Utilising inter-Random Amplified Polymorphic DNA, assess genetic variability in indigenous medicinal plant Heart-leaved Moonseed (*Tinospora cordifolia*)

Abstract

The Ayurvedic medical system, which uses herbal preparations for its traditional remedies, has been used in India since ancient times. One of the powerful immunomodulators is *Tinospora cordifolia*, also known as AMRITA, which is credited to this miracle medication for giving its user youth, longevity, and vigour. RAPD has been widely and successfully used to identify several species of medicinally significant plant. The need for research into and protection of therapeutic plant species is growing. The perseverance of the evaluation was to assess the genetic diversity among 10 accessions of *T. cordifolia* obtained from various districts in Tamil Nadu. 18 decamer primers were used in the analysis. Nine of them had repeatable, different polymorphism patterns in their primers. RAPD investigation, it was discovered that the DNA recovered from several *T. cordifolia* accessions exhibited random amplification and various levels of genetic polymorphism. In the sample from Salem district the most genetic polymorphism was found.

Key words: *Tinospora cordifolia*, Tamil Nadu, RAPD, Genetic diversity, Medicinal plants

Introduction

Since ancient times, people have employed plants as medicine. The Ayurvedic medical system, which uses herbal preparations for its traditional remedies, has been used in India since ancient times. The therapeutic benefits of these medicinal plants have been thoroughly investigated in a number of scientific fields, including pharmacology, cosmetics, fragrance, cuisine, etc (Dubey et al., 2004). The term "Return to Nature" can be used to describe the recent shift in Indian medicine from allopathic to herbal therapy. Since millennia, medicinal plants have been highly valued for their curative qualities in treating ailments and preventing disease. Plants have the capacity for both natural healing and acting as potential antibacterial agents. According to estimations from the World Health Organization (WHO), 80% of people worldwide depend on traditional ayurvedic medicines to maintain a healthy way of living. (Foster et al., 2005).

T. cordifolia is also referred as "Guduchi" (Singh, 2008), although it is most often referred to as "Giloya" a mythological Hindu term that refers to the holy concoction that

prevents the godly spirits from ageing and keeps them youthful eternally. *T. cordifolia*, a herbaceous vine that grows 300 metres above sea level and found throughout the tropical Indian subcontinent and China, is a member of the Menispermaceae family. Although *T. cordifolia* is a resilient plant that can be cultivated in virtually any temperature, it loves a warm environment for the best development. The greatest time to grow this plant is during the rainy season, which is from July to August. This medicinal plant has the amazing capacity to generate aerial roots that may extend 10 metres down and 10 metres up from the earth. These aerial roots are crucial to the plant's ability to grow again. This plant doesn't actually die; instead, it either drops to the ground or expands to a new "life line," reinstating itself. *T. cordifolia* is a climber that needs help from the outside to flourish. Neem is known as NEEM GILOY when it helps *T. cordifolia* flourish. Giloy and neem share a similar chemical makeup and exhibit superior medicinal qualities when combined (Sharma et al., 2008).

T. cordifolia has a number of documented medical applications in Ayurveda. According to "Ayurveda," the nutrient-rich starch extracted from *T. cordifolia's* roots and stems can treat diarrhoea and dysentery. Ayurvedic "Rasayanas" derived from T. cordifolia strengthen the immune system and increase the body's resistance to numerous illnesses (Singh et al., 2003). The fresh plant components are thought to be more effective at treating illnesses. The plant's aqueous extract, known as Indian quinine, is effective in lowering high body temperatures brought on by a cold or dyspepsia. Along with these other uses, it is widely used for spermatorrhea, impotence, sclerotic disorders, urinary tract infections, acidosis, simple debility, and sexually transmitted illnesses. In addition to being used to cure leprosy and malaria, *T. cordifolia* roots also serve as natural sedatives (Kapoor, 2001).

A contemporary polymerase chain reaction-based approach for identifying genetic changes across and within the same species is called random amplified polymorphic DNA (RAPD). The DNA sequences of RAPD markers are amplified using a single, short, and randomly chosen oligonucleotide primer (usually 10 bases long). RAPD has been widely and successfully used to identify several species of medicinally significant plant. The need for research into and protection of therapeutic plant species is growing. The following goal have been set for the current study by taking into account the significance of *T. cordifolia* as a medicinal plant and RAPD as a potent technique to evaluate the genetic polymorphism of medicinal plants.

