# Identification of *Tinospora cordifolia* (Willd.) Miers ex Hook F. & Thomas Using RAPD Markers

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Identified germplasm is an important component for efficient and effective management of plant genetic resources. Traditionally, plant identification has relied on morphological characters like growth habit, floral morphology like flower colour and other characteristics of the plant. Studies were undertaken for identification and genetic variation within 15 clones of *Tinospora cordifolia* through random amplified polymorphic DNA (RAPD) markers. Analysis was made using forty decamer primers. Out of them, 15 primers were selected and used for identification and genetic relationships within 15 clones. A total of 138 distinct DNA fragments ranging from 0.2 to 3.2 kb were amplified using 15 selected random primers. The genetic similarity was evaluated on the basis of presence or absence of bands. The genetic distance was very close within the clones. Thus, these RAPD markers have the potential for identification of species and characterization of genetic variation within the population. This study will be helpful to know the genetic background of the medicinal plants with high commercial value, and also provides a major input into conservation biology.

*Key words:* DNA Fingerprinting, Medicinal Plant, RAPD Marker

## Introduction

*Tinospora cordifolia* (Willd.) Miers ex Hook F. & Thomas belongs to the family Menispermaceae which is distributed throughout the tropical Indian subcontinent and China, ascending to an altitude of 300 m (Anonymous, 1976; Kritikar and Basu, 1975). It is a large, glabrous, deciduous climbing shrub. The stem of *Tinospora cordifolia* is rather succulent with long filiform fleshy aerial roots from the branches. The bark is creamy white to grey, deeply left spirally, the space in between being spotted with large rosette-like lenticels. The leaves are membranous and cordate. The flowers are small and yellow or greenish yellow. In auxiliary and terminal racemes or racemose panicles, the male flowers are clustered and the female are usually solitary (Anonymous, 1976; Kritikar and Basu, 1975; Chopra *et al.*, 1956). The drupes are ovoid, glossy, succulent, red and pea sized. The seeds are curved and considered to be a unique character. Fruits are fleshy and single seeded. It is also propagated by stem nodal cuttings. It is widely used in many ayurvedic systems of medicine for its general tonic, anti-periodic, anti-allergic, anti-spasmodic, anti-inflammatory, anti-arthritic, and anti-diabetic properties (Khosa and Prasad, 1971; Chopra *et al.*, 1982; Nayampalli *et al.*, 1982; Bisset and Nwaiwu, 1983; Sarma *et al.*, 1998; Prince and Menon, 1999). It is also used in preparation of "Rasayanas" to improve the immune system and the body resistance against infections. The root of this plant is known for its anti-stress, anti-leprotic and anti-malarial activities (Gupta *et al.*, 1967; Prince and Menon, 1999; Singh *et al.*, 2003). Considering the distribution, growth habit and commercial importances, it is important to characterize the DNA fingerprinting for conservation and utilization of plant genetic resources. Molecular techniques help researchers not only to identify the authentication of the genotypes, but also in assessing and exploiting the genetic variability through molecular markers (Whitkus *et al.*, 1994). Random amplified polymorphic DNA (RAPD) analysis has proved to be useful for identification of the genotype and estimating genetic diversity, particularly to assist in the conservation of rare species and plant genetic resources (Williams *et al.*, 1990). The present investigation is to characterize the taxa identification and genetic variation of the different clones of *Tinospora cordifolia*, a commercially important medicinal plant using RAPD markers.

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## Materials and Methods

### *Plant material*

Stem nodal cuttings were collected from 15 individual clones of *Tinospora cordifolia* from the reserve forests (~ 120 $km^2$) of Orissa and voucher specimens were recorded in the central herbarium of Regional Plant Resource Centre, Bhubaneswar (No. Represent 1739, 1740). The nodal cuttings were planted in the greenhouse at Regional Plant Resource Centre, Bhubaneswar, Orissa, India. Young shoots were sprouted from the cuttings after two months of planting. Young leaves were collected from individual clones and used for DNA extraction. The isolation of DNA was made thrice.

### *DNA extraction*

DNA was extracted from young leaves from different clones by the CTAB method (Doyle and Doyle, 1990). Approx. 500 mg of fresh leaves were ground to powder in liquid nitrogen using a mortar and pestle. The ground powder was transferred to a 25 ml-tube with 10 ml of cetyltrimethyl ammonium bromide ($C_{19}H_{42}BrN$) buffer: 2% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris [tris(hydroxymethyl)aminomethane]-HCl, pH 8.0, and 0.2% (v/v) $\beta$-mercaptoethanol. The homogenate was incubated at 60 °C for 2 h, extracted with an equal volume of chloroform/isoamyl alcohol (21:1) and centrifuged at 8000 × *g* for 20 min (Kubata KR-2000 C, Rotor-RA-3R, Tokyo, Japan). DNA was precipitated from the aqueous phase by mixing with an equal volume of isopropanol. After centrifugation at 8000 × *g* for 10 min, the DNA pellet was washed with 70% (v/v) ethanol, air-dried and resuspended in 10 mM Tris, pH 8.0, 0.1 mM EDTA buffer. DNA quantifications were performed by visualizing under UV light, after electrophoresis on 0.8% agarose gel (Sigma, USA). The resuspended DNA was then diluted in TE to 5 ng/$\mu$l concentration for use in amplification reactions.

