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Original Research Article

Variants of *Tribulus* species – a scientific study through DNA RAPD – molecular characterization

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ABSTRACT

Gokshura a well-known drug in *Ayurveda* which is extensively used in many disease conditions like dysuria, asthma, diabetes, cough, oedema, cardiac disorders etc. *Tribulus terrestris* (Family – Zygophyllaceae) is an official source of *Gokshura* as per API. Five species of genus Tribulus are found throughout India with a slight morphological difference. In this study, three different species of Tribulus genus from different regions were subjected for molecular characterization by RAPD method. Analysis showed that three different samples gave clearly similar banding pattern with each of the random primers used and 80% similarity between the three samples were observed when the results were subjected to band scoring and analysis with clustering. Even through the micromorpholgical observations showed differentiating characters in mature carpels and intrastaminal glands of the selected species.

Introduction

"*Gokshura*", one of the components of *Dashamoola* (compound of ten drugs) widely used in *Ayurveda* singly as well as in compound. Although *Tribulus terrestris*, Zygophyllaceae family; is an official source of *Gokshura* as per API; Tribulus L. being highly polymorphic genus has about 30 species in the tropical and subtropical countries of the world; five species are found in India [1,2]. Exact determinations of its species are still considered difficult primarily due to the publications of a considerable number of specific epithets that spread over wide a range of variations[3]. Although the overall vegetative and reproductive features of this genus are widely used in the classification of species, mature carpels are considered to be the most reliable character for determinations[4]. Nevertheless, intrastaminal glands are also, sometimes, considered important for the delimitation of species in this genus, at least in the group level[5-6].

The characters of typical specimens with mature fruits are generally constant in a species growing in a relatively stable environment. However, in unstable environment, or species growing in extra arid climate, the characters often tend to change, particularly in the shape and ornamentation of the mericarps (spines and wings). During the course of development of fruits, the spines or wings on the mericarps show significant variations. Determinations of plant specimens collected at various stages of development of fruits often confuse taxonomists and force them to take their own judgments. This will eventually result in several confusions and false interpretations of names of the same species growing in different geographical locations [7]. In the present study, three samples of Tribulus genus collected from different geographical regions of India were subjected for molecular characterization (DNA fingerprints) by RAPD method.

Materials and methods

Collection of the samples

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The *Tribulus* genus samples were collected from following different regions

1. Chitradurga fort, State - Karnataka
2. Sikka thermal power station, Jamnagar, State – Gujarat
3. Kayathar, District – Tuticorin, State – Tamilnadu

Above three samples were botanically identified and confirmed by Assistant Director and Senior Botanist, Repository for medicinal resources, FRLHT, Bangalore and V. Chelladurai, Research Officer (Retired) – Botany (Scientist C), CCRAS, Govt. of India; and coded as following.

1. *Tribulus lanuginosus* L. (TL)
2. *Tribulus terrestris* L. (TT)
3. *Tribulus subramanyamii* P. Singh, Giri & V. Singh (TS)

Molecular Characterization (DNA Fingerprints)

Fresh leaves were used for molecular characterization and DNA fingerprints were obtained by standard and most convenient RAPD method. The RAPD reaction was performed following standard procedures at Aristogene Biosciences Pvt. Ltd, Bangalore[8].

DNA isolation

Young leaves were selected, cut into small pieces without cutting the veins; washed with distilled water and ethanol. 500 mg of leaves were cut into small pieces and homogenized using tissue homogenizer and dry ice. 5 ml of lysis buffer was added. The tubes were incubated at 65˚C for 1 hour in a dry bath with intermittent mixing and centrifuged at 10000 rpm for 10 minutes to separate out the unlysed cells. Supernatant was transferred to a fresh 30 ml centrifuge tube carefully. Equal volume of Chloroform was added, mixed well and centrifuged this at 10000 rpm for 15 minutes. The aqueous layer was pipetted out into the fresh 30 ml centrifuge tube without taking the interface. Equal volume of Isopropanol and 1/10th volume of 3M Sodium acetate was added and mixed well. Then it was incubated at room temperature for 5-10 minutes and centrifuged at 10000 rpm for 10-15 minutes. The supernatant was discarded. The pellet was washed with 500 µl of 70% ethanol. The pellet air dried and suspended in 500 µl of 1X Tris- EDTA buffer. To remove inhibitors for PCR; further the DNA sample was column purified.

