and purity index of *Selaginella* obtained from five different species using different protocols.

Plant sample	Modified Rogers and Benedich protocol (1994)		Original Rogers and Benedich protocol (1994)		Murray and Thompson protocol (1980)	
	Quantity (μg/ml)	Purity index (A_{260}/A_{280} ratio)	Quantity (μg/ml)	Purity index (A_{260}/A_{280} ratio)	Quantity (μg/ml)	Purity index (A_{260}/A_{280} ratio)
Selaginella delicatula	1164	1.84	486	1.92	294	1.36
Selaginella bryopteris	924	1.69	390	1.71	246	1.41
Selaginella plana	1836	2.01	696	1.96	366	1.60
Selaginella monospora	846	1.71	318	2.65	276	1.53
Selaginella repanda	912	1.87	450	2.08	234	1.34

Selaginella obtained using the original protocols of Rogers and Benedich (1994), Murray and Thompson (1980) and the modified protocol of Rogers and Benedich (1994) are given in Table 1.

For RAPD analysis, initial PCR amplification of genomic DNA from the five species of *Selaginella* was done. Among the nine random primers tried, two primers from OP series (OPD 5 and OPG 2) were selected for RAPD analysis which could amplify the template DNA from the five leaf samples of *Selaginella* using PCR. From the study we obtained a number of RAPD bands from two different primers for the samples of *Selaginella* studied (Table 2).

DISCUSSION

A cost effective DNA extraction procedure greatly facilitates genetic diversity analysis. The present study demonstrates that the DNA extraction procedure significantly affects yield and quality in *Selaginella*, as well as the

TABLE 2. Primers used in RAPD analysis.

Primer	Sequence	Total number of amplified bands	Total number of polymorphic bands	Percentage of polymorphic bands (%)
OPA A17	5′- GAG CCC GACT - 3′	Absent		
OPB 4	5′- GGA CTG GAGT - 3′	Absent		
OPC 11	5′- AAA GCT GCG C - 3′	Absent		
OPD 5	5′- TGA GCG GAC A - 3′	26	21	84.7
OPG 2	5′- GGC ACT GAG G- 3′	19	9	47.3
OPG 19	5′- GTC AGG GCA A- 3′	Absent		
OPK 10	5′- GTG CAA CGT G - 3′	Absent		
OPC 2	5′- GTG AGG CGT C - 3′	Absent		
OPB 13	5′- TTC CCC CGC T - 3′	Absent		

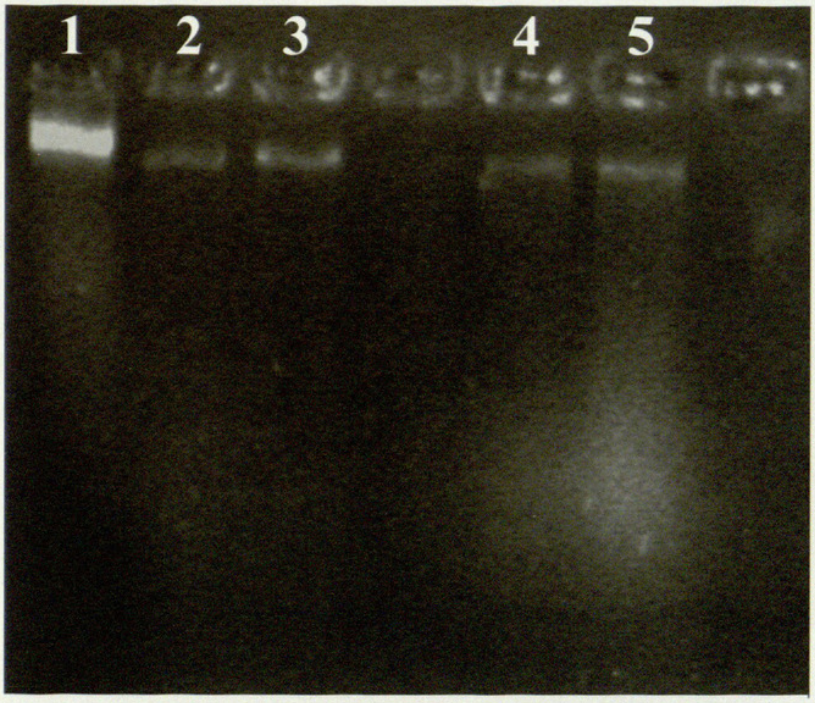

FIG. 1. Electrophoretic analysis of total DNA from leaves of *Selaginella plana*, *Selaginella monospora*, *Selaginella delicatula*, *Selaginella repanda*, and *Selaginella bryopteris* extracted by the modified protocol of Roger and Benedich (1994).