### *Primer screening*

Forty decamer primers, corresponding to kits A, B and N from Operon, Advanced Biotechnologies Inc., Alameda, USA were initially screened using one individual clone to determine the suitability of each primer for the study. Primers were selected for further analysis based on their ability to detect distinct, clearly resolved and polymorphic amplified products within the population. To ensure reproducibility, the primers generating no, weak, or complex patterns were discarded.

### *PCR amplification*

A set of forty 10-base primers (Operon, Advanced Technologies) were used for polymerase chain reaction (PCR). Amplification reactions were performed in 25 $\mu$l: 2.0 $\mu$l of 1.25 mM each of dNTP's, 15 ng of the primer, 1 × Taq polymerase buffer, 0.5 U of Taq DNA polymerase (Genei, Bangalore, India) and 20 ng of genomic DNA. DNA amplification was performed in a PTC-100 DNA Thermal Cycler (M J Research Inc., Watertown, MA, USA) programmed for 45 cycles: 1$^{st}$ cycle of 3.5 min at 94 °C , 1 min at 37 °C and 2 min at 72 °C; then 44 cycles each of 1 min at 94 °C, 1 min at 37 °C, 2 min at 72 °C followed by one final extension cycle of 7 min at 72 °C. Amplified products were electrophoresed in a 1.2% (w/v) agarose gel with 1 × TAE buffer, stained with ethidium bromide, and photographed under ultraviolet (UV) light. Gel photographs were scanned through a Gel Doc System (Gel Doc. 2000, Bio-Rad, USA) and the amplification product sizes were evaluated using the software quantity one (BioRad). The sizes of the amplification products were estimated from a 3.0 kb (100 bp to 3.0 kb) ladder (MBI Fermentas Inc., New York, USA). All the reactions were repeated at least two times.

### *Data analysis*

Data were recorded as presence (1) or absence (0) of band products from the examination of photographic negatives. Each amplification fragment was named by the source of the primer (Operon, Advanced Biotechnologies Inc.), the kit letter or number, the primer number and its approximate size in base pairs. Bands with similar mobility to those detected in the negative control, if any, were not scored. Similarity indexes were estimated using the Dice Coefficient of similarity (Nei and Li, 1979). The similarity matrices were used to generate a tree using unweighted pair-group method arithmetic average (UPGMA).

## Results and Discussion

Out of forty primers tested, 15 decamer primers showed good polymorphism within the 15 clones of *Tinospora cordifolia.* The reproducibility of the

Table I. Total number of amplified fragments and number of polymorphic fragments generated by PCR using selected decamer primers.

| Name of primer | Sequence of the primer (5′–3′) | Total number of amplification products | Number of polymorphic products | Size range [kb] |
|---|---|---|---|---|
| OPA-02 | 5′-TGCCGAGCTG-3′ | 10 | 3 | 0.7 –2.2 |
| OPA-03 | 5′-AGTCAGCCAC-3′ | 12 | 2 | 0.32–2.44 |
| OPA-05 | 5′-AGGGGTCTTG-3′ | 11 | 2 | 0.2 –2.5 |
| OPA-10 | 5′-GTGATCGCAG-3′ | 11 | 2 | 0.6 –2.85 |
| OPA-11 | 5′-CAATCGCCGT-3′ | 8 | 3 | 0.4 –1.5 |
| OPA-18 | 5′-AGGTGACCGT-3′ | 9 | 2 | 0.45–2.6 |
| OPB-06 | 5′-TGGTCTGCCC-3′ | 8 | 2 | 0.34–3.0 |
| OPB-08 | 5′-GTCCACACGG-3′ | 10 | 3 | 0.4 –2.65 |
| OPB-11 | 5′-GTAGACCCGT-3′ | 6 | 1 | 0.5 –3.0 |
| OPB-16 | 5′-TTTGCCCGGA-3′ | 8 | 0 | 0.28–3.2 |
| OPN-01 | 5′-CTCACGTTGG-3′ | 11 | 2 | 0.4 –2.8 |
| OPN-03 | 5′-GGTACTCCCC-3′ | 13 | 3 | 0.6 –3.2 |
| OPN-05 | 5′-ACTGAACGCC-3′ | 4 | 1 | 0.8 –1.4 |
| OPN-06 | 5′-GAGACGCACA-3′ | 7 | 2 | 0.3 –2.5 |
| OPN-16 | 5′-AAGCGACCTG-3′ | 10 | 2 | 0.5 –2.6 |

