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MOLECULAR IDENTIFICATION OF *Tinospora sinensis* BY ITS2 SEQUENCE ANALYSIS

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Abstract- Intergenic spacer region 2 (ITS2) between 5.8S RNA and 28S RNA is well established genomic region for molecular identification of plant as potential universal barcode. The Present studies deals with molecular identification of Tinospora sinensis, by a unique ITS2 region. Briefly, the plant genomic DNA isolated by CTAB method and targeted for amplification of ITS2 region by eukaryotic conserved primers i.e. RM5.8s1F and RM 28s R for generation of 400 bp amplicon followed by DNA sequencing. Sequence analysis by BLAST search on NCBI database and phylogenetic studies suggests 96% identity with published sequence of Tinospora sinensis, i.e. intraspecies identity and 92% identity with other species of Tinospora genera i.e. T. malabarica. Phylogenetic analysis by MEGA4.1 suggest more close relationship with Tinospora sinensis Thus, this is a first report of ITS2 based molecular detection of Tinospora sinensis from Indian soil.
Key words- Tinospora sinensis, ITS 2 and molecular identification

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Introduction

Tinospora is among the largest and most geographically widespread genera within MenispermaceaeJuss, with distributions throughout tropical and subtropical regions of Asia, Africa, and Australia. Among the approximately 33 species distributed worldwide, three species, T. cordifolia, T. sinensis, and *T. crispa,* have ranges on the Indian subcontinent. *T. cordifolia* is found exclusively in India, Bangladesh, and Sri Lanka; *T. sinensis* and *T. crispa* have more extensive ranges throughout Southeast Asia. The fourth species, *T glabra*, occurs on the Andaman Islands, India Territory. All these species that occur in India are sold in the market under one name, T. cordifolia, thus the commercially available plant material is often a mixture of these species.

T. cordifolia is mentioned in ancient Ayurvedic literature as a constituent of several formulations used for the treatment of general debility, dyspepsia, and urinary diseases. The plant also has antispasmodic, anti-inflammatory, and antipyretic properties and is used in the treatment of gout, jaundice, and rheumatism [1-3]. *T. cordifolia* and *T. sinensis* are morphologically similar and often thought to be the same species [4]. The Morphological characters useful in identification include presence/absence of indumentum, tuberculate stems, leaf domatia, and floral and endocarp characters.Accurate authentication is constantly necessary to prevent the adulteration of target plant with other plant species. For the standardization of botanical preparations chromatographic techniques (HPLC, TLC, HPTLC, UV spectroscopy, mass spectroscopy, gas chromatography, infrared and NMR spectroscopy) have limitations because the compositions and relative amount of chemicals in a species varies with growing conditions, harvesting periods, post-harvest processes and storage conditions. This can be misleading if the samples are deliberately adulterated with a marker compound. Also, it is difficult to distinguish closely related species due to similar chemical compounds. Ordinary chemical authentication was not reliable enough to produce easy to- interpret results. Therefore, it is necessary to develop a more effective, accurate, reliable and sensitive technology for the authentication of medicinal plants. The DNA-based markers have now become a popular means for the identification and authentification of plants

because genetic composition is unique for each individual irrespective of the physical form and is less affected by age, physiological condition, environmental factors, harvest, storage and processing. The DNA extracted from leaves, stems or roots of a herb all carry the same genetic information and extracted DNA can be stored for longer duration as they are stable [5,8]. Nuclear ribosomal RNA genes and internal transcribed spacer 2(ITS2) sequence has become favoured marker in evolutionary studies at different taxonomic levels. In recent days, these sequence variations are used to develop specific markers for the identification and authentication of raw drugs and herbal formulations [6-10]. The objective of the present investigation was to develop molecular markers for distinguishing *Tinospora cordifolia*, and *Tinospora sinensis* based on their nuclear DNA ITS2 sequence.

Materials and methods

Plant material

Field sample of Tinospora Sinensis was collected from Latur, Maharashtra in India. The authenticity of the samples was confirmed by qualified taxonomist.