Column purification

The column was placed in collection tube, 400 µl of equilibration buffer was added to the column and centrifuged at 10000 rpm for 1 minute. Collected buffer was discarded. 400 µl of equilibration buffer was added to the DNA samples, mixed and loaded into the column (This step was repeated till the DNA sample was completed). 500 µl of wash buffer 1 was added, centrifuged at 10000 rpm for 1 minute and buffer was collected. 500 µl of wash buffer 2 was added, centrifuged at 10000 rpm for 1 minute and buffer was collected. The column was centrifuged with empty collection tube to completely remove the wash buffer for 2 minutes. 50 µl of elution buffer was added to the column placed in new collection tube. Incubated at room temperature for 2 minutes and centrifuged at 10000 rpm for 1 minute and eluted sample was saved (elution 1). Previous step was repeated (DNA may elute in this fraction also) (elution 2). Quantization of eluted DNA samples was done by loading into the Agarose gel.

Table 1: RAPD PCR Sequences of primers used

Sr. No	Primers	Sequence
1.	OPA-02	TGCCGAGCTG
2.	OPD-02	GGACCCAACC
3.	OPC-07	GTCCCGACGA
4.	OPB-10	CTGCTGGGAC
5.	OPC-06	GAACGGACTC

*Random Primer used were

Table 2: Reactions were set up with PCR master mix and respective random

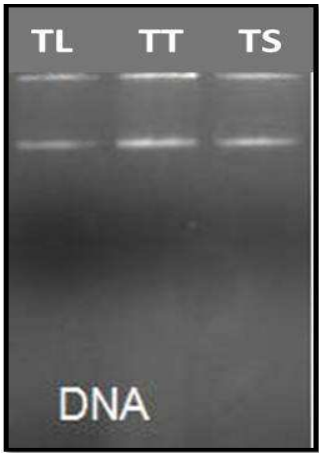
		Master mix
Components	1X	15X
D.D.H20	18 µl	270 µl
2X PCR Master Mix	20 µl	3000 µl
***Random Primer**	1 µl	15 µl
Template	1 µl	15 µl
Total Volume	40 µl	600 µl

Table 3: PCR cycle condition

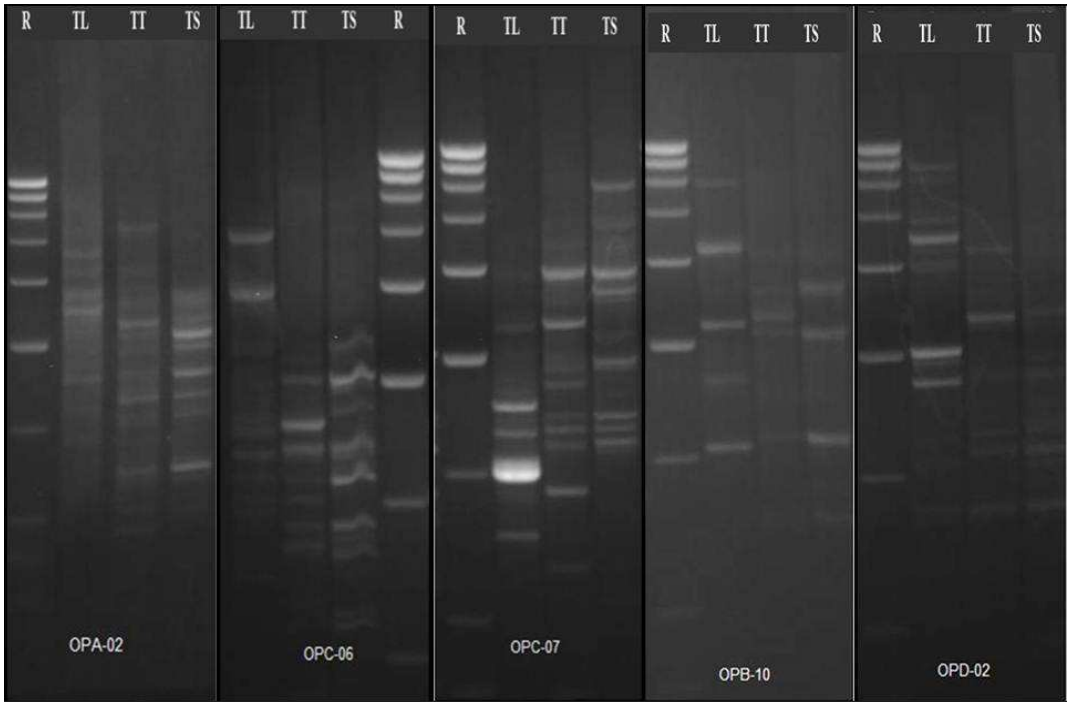
Temperature	Time	No. of cycles
94˚C	5 minutes	1
94˚C	30 seconds	40
45˚C	1 minute	
72˚C	1 minute 30 seconds	
72˚C	7 minutes	1