amplification product was tested by DNA from three independent extractions of the 15 clones. Most of the amplification reactions were duplicated. Only bands that were consistently reproduced across amplifications were considered for the analysis. Bands with the same mobility were considered as identical fragments, receiving equal values, regardless of their staining intensity. When multiple bands in a region were difficult to resolve, data for that region of the gel was not included in the analysis. As a result, 15 informative primers were used to evaluate the degree of polymorphism within clones. The selected primers generated distinctive products in the range of 0.2–3.2 kb. Maximum and minimum number of bands were produced by the primers OPN-03 (13) and OPN-05 (4), respectively (Table I). A total of 138 amplified fragments was scored across 15 clones for the selected 15 primers, and were used to estimate genetic relationships within the clones. The patterns of RAPD produced by the primers OPA-03, OPA-10, OPN-03 and OPN-05 are shown in Fig. 1. The identification and genetic variation through RAPD markers has been highlighted in a number of plant species (Bretting and Widrelechner, 1995; Jain *et al.*, 2003; Rout *et al.*, 2003; Samal *et al.*, 2004). The similarity matrix obtained after multivariate analysis using Nei and Li's coefficient (Table II) shows that there was the close variation among the 15 clones. Vainstein *et al.* (1993) reported that the genetic similarities are small within the rose groups (hybrid tea, floribunda, polyantha

Table II. Similarity matrix for Nei and Li's coefficient among the 15 clones of *Tinospora cordifolia*.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1.0 | | | | | | | | | | | | | | |
| 2 | 0.94 | 1.0 | | | | | | | | | | | | | |
| 3 | 0.88 | 0.91 | 1.0 | | | | | | | | | | | | |
| 4 | 0.90 | 0.90 | 0.98 | 1.0 | | | | | | | | | | | |
| 5 | 0.94 | 0.93 | 0.90 | 0.88 | 1.0 | | | | | | | | | | |
| 6 | 0.96 | 0.97 | 0.91 | 0.91 | 0.98 | 1.0 | | | | | | | | | |
| 7 | 0.97 | 0.94 | 0.89 | 0.92 | 0.98 | 0.96 | 1.0 | | | | | | | | |
| 8 | 0.96 | 0.92 | 0.88 | 0.86 | 0.95 | 0.94 | 0.96 | 1.0 | | | | | | | |
| 9 | 0.97 | 0.92 | 0.90 | 0.89 | 0.93 | 0.95 | 0.96 | 0.98 | 1.0 | | | | | | |
| 10 | 0.96 | 0.93 | 0.88 | 0.91 | 0.98 | 0.90 | 0.94 | 0.96 | 0.97 | 1.0 | | | | | |
| 11 | 0.94 | 0.93 | 0.89 | 0.90 | 0.94 | 0.96 | 0.97 | 0.98 | 0.93 | 0.94 | 1.0 | | | | |
| 12 | 0.97 | 0.95 | 0.89 | 0.88 | 0.93 | 0.92 | 0.98 | 0.96 | 0.94 | 0.98 | 0.96 | 1.0 | | | |
| 13 | 0.98 | 0.92 | 0.88 | 0.91 | 0.94 | 0.97 | 0.95 | 0.97 | 0.98 | 0.94 | 0.92 | 0.95 | 1.0 | | |
| 14 | 0.93 | 0.98 | 0.91 | 0.94 | 0.95 | 0.93 | 0.92 | 0.89 | 0.94 | 0.93 | 0.95 | 0.94 | 0.96 | 1.0 | |
| 15 | 0.87 | 0.91 | 0.89 | 0.90 | 0.98 | 0.90 | 0.97 | 0.92 | 0.95 | 0.97 | 0.92 | 0.97 | 0.95 | 0.97 | 1.0 |

