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Research Article

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DNA Profiling of Biotypes of *Trichosanthes Tricuspidata*; an Important Ethno medicinal Cucurbit Using RAPD, ISSR and Scot Molecular Markers

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bstract: *Trichosanthes tricuspidata* Lour., is an important ethnomedicinal plant belonging to the Cucurbitaceae family. It is known / various vernacular names like Red ball snake gourd in English, Lal Indrayan in Hindi, Kalayar in Malayalam, and Avaduta in elugu. There are several studies about the medicinal applications of *Trichosanthes tricuspidata*, but there is scanty information)out molecular level identification of different biotypes of *Trichosanthes tricuspidata*. Hence, the present study was undertaken to evelop a protocol for genetic analysis of three biotypes of *Trichosanthes tricuspidata* collected from wild areas of Warangal, arimnagar and Adilabad districts of Telangana State, India by using Randomly Amplified Polymorphic DNA (RAPD), Inter Simple equence Repeat (ISSR) and Start Codon Targeted (SCoT) primers-based PCR method. Genomic DNA was isolated from 1-gram esh leaf material of three biotypes of *T. tricuspidata*. The primers OPA-2 of RAPD, S-5 of ISSR and S-8 of SCoT has shown good nplification with generation of monomorphic DNA bands ranging from 250 bp to 1000 bp by OPA-2 of RAPD primer; 500 bp to 500 bp by S-5 of ISSR primer and 1450 bp by S-8 of SCoT primer. In conclusion, no genetic variation was detected in three otypes, as the generated DNA bands from OPA-2 of RAPD, S-5 of ISSR and S-8 of SCoT primer-based amplifications were onomorphic. The developed protocol of genetic analysis of three biotypes of *T. tricuspidata* by RAPD, ISSR and SCoT primers)uld be used in near future for molecular level studies.

Keywords: *Trichosanthes tricuspidata*; DNA profiling, RAPD, ISSR & SCoT markers

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1. INTRODUCTION

The family Cucurbitaceae comprises of 130 genera and about 800 species. Some of the important genera are *Benincasa*, *Bryonopis*, *Citrullus*, *Corallocarpus*, *Cucumis*, *Cucurbita*, *Lagenaria*, *Luffa*, *Momordica*, *Trichosanthes, Sechium* etc.[1] The seeds and parts of fruit of cucurbits possess purgative, emetic, ant-diabetic, anti-helmintic properties due to its wide range of secondary metabolites.[2,3] The wild medicinal cucurbits of Eastern Ghats of peninsular India has several medicinal applications.[4] Mostly, fruits and vegetative parts like roots and leaves are reported to possess therapeutic properties[5] Among the different genera; *Trichosanthes* with approximately 100 species is considered as the largest genera of Cucurbitaceae family with wide distribution across the tropical Australia, China, Fiji, India, Japan, Malaysia, New Guinea, Sri Lanka, Vanuatu and Eastern Pacific countries.[6-8] In India, 22 species of *Trichosanthes* are distributed across South, North-Eastern India and Eastern Himalayas[9] *Trichosanthes* genus was recognized long back for its medicinal compounds and species of this genus can be easily recognized by their perennial, dioecious nature, tendrillar climbing habit, distinct petals of fimbriate.[10] or fringed type[11] and possess brightly coloured fruits.[12] Recently, the potential properties regarding the pharmacological applications of different species of *Trichosanthes* was reported.[13] *T. cucumerina* also possess anti-bacterial activity.[14,] Another *Trichosanthes* species, *T. dioica* demonstrated anti-hyperglycaemic and anti-hyperlipidaemic activities.[15] *Trichosanthes tricuspidata* Lour., is another important species in Cucurbitaceae family. The plant is distributed across Australia, China, Malaysia, Japan and India. It is known in English as Red ball snake gourd and also in various vernacular names such as Kaundal (Marathi), Avaduta (Telugu), Lal Indrayan (Hindi), Khe Ka Daeng (Thai) and Indreni (Nepal).[16]