DNA extraction

For extraction of DNA fresh leaf samples were used, for the standardization of extraction protocol three different protocols were used namely, DNAzol method, CTAB method, and CTAB DNA recovery by DNAZol method. Among these methods CTAB method was found more effective method for the plant samples because it gave better results both quantitative and qualitative analysis of extracted DNA, when compared to other two methods. It was also found that the DNA that is sometimes lost during washing steps of CTAB method can be recovered by CTAB DNA recovery by DNAZol method.

Screening of primers by survey of literature

A large number of research articles were studied and suitable primers were screened for the amplification of ITS2 region in plants of Tinospora genus, these primers screened [2] were ordered from Integrated DNA technologies and the primers are as following:

RM 5.8S1F – 5'-TGA AGA ACG TAG CGA AAT GCG-3'(Tm = 55.8^0 C, MW = 6,528.3)

RM 5.8S2F – 5'-AAC CAT CGA GTC TTT GAA CGC A-3' (Tm = 57. 0^0 C, MW = 6,703.4)

RM 28SR – 5' -ACT CGC CGT TAC TAG GGG AA-3'(Tm = 57. 8 c, MW = 6,142.0) [2]

PCR STANDARDISATION

Gradient PCRs were carried out for two *Tinospora sinensis* for analysing the optimum annealing point, with two sets of primers RM 5.8S1 F & RM 28S and RM 5.8S2 F & RM 28S R. For a full PCR reaction of 100 ul volume, PCR reagents were added as follows: 10X PCR buffer, MgCl2 0.9 mM, dNTP mix 200uM , Forward and Reverse primers 100 pmoleseach, 3.0 U of Taq Polymerase with total plant genomic DNA, 920ngs. Final volume was adjusted by nuclease free water. PCR productswere visualized by agarose gel electrophoresis. Briefly, 2 % gel was prepared in 1X TAE buffer with EtBr final concentration of 300 ng/ml in 50 ml gel. Low range DNA ruler plus with total 16 bands, 3rd and 4th bands from bottom of the gel corresponds to 300 bp and 400 bp DNA products. 6X Orange dye, corresponds to 50 bp product was used as tracking dye. Gel was allowed to run at 75 volts for 35 minutes in Biorad Sub Cell GT submarine electrophoresis tank, with Biorad Universal Power pack. After 35 minutes, results were recorded in Biorad Gel documentation system and analyzed by Discovery Series software and stored for future use.

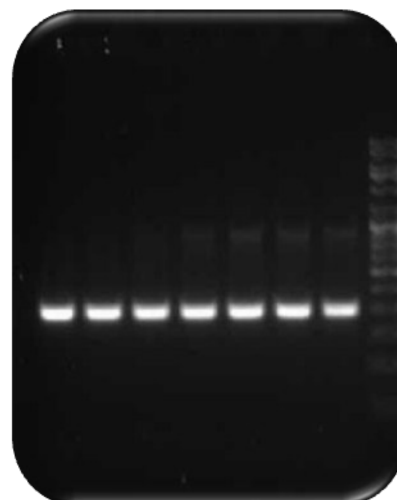

Fig. 1- Tinospora sinensis (Latur isolate) approximate 400 bp PCR product was visualized on 2 % agarose gel. Lane 1: Molecular weight marker: Low range DNA ruler. Desired PCR product of 350 bp size was observed. (MW; L2=55.0 ^{0}C; L3= 54.3 ^{0}C; L4=53 ^{0}C; L5= 51.2 ^{0}C; L6= 49.0 ^{0}C; L7= 47.1^{0}C; L8=45.8^{0}C).