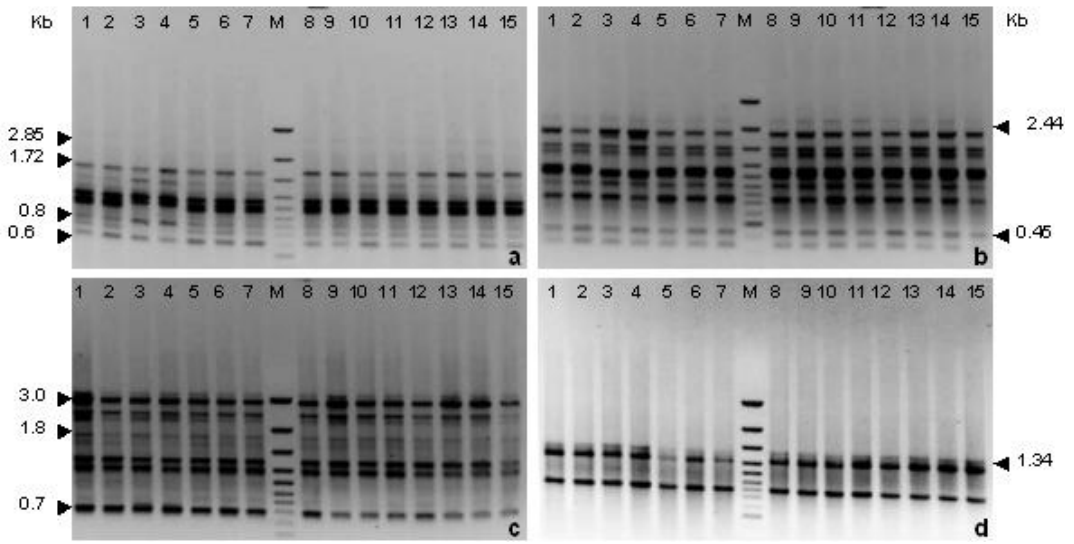


Fig. 1. RAPD patterns of 15 clones of *Tinospora cordifolia* generated by primer (a) OPA-10 (5′-GTGATCGCAG-3′), (b) OPA-03 (5′-AGTCAGCCAC-3′), (c) OPN-03 (5′-GGTACTCCCC-3′) and (d) OPN-05 (5′-ACTGAACGCC-3′). 1–15, different clones; M, kb molecular weight ladder.

and miniature) by using 28 DNA fragments from microsatellite fingerprints. Our results indicate that the mean levels of genetic variation were low among the individuals. The low differences in number of individuals estimated by RAPD markers in this study are similar to the result obtained by Rajaseger *et al.* (1997) in RAPD studies of *Ixora coccinea* and *I. javanica.* They found that the taxa-specific RAPD bands can be utilized to define the identification. It was observed that the clone showed a good number of amplification bands in the primers OPA-03 and OPN-03, but lesser number of amplified fragments in the primer OPN-05 (Fig. 1). The cluster analysis indicates that 15 clones of *Tinospora cordifolia* formed two major cluster groups (Fig. 2). Group A represents two clones (3 and 4). Group B represents thirteen clones. The major cluster group B again is divided into two minor subclusters, *i.e.* B1 and B2. Subgroup B1 represents only two clones and B2 represents eleven clones. The subgroup B2 showed two minor subclusters, *i.e.* C1 and C2. The minor subcluster C1 represents three clones and C2 represents 8 clones. The subcluster group C2 divided further into two subclusters, *i.e.* D1 and D2. Both the subclusters having four clones each (Fig. 2). However, all the clones share more than 90% RAPD markers. Clones 3 and 4 were different from the other thirteen clones. The dendro-

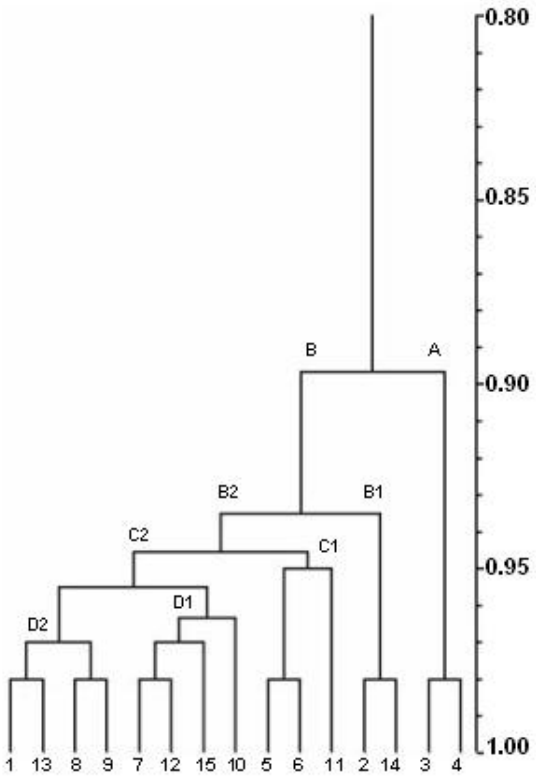


Fig. 2. Dendrogram of cluster analysis of RAPD markers illustrating the genetic relationships among 15 clones of *Tinospora cordifolia.*

gram also represents the close distances among the clones occurring in adjacent tips of the classification according to numerical taxonomy (Sneath and Sokal, 1973). It indicates that the RAPD markers provide a more reliable method for identification of species or clones than morphological characters.

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