The roots of *T. tricuspidata* have exhibited anti-oxidative effect on sildenafil induced migraine in albino mice, antipyretic effect on albino rats.[17] The fruit extract of *T. tricuspidata* showed moderate larvicidal effect on mosquito *Culex quinquefasciatus* Say.[18] The cytotoxic effect of cucurbitacin from pericarps of *T. tricuspidata* fruits was investigated-trichosanthin isolated from *T. tricuspidata* induced apoptosis in Leukaemia K56 cells and Trichosanthin is undergoing trials as a possible remedy for AIDS.[19] *T. tricuspidata* is little experimented plant with immense medicinal potential.[20] The seeds are soaked in wine which is used to treat stomach ache and is also used as purgative.[21] As per the above cited literature, there are several studies about the immense medicinal applications *of T. tricuspidata*, but there is scanty information about molecular studies with regard to genetic identification of different biotypes of *T. tricuspidata*. Hence, a study is undertaken to identify the genetic similarity or genetic diversity among different biotypes available in the North Telangana region. In the molecular analysis dominated era, genetic similarity or diversity of different biotypes of a particular species can be accurately assessed by carrying out DNA analysis using molecular markers like RAPD, ISSR, SNP's, AFLP and SSR. Utilization of these markers for genetic analysis are reported in *Capsicum*,[22] *Vicia*;[23] *Luffa*, [24] *Rauwolfia* [25] etc.Molecular markers have advantages over biochemical and phenotypic markers, as the technique is simple, cost effective and does not involve the utilization of radioactive probes.[26] In this study we report the application of RAPD, ISSR and SCoT primer based PCR analysis of three biotypes of *T. tricuspidata*.

2. MATERIAL & METHODS

2.1 Plant material

The wild plants of *Trichosanthes tricuspidata* Lour., were collected from three different locations, *viz.,* i) Laknepally (V) Narsampet (M) Warangal (D) Telangana (S), India (18.5962 °N,79.2902 °E) called as biotype-1, ii) Khanapur (V) Jagityal (M), Karimnagar (D), Telangana (S), India (18.5962 °N,79.2902 °E) called as biotype-2 and iii) Nirmal (V&M), Adilabad (D), Telangana (S), India (19.6641 °N,78.5320 °E) called as biotype 3. The plant material was authenticated as *Trichosanthes tricuspidata* Lour., (Voucher No.192) by Dr. Md. Mustafa, Department of Botany, Kakatiya University Warangal, Telangana, India.

2.2 Genomic DNA Isolation

Genomic DNA was isolated from fresh leaves (1 g) of *T. tricuspidata* biotypes 1,2,3 by C-TAB method.[27] The DNA pellet was dissolved in 1 X TE buffer followed by its quantitative determination by spectrophotometric method. About 20µl (25 ng) of DNA of each biotype was utilized for molecular analysis studies. [28]

2.3 RAPD, ISSR and SCoT primers-based PCR Analysis

RAPD analysis was performed as per the method of Williams *et al*[29] by using RAPD primers (Table-1). ISSR analysis was performed as per the method of Pradeep *et al.*,[30] by using ISSR primers (Table-2). SCoT analysis was performed as per the method of Rohela *et al.,*[31] by using SCoT primers (Table-3). PCR mixture was prepared using the following quantities of ingredients given in Table-4, Table-5 and Table-6 for RAPD, ISSR and SCoT analysis, respectively. Amplification was carried out in a thermocycler (GeneAmp PCR System 9700, Applied Biosystems California, USA). The programming conditions for RAPD, ISSR and SCoT analysis are given in Table-7, Table-8. The amplified DNA was subjected to electrophoretic separation on 1.5% Agarose gel by using 1X TAE buffer with a marker DNA (0.25-10kb).

3. STATISTICAL ANALYSIS

The empirical data obtained in the present study were analyzed using SPSS version 17 (SPSS Inc., Chicago, USA). Statistical hypothesis test, the Students "t" test was used to determine means of two sets of data are significantly are different from each other. The data were presented as mean ± SE. Probability value of not less than 0.05 was considered statistically significant.