Materials and Methods

Plant Collection

Tinospora cordifolia leaves were collected in Tamil Nadu, India, from a variety of biological zones and altitudes ranging from 8 to 378 m MSL. Entirely of the plant samples were carefully categorised and brought back from the fields in ice boxes. The current research includes ten accessions of *T. cordifolia* from Trichy, Dindigul, Ariyalur, Ranipet, Vellore, Cuddalore, Coimbatore, Nagapattinam, Dindigul, Thirupathur (Table 1). Altitude was used as a reference point among other factors while examining genetic variation.

Table 1. Samples from various Tamil Nadu locations collected for RAPD analysis

S. No.	Place	District	Latitude	Longitude	Altitude (MSL)	Code
1.	Thittacheri	Nagapattinam	$10^0$52'09"N	$79^0$47'08"E	8 m	TCNT
2.	Virudhachalam	Cuddalore	$11^0$30'52"N	$79^0$19'31"E	33 m	TCCV
3.	Jayankondam	Ariyalur	$11^0$12'45"N	$79^0$21'45"E	61 m	TCAJ
4.	Kalavai	Ranipet	$12^0$46'01"N	$79^0$25'01"E	132 m	TCRK
5.	Thalaivasal	Salem	11035'10"N	78045'30"E	162 m	TCST
6.	Vedasandur	Dindigul	$10^0$31'50"N	$77^0$56'54"E	212 m	TCDV
7.	Gudiyatham	Vellore	$12^0$56'12"N	$78^0$52'46"E	268 m	TCVG
8.	Kinethukadavu	Coimbatore	$10^0$48'39"N	$77^0$01'20"E	308 m	TCCK
9.	Vaniyampadi	Thirupathur	$12^0$41'23"N	$78^0$37'09"E	341 m	TCTV
10.	Palani	Dindigul	$10^0$26'57"N	$77^0$30'58"E	378 m	TCDP

Extraction of genomic DNA using the modified CTAB technique

Tinospora cordifolia leaves yielded DNA (5gms). Using a pre-cooled mortar and pestle, leaves were pounded into a fine powder and then dissolved in liquid nitrogen. The powder was added to a centrifuge tube with 7 ml of preheated (65^0 C) DNA extraction buffer, and the mixture was forcefully agitated to create a homogeneous suspension. The suspension was incubated at 65^0 C for an hour, with inversion mixing performed every ten minutes. The mixture was extracted for around 15 minutes using an equal amount of phenol and chloroform (24:1). After incubation, centrifuged the samples at 10,000 rpm for 10 minutes. Transferring the top aqueous layer with care to a different centrifuge tube 0.6 litre of cold ethanol was added to this and stirred by inverting. Overnight, the samples were incubated at -20°C. The next morning, the samples were centrifuged for 10 minutes at 10,000 rpm after being mixed by inverting them. The particle was washed three times with 70% ethanol after the supernatant was discarded. Overnight, The pellet was dried by air. After drying, the dissolved the pellet in 50 l of TE.

PCR-RAPD- amplification

The Gene Amp PCR System 9700 R (Applied Biosystems, Carlsbad, USA) was used for the PCR amplification, with the following amplification parameters: initial denaturation at 94°C for 5 min, followed by 45 cycles of denaturation at 94°C for 1 min, primer annealing at 37°C for 1 min, amplification at 72°C for 2 min, and a final extension at 72°C for 5 min. Amplified DNA was electrophoresed in a 1.4% agarose gel using a 1 TAE buffer electrophoresis at 50 V for three hours, and ethidium bromide was used to observe the electrophoresis. Gene RulerTM, a 1-kb DNA ladder, was applied as usual.