Note: R-mid range ruler ranging from 0.1, 0.2, 0.3, 0.6, 1, 1.5, 2, 2.5, 3 and 3.5kb

Observation and results



Quantification of DNA Samples

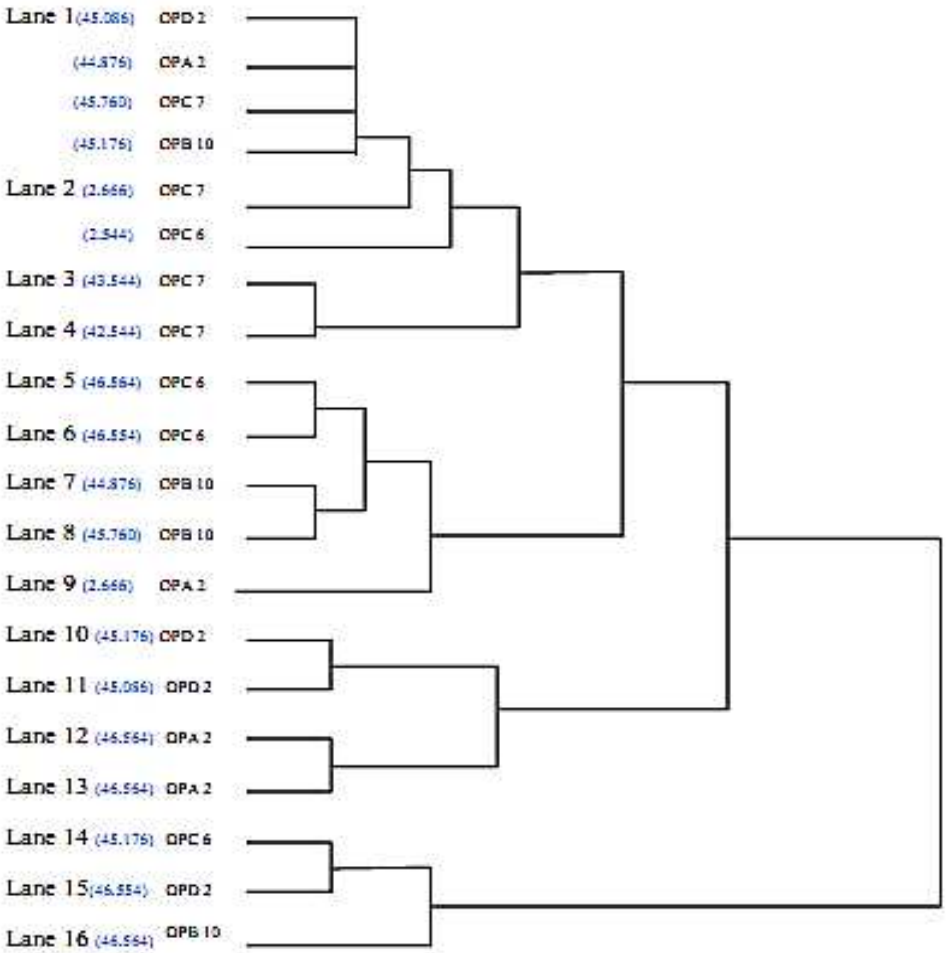


OPD 2, OPA 2, OPC 7 & OPB 10 - Lane 1, OPC 7, OPC 6 - Lane 2, OPC 7 - Lane 3, OPC 7 - Lane 4, OPC 6 - Lane 6, OPB 10 - Lane 7, OPB 10 - Lane 8, OPA 2 - Lane 9, OPD 2 - Lane 10, OPD 2 - Lane 11, OPA 2 - Lane 12, OPA 2 - Lane 13, OPC 6 - Lane 14, OPD 2 – Lane 15, OPB 10 - Lane 16.
Product size: TT – 295 bp, TL – 300 bp and TS – 284 bp

Comparing the similarity and diversity of sample sets with Jaccard coefficient

Jaccard coefficient is a statistic used for comparing the similarity and diversity of sample sets. The gels were scored by using binary coding method and uploaded to the software. Presence of band is noted as '1' and absence of the band is considered as '0'. The data was uploaded to the statistical software Bioconductor R, where the analysis was done based on the clustering method.

Cluster samples TT, TL and TS



OPD2, OPA2, OPC7 & OPB10- lane 1, OPC 7, OPC 6 –Lane 2, OPC7-Lane 3, OPC 7 –Lane 4, OPC6-Lane6, OPB10- Lane 7, OPB 10-Lane 8, OPA2- Lane 9, OPD2 –Lane 10, OPD2-Lane 11, OPA2-Lane 12, OPA2 –Lane 13, OPC6- Lane 14, OPD2-Lane 15, OPB10- Lane 16.

Note

• Marker (M): PhiX/Hae III/Marker is DNA ruler ranging from 0.1 to 3.5 kb

• Fragment sizes: A260 was checked and 50ng were loaded on to the gel.

Discussion

Medicinal plant species were authenticated by RAPD analysis. The advantages of RAPD technique include their simplicity, rapidity and low amount of genomic DNA required. RAPD marker provides a wide variation of amplification conditions as it was clearly visible up to a nailing temperature of 38^0 C and results were not affected with changes in the origin of the primer. Plant DNA extraction was done using CTAB method. The DNA was further purified using spin columns. RAPD was done with 5 primers which showed clear differentiation between samples TL, TS and TT. Each primer