Table 1: List of RAPD primers used in DNA profiling

S. No	Primer Code	Primer Sequence (5'-3')
1	OPA-1	5'-CAGGCCCTTC-3'
2	OPA-2	5'-AGTCACAC-3
3	OPA-3	5'-TGGGCGTCAA-3'
4	OPA-4	5'-ATTCGGTGA -3'
5	OPA-5	5'-AGGTCCG-3'
6	OPA-6	5'-TGCGAGCTG-3'
7	OPA-7	5'-GGCATGACT-3'
8	OPA-8	5'-TGGGCGTCAA-3'
9	OPA-9	5'-CCAGCAGCTT-3'
10	OPA-10	5'-GACTGCACAC-3'

Table 2. List of ISSR Primers Used in DNA profiling

S. No	Primer code	Primer Sequence (5'-3')
1	S 1	5'-AGAGAGAGAGAGAGAGC-3'
2	S 2	5'-AGAGAGAGAGAGAGAGG -3'
3	S 3	5'-GAGAGAGAGAGAGAGAT -3'
4	S 4	5'-GAGAGAGAGAGAGAGAC-3'
5	S 5	5'-TCTCTCTCTCTCTCTC -3'
6	S 6	5'-TCTCTCTCTCTCTCTC -3'
7	S 7	5'-AGAGAGAGAGAGAGAGT--3'
8	S 8	5'-AGAGAGAGAGAGAGAGTC-3'
9	S 9	5'-GAGAGAGAGAGAGAGAT--3'
10	S 10	5'-GAGAGAGAGAGAGAGA-3'

Table 3: List of SCoT primers used in DNA profiling

S No	Primer Code	Primer Sequence 5'-3'
1	S 1	CAACAATGGCTACCACCA
2	S 2	CAACAATGGCTACCACCC
3	S 3	CAACAATGGCTACCACCG
4	S 4	CAACAATGGCTACCACCT
5	S 5	CAACAATGGCTACCACGA
6	S 6	CAACAATGGCTACCACGC
7	S 7	AACGGTGCACCACGG
8	S 8	ACGACATGGCGACCAAC G
9	S 9	ACCATGGCTACCACCGAC
10	S 10	CCATGGAATCGA

Table 4. RAPD-PCR Mixture

S. No	Chemical	Quantity (µl)
1	50 ng of sample DNA	2.0
2	2.5 µM dNTPs	2.0
3	*Taq* buffer (100 mM Tris hydrogen chloride, pH 8.3, 500 mM Potassium chloride and 0.1% Gelatin)	2.0
4	25 mM Magnesium chloride	2.0
5	RAPD primer (Bioserve, India)	3.0
6	0.5 unit of *Taq* Polymerase	1.0
7	Autoclaved Milli Q water (PCR – grade water)	8.0
	Total Volume	20.0

µl-microliter

Table 5. ISSR - PCR Mixture

S. No	Chemical	Quantity µl)
1	50 ng of sample DNA	2.0
2	1X PCR Master mix (GCC Biotech)	15.0
3	10 p mole of ISSR primer (Bioserve, India)	3.0
	Total Volume	20.0

µl-microliter

Table 6. SCoT - PCR Mixture		
S. No	**Chemical**	**Quantity (µl)**
1	50 ng of sample DNA	2.0
2	1X PCR Master mix (GCC Biotech)	15.0
3	10 p mole of SCoT primer (Bioserve, India)	3.0
	Total Volume	20.0

µl-microliter

Table 7. RAPD- Thermocycler Programming Conditions			
Steps	**Temperature (°C)**	**Duration**	**Cycles (No.)**
Initial denaturation	94	5.0 min.	1.0
Denaturation	94	30 sec.	35.0
Annealing	37	45 sec.	35.0
Extension	72	2.0 min.	35.0
Final extension	72	7.0 min.	1.0