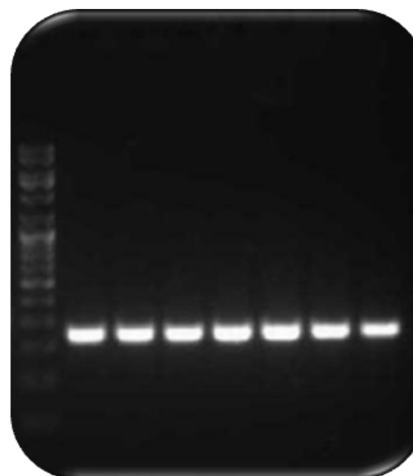

Fig. 2- PCR product of Tinospora sinensis (Latur isolate) approximate 350 bp PCR product was visualized on 2 % agarose gel, Lane 1: Molecular weight marker: Low range DNA ruler. Desired PCR product of 350 bp size was observed. (MW; L2=55.0 ^{0}C; L3= 54.3 ^{0}C; L4=53 ^{0}C; L5= 51.2 ^{0}C; L6= 49^{0}C; L7= 47.1^{0}C; L8=45.8^{0}C)

Scale up PCR and sequencing

Scale up of 400bp PCR product was done for further analysis at standardized annealing temperature 55^{0}C. Scale up PCR product was sent for DNA sequencing by Sanger's modified dideoxy chain termination method and raw data was received for detail analysis of DNA sequence of ITS2 region.

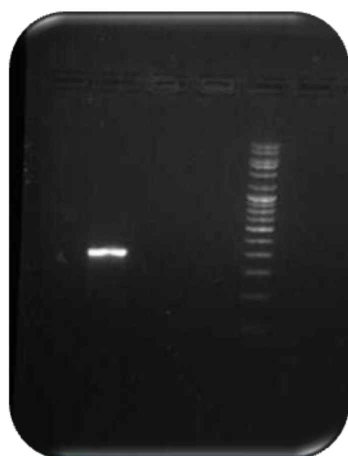

Fig. 3- scale up PCR: Tinospora sinensis (Latur isolate) approximate 400 bp PCR product was visualized on 2 % agarose gel. Lane 1: Molecular weight marker: Low range DNA ruler.

Phylogeny

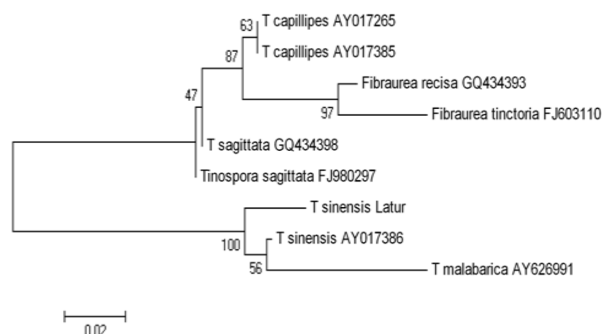

Fig. 4- The bootstrap consensus tree inferred from 1000 replicates. Number of nodes represents percent support for 1000 replication by Neighbour Joining Bootstrap test of phylogeny. Bar represents 0.02 percent sequence divergence.

Results and discussion

The Data derived from forward and reverse primer sequences were analyzed for data analysis. Sequence data was read by BioEdit and primarily analyzed by NCBI BLAST for comaparative studies with existing sequences in gene bank. ClustalW and MEGA4.1. Other online server i.e. ITS2 database was used for annotation of ITS2. And then the sequence was annotated by using ITS2 Database tool Annotate. Partial 5.8s RNA- complete ITS2- partial 28s RNA sequence is deposited for NCBI nucleotide database, with Accession No JN991264.

Phylogenetic tree constructed using MEGA4.1 software. Phylogeny shown relative distance between selected plants by Bootstrap NJ test of phylogeny. Our studied plant i.e. *Tinospora sinensis f*rom Latur (Ac.NoJN991264) is very closely matched with reported T. sinensis AY017386

Sequence analysis by BLAST search on NCBI database and phylogenetic studies suggests 96% identity with published sequence of *Tinospora sinensis,* i.e. intraspecies identity and 92% identity with other species of Tinospora genera i.e. T. malabarica. Phylogenetic analysis by MEGA4.1 suggest more close relationship with *Tinospora sinensis* Thus, this is a first report of ITS2 based molecular detection of *Tinospora sinensis* from Indian soil.

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