Table 2 Primers list

S. No.	Codes	SEQUENCE	Tm^0c
1.	OPA - 01	5’ GA GT CT CA GG 3’	32.00
2.	OPC - 02	5’ GG TC TA GA GG 3’	32.00
3.	OPC - 14	5’ GG AG TA CT GG 3’	32.00
4.	OPA - 03	5’ AA GC CT CG TC 3’	32.00
5.	OPA - 09	5’ GT CC CG AC GA 3’	32.00
6.	OPC - 04	5’ AG CC AG CG AA 3’	32.00
7.	OPC - 15	5’ TC TG TG CT GG 3’	32.00
8.	OPA - 13	5’ GA CC GC CT TG T 3’	32.00
9.	OPA - 17	5’ GG GT AA CG CC 3’	32.00
10.	OPA - 20	5’ GT TT GC GA TC C 3’	32.00
11.	OPG - 11	5’ GT GA GA CG GA 3’	32.00
12.	OPC - 11	5’ AC CG AG CG AT 3’	32.00
13.	OPG - 02	5’ TG TG TG CT AA 3’	32.00
14.	OPA - 04	5’ GA TT GC CA AG T 3’	32.00
15.	TC - 01	5’ GG CT AA CG GC 3’	32.00
16.	TC - 02	5’ GG AC TG CA GA 3’	32.00
17.	TC - 03	5’ AG TC AG CC AC 3’	32.00
18.	OPG - 24	5’ CA GG CC CT TC 3’	32.00

The presence or lack of bands was used to assess genetic similarities. DNA bands may be seen and rated as present (1), absent (0), or not present, and a binary matrix was created. MVSP software was used for data processing, and genetic diversity was investigated.

RESULTS AND DISCUSSION

Researchers have identified 152 species in India that might be used to create ayurvedic medications, and *T. cordifolia* is one of these potentially useful plants (Srivastava et al., 2011). RAPD markers have the capacity to detect and define the genetic diversity of plants (Rout, 2002). Proper plant management of its genetic resources is aided by the many genetic components found in the crop and detected by markers reported Saha and co-workers in 2010. Despite the availability of several molecular markers, RAPD markers are explored in the current research because of their implications.

Sample collection

T. cordifolia stems were gathered in ten different places in Tamil Nadu, ranging from 8.00 to 378.00 meters above MSL (Table 1) over the years of 2022. Plant samples were collected from Thittacheri village of District Nagapattinam at the peak of 8m above MSL (Mean Sea Level), one town from municipality Cuddalore i.e Virudhachalam at the peak of 33m above MSL one town from municipality Ariyalur i.e Jayankondam at the peak of 61m above MSL one town from municipality Ranipet i.e Kalavai at the peak of 132m above MSL, one town from municipality Salem i.e Thalaivasal at the peak of 162m above MSL, one town from municipality Dindigul i.e Vedasandur at the peak of 212 m above MSL, one village from District Vellore i.e Gudiyatham at the peak of 268m above MSL, one town from municipality Coimbatore i.e Kinethukadavu at the peak of 308m above MSL, one village from District Thirupathur i.e Vaniyampadi at the peak of 341m above MSL, and one village from District Dindigul i.e Palani at the peak of 378m above MSL. The RAPD analysis and antibacterial properties of *T. cordifolia* have both been the subject of several investigations. For instance, Mohapatra and Rout, 2005 reported fresh plant samples were gathered from Kanyakumari District Tamil Nadu, while leaves of *T. cordifolia* were taken from Ambasamudram , Tirunelvi, Sankarankoil, Tenkasi and Alangulam, in District Tamil Nadu (Britto et al., 2010).

Tinospora cordifolia molecular profiling with RAPD markers

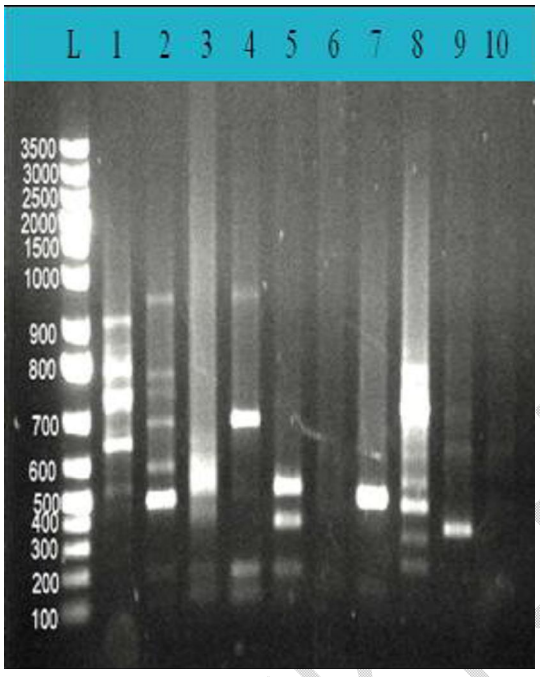
The genetic link and degree of relatedness among the several T. cordifolia accessions were discovered using RAPD primers. 18 decamer primers were first screened for polymorphism analysis, including TC - 01, TC - 02, TC - 04, OPA - 01, OPA - 03, OPA - 04, OPA - 09, OPA - 13, OPA - 17, OPA - 20, OPC - 02, OPC - 04, OPC - 11, OPC - 14, OPC - 15, OPC - 24, and OPG - 11 (Table 2). Nine primers were chosen from among them based on the highest level of polymorphism found in all DNA samples extracted from *Tinospora*