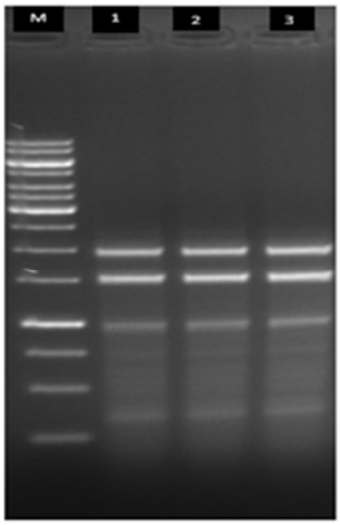
^{0}C-centigrade, min-minutes, sec-seconds

Table 8. ISSR & ScoT- Thermocycler Programming Conditions			
Steps	**Temperature(°C)**	**Duration**	**Cycles (No.)**
Initial denaturation	94	5.0 min	1.0
Denaturation	94	30 sec	35.0
Annealing	50	45 sec	35.0
Extension	72	2.0 min	35.0
Final extension	72	7.0 min	1.0

^{0}C-centigrade, min-minutes, sec-seconds

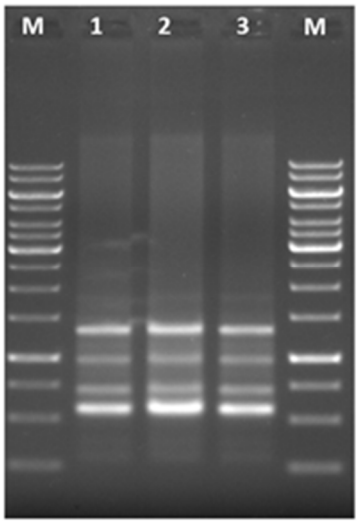
4. RESULTS AND DISCUSSION

The Genomic DNA (25ng) isolated from leaves (1 g) of *T. tricuspidata* biotypes 1,2,3 of the ten (10) RAPD primers employed, OPA-2 primer generated scorable monomorphic DNA bands in the range of 250 bp to1000 bp (Fig.1). Of the ten (10) ISSR primers employed, ISSR -5 primer generated scorable monomorphic DNA bands in the range of 500bp to 1500bp (Fig.2). Of the ten (10) SCoT primers employed, SCoT-8 primer generated a single scorable monomorphic DNA band of 1450bp in size (Fig.3). These primers viz., OPA-2, ISSR -5, SCoT-8 have given reproducible results, hence found suitable for molecular analysis studies to determine the genetic similarity among the different biotypes of *T. tricuspidata*. RAPD markers were reported for both genetic diversity and genetic fidelity for identification of elite biotypes in *Terminalia arjuna*.[32] Similar observations regarding the genetic similarity and diversity studies in a tree plant species was reported.[33,34] ISSR markers were also reported earlier for DNA profiling studies of *Ocimum* species.[35] SCoT markers has proved to be revealing, effective, successful and reproducible for genetic stability of plants.[36,37] SCoT is gene targeted a simple cost effective DNA based markers these primer target the short conserved region ATG *i.e.* translation start codon of plant genes.[38]



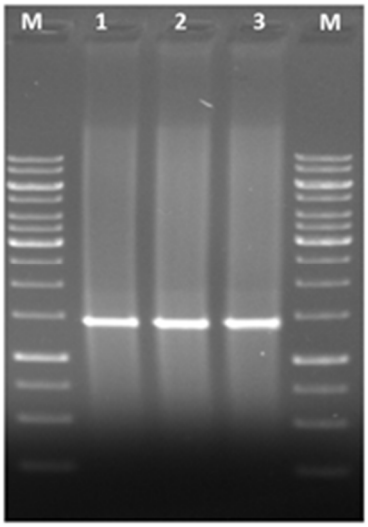
Lane M: Marker DNA- 250 bp-10,000 bp
Lane 1: DNA bands ranging from 250 bp to 1000 bp in biotype 1
Lane 2: DNA bands ranging from 250 bp to 1000 bp in biotype 2
Lane 3: DNA bands ranging from 250 bp to 1000 bp in biotype 3