efficiency of RAPD amplification. Upon gel electrophoresis a clear continuous band of DNA was obtained showing that the quality of DNA had improved and was consistently suitable for PCR amplification for RAPD analysis (Fig. 1). After PCR optimization for RAPD analysis, the generated amplification products were found to be of good quality and could be used to discriminate the genetic polymorphisms present in different species of *Selaginella* used in the study. Yield of genomic DNA was increased by certain modifications of the protocol. The modification involved increasing the time of incubation in 1X CTAB buffer. CTAB being a cationic detergent, it can form a CTAB–nucleic acid precipitate at room temperature, when the salt concentration is lower than 0.5 M. We observed that increasing the incubation time of the chloroform-isoamyl alcohol extract in precipitation buffer (1X CTAB solution) may cause more nucleic acids to be selectively precipitated, thus increasing the net yield.

We also observed that, in general, the Rogers and Benedich (1994) protocol yielded higher quantities of DNA compared to the protocol of Murray and

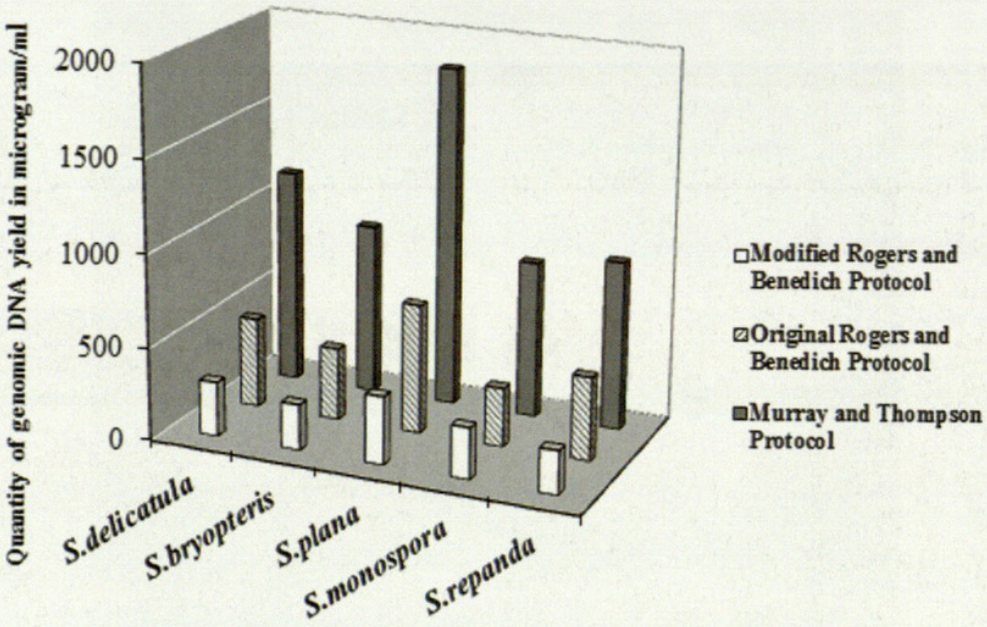

FIG. 2. Graphical representation showing the quantity of DNA obtained from different protocols for the five different species of *Selaginella*.

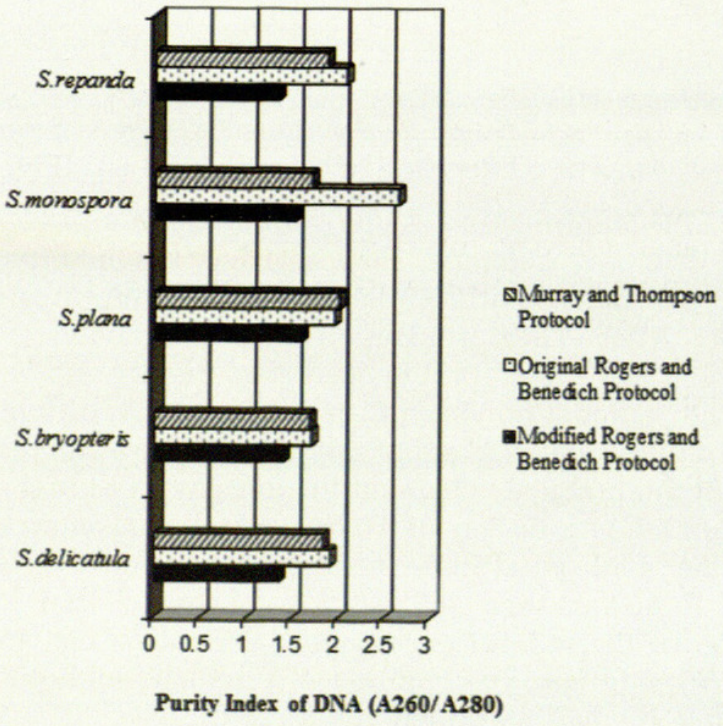

FIG. 3. Graphical representation showing the purity index of DNA obtained from different protocols for the five different species of *Selaginella*.