leaves collected from various places in Himachal. This was also utilised to identify and examine the genetic connections among the 10 plant accessions. The resultant amplified fragments were examined for genetic variants and relationships based on the bands are present or not. DNA bands were either present means indicted as one (1) or absent means indicted as zero (0), depending on data. After RAPD, the bands were seen under a UV transilluminator, and gel documentation system photographs were made, as shown in Figure 3. (A-H). All of the analysed samples revealed polymorphism. Less polymorphic primers included OPC – 15, OPC - 04, OPA -13 and OPA - 09 whereas markers OPA – 03 and OPC - 14 had fair to outstanding polymorphism, as did primers OPA-01 and OPC-2. Further cluster analysis was performed using the RAPD primer OPA-01. Table 3 provides an overview of primer codes, sequences, number of amplified alleles, number of polimorphic alleles, number of monomorphic alleles, polymorphic per cent and amplification status.

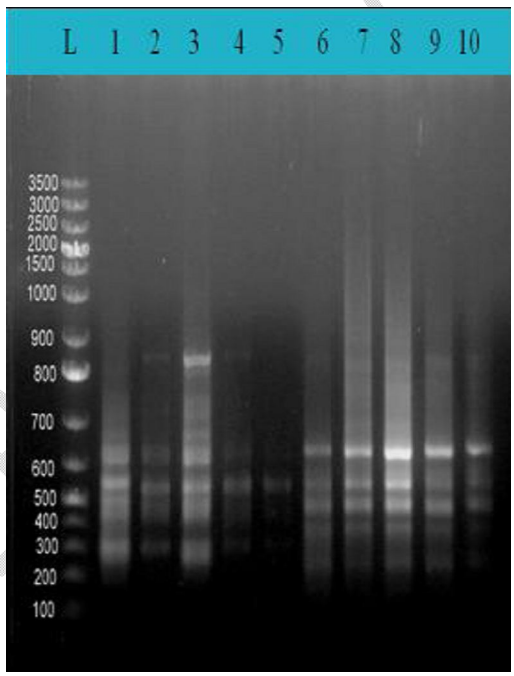
Table 3. *T. cordifolia* sample RAPD analysis using primers

S. No.	CODES	No. of amplified alleles	No. of polimorphic alleles	No. of monomorphic alleles	polymorphic %	Amplification Status
1	OPA - 01	6	6	0	100	Amplified
2	OPC - 02	5	4	1	80	Amplified
3	OPC - 14	6	6	0	100	Amplified
4	OPA - 03	4	4	0	100	Amplified
5	OPA - 09	5	5	0	100	Amplified
6	OPC - 04	4	4	0	100	Amplified
7	OPC - 15	4	4	0	100	Amplified
8	OPA - 13	4	4	0	100	Amplified
9	OPA - 17	-	-	-	-	No Amplification
10	OPA - 20	-	-	-	-	No Amplification
11	OPG - 11	-	-	-	-	No Amplification
12	OPC - 11	-	-	-	-	No Amplification
13	OPG - 02	-	-	-	-	No Amplification