Fig.1: Agarose Gel Electrophoretic separation of PCR-RAPD: OPA-6 amplified products of leaf genomic DNA of *T. tricuspidata* biotypes 1, 2 & 3



Lane M: Marker DNA- 250 bp-10,000 bp
Lane 1: DNA bands ranging from >500 bp to 1,500 bp in biotype 1
Lane 2: DNA bands ranging from >500 bp to 1,500 bp in biotype 2
Lane 3: DNA bands ranging from >500 bp to 1,500 bp in biotype 3
Lane M: Marker DNA- 250 bp-10,000 bp

Fig. 2: Agarose Gel Electrophoretic separation of PCR-ISSR: S-5 amplified products of leaf genomic DNA of T. tricuspidata biotypes 1, 2 & 3



Lane M: Marker DNA- 250 bp-10,000 bp
Lane 1: DNA band of 1450 bp in biotype 1
Lane 2: DNA band of 1450 bp in biotype 2
Lane 3: DNA band of 1450 bp in biotype 3

Fig.3: Agarose Gel Electrophoretic separation of PCR-SCoT: S-8 amplified products of leaf genomic DNA of *T. tricuspidata* biotype 1, 2 & 3

4.1 Fruit Phenotype

The fruit phenotype of *T. tricuspidata* biotypes 1,2,3 is given in Table 9. In this study no variation was observed in six parameters analyzed in *T. tricuspidata* biotypes 1,2,3. The number of fruits per plant, fruit weight (g), fruit diameter (cm), fruit length (cm) and number of seeds ranged from 19.0 ± 0.0 to 21.0 ± 0.0, 74.0 ± 0.08 to 78.8 ± 0.4, 7.56 ± 0.09 to 7.66 ± 0.11, 2.64 ± 0.12 to 2.86 ± 0.14, 72.1 ± 0.22 to 73.5 ± 0.12, respectively in *T. tricuspidata.* However, Tripathy *et. al*.,[39] reported morphological variations in *T. tricuspidata* inhabiting Similipal Biosphere Reserve (SBR), Odisha(S) forest which is spread over an area of 5,569 km^2. Geographically impacted variation in phenotype traits may be attributed to genetic and environmental factors.[40]

Table 9: Fruit phenotype of *Trichosanthes tricuspidata* biotypes 1, 2 and 3.

Parameter	*Trichosanthes tricuspidata*		
	biotype-1	biotype-2	biotype-3
No. of fruits	20.0 ±0.0	19.0 ±0.0	21.0 ±0.0
Fruit weight (g)	78.8±0.4a	74.9±0.21c	74.0 ±0.08 c
Fruit diameter (cm)	7.66±0.1e	7.6±0.14e	7.56±0.09d
Fruit length (cm)	2.73±0.6h	2.64±0.12g	2.86±0.14a
No. of seeds	73.5±0.12b	72.1±0.22a	73.5±0.12b
Weight of seeds (g)	37.1±0.21c	37.2±0.33c	37.1±0.22c
Fruit colour	Red	Red	Red

Data scored for 3 consecutive years viz., 2015, 2016 & 2017. Data represents an average of three replicates. Mean ± standard error (SE); Mean followed by the same superscript in a row is not significant at p value 0.05g-gram; cm-centimeter,

5. CONCLUSION

Primers of OPA-2, ISSR-5 and SCoT-8 has generated amplified products with monomorphic DNA bands in the range of 250bp to 1000 bp, 500bp to 1500 bp and 1450 bp, respectively in *T. tricuspidata* biotypes -1,2,3 hence no genetic variation was detected. The protocol thus developed could be used efficiently for molecular studies related to *Trichosanthes* species.

6. FUNDING/ACKNOWLEDGEMENTS

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7. AUTHORS CONTRIBUTION STATEMENT

RG Developed analytical methods of this work, TP&DK performed the DNA isolation of this work and CT to investigate and supervise the findings of this work.

8. CONFLICT OF INTEREST

Conflict of interest declared none.

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