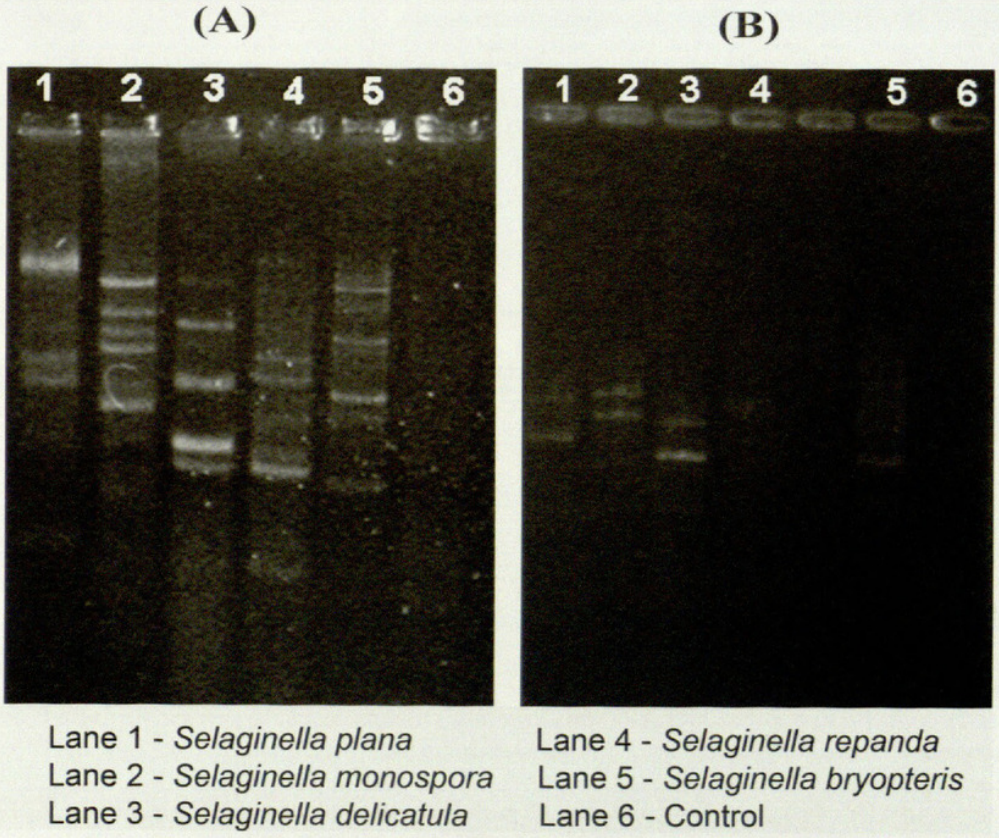

FIG. 4. PCR amplification profile of five different species of *Selaginella* using the primer OPD 5 using genomic DNA extracted from two different protocols, (A) modified Roger and Benedich (1994), and (B) original Roger and Benedich (1994).

Thompson (1980). The modified Rogers and Benedich (1994) protocol, on the other hand, yielded better quantity (2–3 times more) and quality genomic DNA than the original protocol of Rogers and Benedich (1994) (Fig. 2 and Fig. 3). The isolation protocols of Dellaporta *et al.* (1983) and Doyle and Doyle (1987) did not yield quantifiable amounts of genomic DNA.

The present study revealed that though the modified protocol of Rogers and Benedich (1994) required longer time (nearly three days) than the other methods followed, better quality DNA was extracted using this protocol (Fig. 1). Since the quantity and purity of extracted genomic DNA plays a crucial role for analysis of molecular diversity and optimization of different parameters for PCR (Weeden *et al.*, 1992; Staub *et al.*, 1996), the modified DNA extraction protocol of Rogers and Benedich proved to be most useful, as both the quality and quantity of genomic DNA significantly increased, yielding better results in RAPD-PCR analyses compared to the original DNA extraction protocol of Rogers and Benedich (Fig. 4 a and b).

Further analyses using more RAPD primers are necessary to obtain molecular markers for distinguishing the different subgenera of *Selaginella*, which would give valuable information regarding genetic diversity of *Selaginella* species.

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