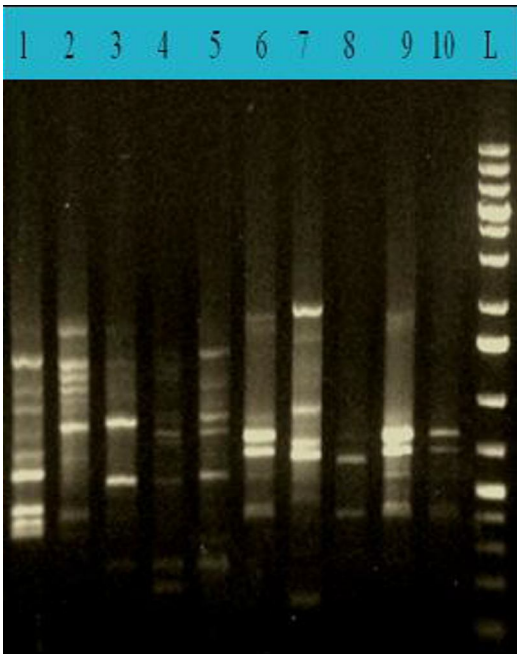
14	OPA - 04	-	-	-	-	No Amplification
15	TC - 01	-	-	-	-	No Amplification
16	TC - 02	-	-	-	-	No Amplification
17	TC - 03	-	-	-	-	No Amplification
18	OPG - 24	-	-	-	-	No Amplification