showed entirely different pattern for the three samples. Prominent band obtained at 0.6 Kb with OPD – 02 primer for sample TT. Prominent band obtained at 0.4 Kb with OPD – 10 and 15 primer for sample TL and TS respectively. According to the dendrogram, lanes 1 and 4 shared similarity between samples TT and TL, lanes 3 and 5 shared similarity between samples TT and TS. Similarity between samples TL and TS is seen in lanes 8 and 10. The binary scoring of primers OPA 2, OPD 2, OPC 6, OPC 7 and OPB 10 were observed for parallel characters. Product size of TT – 295 bp, TL – 300 bp and TS – 284 bp; output product similarities showed 80% of similar characters and 20% different characters between three samples TT, TL and TS. Samples TT and TL showed 90% similarity, samples TT and TS showed 85% similarity; whereas samples TL and TS showed 80% similarities. Thus the DNA

fingerprint result showed most of all similar genomic characters, expressed in pharmacognostical study. From the aspects of categorization, DNA fingerprints of selected samples can contribute significant scientific information, which further need to be elaborated up to bar coding system.

Conclusion

In case of same family members with same genus, many species create same identical morphology. When it is scientifically subjected to the pharmacognosy, phytochemistry and the advanced DNA finger printing give the specific differentiating and similarity characters which is scientifically necessary in this type of works. In RAPD analysis, samples *Tribulus terrestris*, *Tribulus lanuginosus* and *Tribulus subramanyamii* gave clearly similar banding pattern with each of the random primers used. When the results were subjected to band scoring and analysis with clustering, 80% similarity between samples *Tribulus terrestris*, *Tribulus lanuginosus* and *Tribulus subramanyamii* were observed.

Conflict of interest statement

We declare that we have no conflict of interest.

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