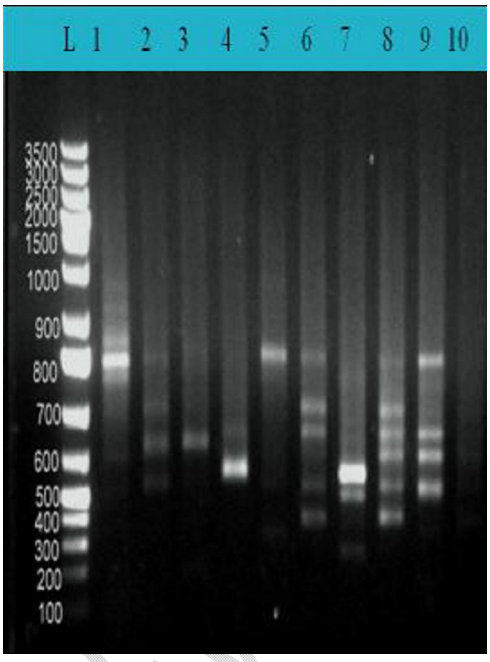
I* OPA 01



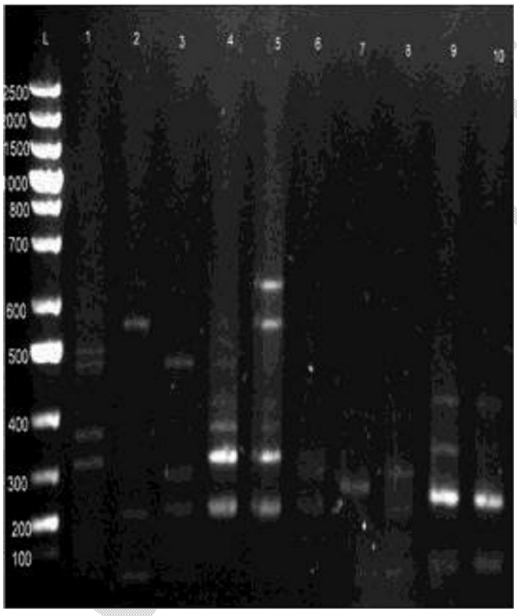
II* OPC 02



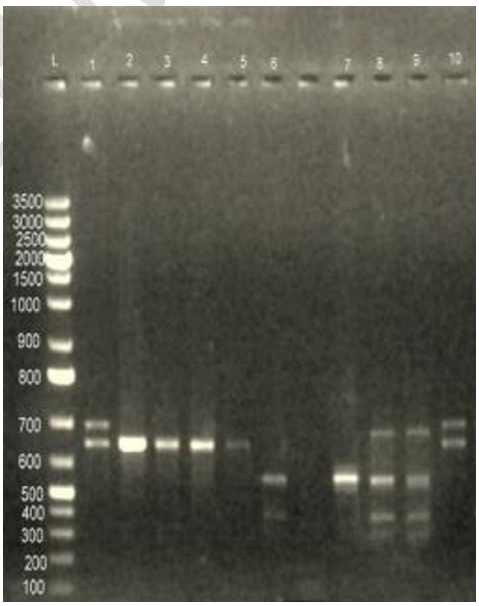
III* OPC14



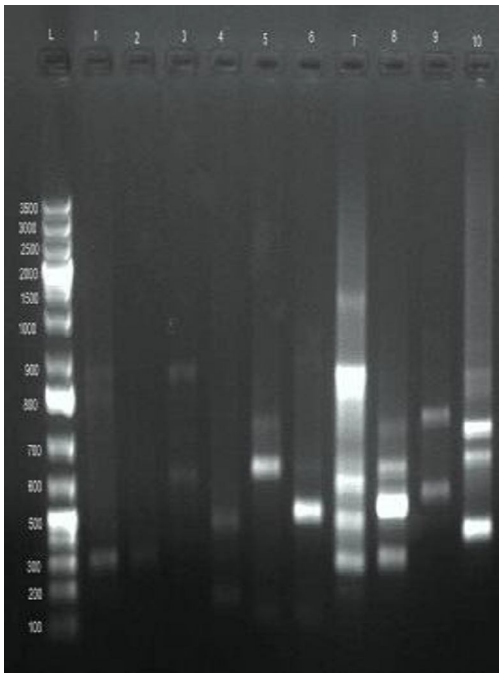
IV* OPA03



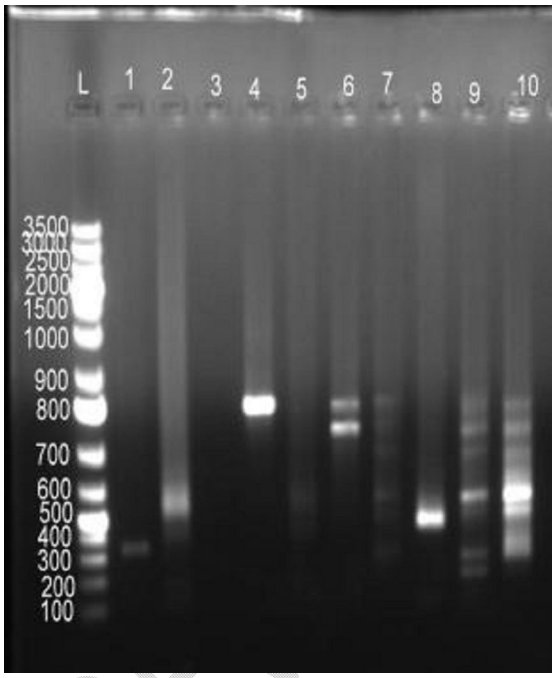
V* OPA - 13



VI* OPA - 04



VII* OPC - 04



VIII* OPC - 01

Figure 1: I and II demonstrate tha samples 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 underwent random amplification using the RAPD primers OPA01 and OPC2, which demonstrates the greatest polymorphism; C and D demonstrate that samples 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 underwent random amplification using the RAPD primers OPC14 and OPA03, which demonstrates the finest polymorphism; OPA-13, OPA-4, OPC-4, and OPC-1 RAPD primers, random amplification was performed in samples 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10, and the results indicated minimal polymorphism (E, F, G, and H).

***L (Ladder)** Sample from Lane 1 comes from Thittacheri,Nagapattinam; Sample from lane 2 comes from Virudhachalam, Cuddalore; Sample from Lane 3 comes from Jayankondam, Ariyalur; Sample from Lane 4 comes from Kalavai, Ranipet; Sample from Lane 5 comes from Thalaivasal, Salem ; Sample from Lane 6 comes from Vedasandur, Dindigul; Sample from Lane 7 comes from Gudiyatham, Vellore; Sample from Lane 8 comes from Kinethukadavu, Coimbatore; Sample from Lane 9 comes from Vaniyampadi, Thirupathur; Sample from Lane 10 comes from Palani, Dindigul

Initial RAPD results showed that 9 primers RAPD, namely OPC 2, OPA 01, OPC 14, OPA 13, OPA 03, OPA 09, OPC 1 and OPC 4 with 10 distinct samples of *T. cordifolia* accessions obtained from several districts in Tamil Nadu, exhibited amplification and varying

degree of genetic polymorphism. In the sample taken from Thalaivasal in the District of Salem, primer OPA01 demonstrated good polymorphism, while samples taken from other locations revealed significant genetic variety. Figure 1A displayed all of the distinct bands produced by amplifying using OPA 01 primer. Using Alfaview software, the amplified fragment sizes were estimated and discovered to be between 200 and 900 bp. Thus, the genetic relationship and degree of polymorphism of the various *T. cordifolia* samples were assessed using the illuminating OPA-01 primer as well as the sample obtained from Salem had the highest level of polymorphism.

Using RAPD markers, the genetic diversity of 15 Bhubaneswar-collected *T. cordifolia* clones was evaluated. There were 40 decamer primers used. To determine the genetic links among 15 clones, 15 primers were utilised, and these primers produced unique fragments of DNA (Rout, 2006). Using RAPD markers, Shinde et al. (2010) discovered limited amount of *T. cordifolia*'s genetic diversity. Twenty Western Himalayan *T. cordifolia* samples were chosen for genetic analysis using RAPD markers. Out of the 120 decamer oligonucleotide primers employed, four RAPD primers only OPC-13, OPA-16, OPC-07 and OPC-05 exhibited minimal genetic variation of *T. cordifolia* (Sharma et al., 2006). 40 germplasms were gathered from different parts of India, including Delhi, Kerala, Himachal Pradesh, Assam, and Jammu & Kashmir. Three species of *Tinospora* were polymorphized by five out of nine primers (Ahmad et al., 2009). The unweighted paired group method (UPGM) dendrogram produced by Ishnava et al. (2009) demonstrated the extent of genetic diversity among several T. cordifolia germplasm.

Cluster analysis and Similarity matrix

Ten samples' dendrogram demonstrating their genetic relatedness shows on fig. 2. The samples TCDP, TCST, TCRM1, and TCCK were discovered to be genetically related and to have reduced polymorphism. While distantly linked to samples TCDP and TCST, sample TCDV was genetically distinct from samples TCRM1 and TCCK. Despite being distantly related to sample TCVG, TCTV and TCRK shared genetic similarities. The samples TCDP and TCST were closely linked to TCRK, TCTV, and TCVG. Genetically, TCVG and TCDV were not related. The greatest polymorphism samples, TCNT and TCAJ, were distantly linked to each other and to all other samples. The genetic makeup of TCAJ was distinct from all other samples, demonstrating the most genetic variety among the *T. cordifolia* accessions that had been gathered.

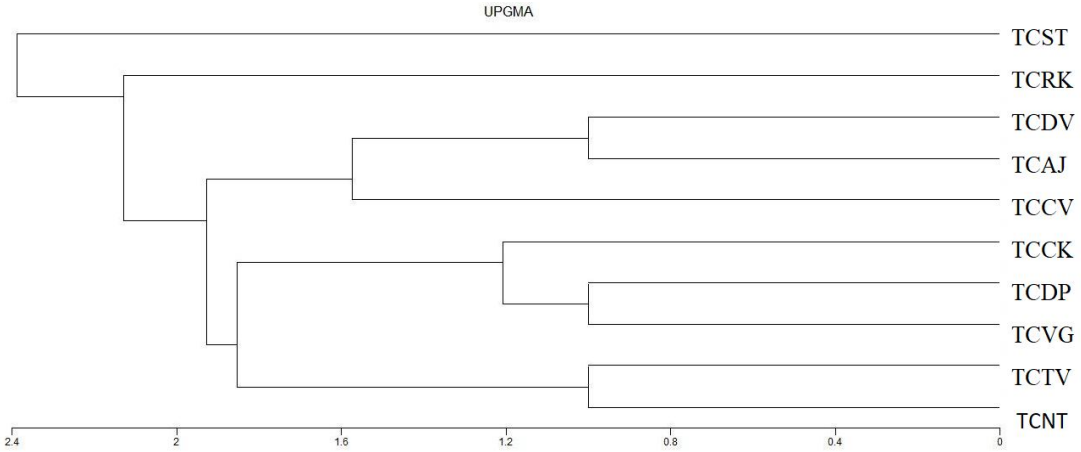


Figure 2. Ten samples' dendrogram demonstrating their genetic relatedness

Conclusion

Ten accessions of *T. cordifolia* were gathered for the current study from diverse locations throughout several districts of Tamil Nadu. The leaf samples of T. cordifolia that were procured from several areas in Tamil Nadu contained PCR amplifiable genomic DNA. Although leaves reacted to the improved CTAB technique of DNA separation, the leaves consistently produced large amounts of DNA. Using RAPD (random amplified polymorphic DNA) markers, the genetic polymorphism of multiple *T. cordifolia* samples was examined. Nine primers out of the 18 primers were capable of spotting differences between the 10 *T. cordifolia* samples that were obtained. Following analysis of RAPD, it was discovered that the DNA recovered from several *T. cordifolia* plants exhibited random amplification and various levels of genetic polymorphism. In the sample from Salem district the most genetic polymorphism was found.

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