Journal of Carcinogenesis
Covering Genesis to Therapeutics

Journal of Carcinogenesis, ISSN: 1477-3163
2025; Vol 24: Issue 7s, https://carcinogenesis.com/

Genomic DNA Isolation and DNA Barcoding of Constituent Plants in Athiyadhi Kashayam for Species Authentication

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ABSTRACT

Siddha formulations form a major component of complementary and alternative medicine (CAM), but adulteration of traded medicinal plants threatens their safety and therapeutic reliability. Since morphological characters are often lost in processed raw drugs, DNA barcoding has emerged as a robust approach for authentication. In this study, we focused on *Athiyadhi Kashayam*, a classical Siddha polyherbal formulation, by authenticating five of its key ingredients—*Ficus racemosa*, *Senna auriculata*, *Cassia fistula*, *Syzygium cumini*, and *Salacia oblonga*. Genomic DNA was isolated from stem bark tissues using a modified CTAB protocol with β-mercaptoethanol. DNA quality and concentration were confirmed by agarose gel electrophoresis, Nanodrop spectrophotometry, and UV visualization. PCR amplification was performed with *ITS* and *rbcL* primers, both yielding successful amplification. The resulting sequences were analysed using BLAST against NCBI GenBank, confirming species identity. Validated sequences were submitted to GenBank, and species-specific barcodes were generated.

The results highlight the effectiveness of *ITS* and *rbcL* markers in authenticating constituent plant species of *Athiyadhi Kashayam*. This study underscores the importance of DNA barcoding as a reliable tool to ensure authenticity of raw drugs, prevent adulteration, and enhance the credibility of traditional formulations. The genomic data generated also contribute to a growing reference database for medicinal plants, supporting regulatory compliance and quality assurance in herbal drug research and industry..

Keywords: *Species authentication, DNA barcoding, Medicinal plants, Athiyadhi Kashayam, ITS marker.*

How to Cite: Dr. C. Meenakshi, Dr. S. Chithra, Dr. M. Ramani, Dr. S. Balamani, Dr. G. Bharath Kumar,Dr. S. Selvakumar, (2025) Genomic DNA Isolation and DNA Barcoding of Constituent Plants in Athiyadhi Kashayam for Species Authentication, *Journal of Carcinogenesis*, *Vol.24, No.7s*, 809-819

1. INTRODUCTION

Medicinal plants play an important role in healthcare systems and it is the foundation of complementary and alternative medicine. In traditional Siddha system of medicine the polyherbal formulations are frequently used to treat various ailments due to their synergistic approach. [1, 2, 3] Currently, people depend more on Siddha mode of treatments which increases the commercialization of herbal drugs. This raise has led to serious concerns towards adulteration, substitution, and misidentification of raw drugs. The process of adulteration reduces the therapeutic effect of the drug and it might induce toxicity, posing signification health risk to consumers. These challenges are especially evident in raw drugs, as they are available in the form of dried powders, where morphological characters used for species identification are no longer evident. [4, 5]

To overcome these limitations, DNA barcoding has emerged as a reliable molecular approach for plant species authentication. This technique uses short, standardized regions of DNA—such as *rbcL*, *matK*, and the internal transcribed spacer (ITS)—to enable rapid and accurate identification of medicinal plants. Previous studies have demonstrated the utility of DNA barcoding in detecting adulteration rates ranging from 20% to nearly 50% in traded herbal raw drugs, highlighting its critical role in ensuring quality control of herbal formulations. By generating barcode reference sequences and linking them to public databases such as GenBank, DNA barcoding also strengthens regulatory frameworks and scientific validation of traditional medicines. [6, 7]

Athiyadhi Kashayam is a classical Siddha polyherbal formulation traditionally prescribed for metabolic and inflammatory disorders. It contains five key medicinal plants—*Ficus racemosa*, *Senna auriculata*, *Cassia fistula*, *Syzygium cumini*, and *Salacia oblonga*—each with well-documented pharmacological activities. Despite its widespread use, authentication of these constituent species at the molecular level has not been extensively documented. [8, 9]

The present study aims to isolate high-quality genomic DNA from the stem bark of the five constituent plants of *Athiyadhi Kashayam* and to perform DNA barcoding using *ITS* and *rbcL* markers. The obtained sequences were analysed through BLAST for species confirmation, submitted to GenBank, and converted into DNA barcodes. This work provides molecular evidence for authenticating *Athiyadhi Kashayam* ingredients and contributes to the genomic documentation of medicinal plants, thereby promoting the safety, reliability, and global acceptance of Siddha formulations.

2. MATERIALS AND METHODOLOGY

2.1 Collection of Sample

In accordance with *Mega Nivarana Bodini Ennum Neerizhivu Maruthuvam's* literature, *Athiyadhi Kashayam* (AK), a blend of *Ficus racemosa, Cassia auriculata, Cassia fistula, Syzygium cumini,* and *Salacia reticulata*, was prepared for this study.[9] The ingredients for the aforementioned *Athiyadhi Kashayam* were purchased from a reputable country pharmacy in Chennai. Every piece of bark was gathered, cleaned with water, allowed to dry in the shade, and then ground into a coarse powder.

2.1.1 Identification and Authentication: The Department of Pharmacognosy, Siddha Central Research Institute, Arumbakkam, Chennai - 600106, recognized and certified the identification and authenticity of all the ingredients of *Athiyadhi Kashayam*. All the ingredients specimen samples were labelled and stored for later use.

2.2 Genomic DNA Isolation

About 100 mg of plant tissue was ground with liquid nitrogen to make fine powder using mortar and pestle. Added 1 ml of preheated CTAB extraction buffer with 20µl of β-mercaptoethanol to the mortar and finely ground. The contents were transferred into a 2 ml centrifuge tube and incubated for 20 to 30 min at 65°C on a water bath. After centrifuging using Refrigerated Centrifuge (Eppendrof, 5418R) the tube at 12,000 rpm for 10 min the supernatant was transferred to a fresh centrifuge tube and added equal volumes of chloroform: isoamyl alcohol (24:1) mixture and mixed gently by inverting tubes till an emulsion was formed. The tubes were centrifuged at 13,000 rpm for 12 min. The clear aqueous phase was transferred to fresh centrifuge tubes and equal volumes of ice-cold isopropanol was added. The sample was incubated overnight at -20°C and centrifuged at 12000 rpm for 3 min. The supernatant was discarded and the pellet was washed with 70% ethanol. DNA pellets were then air-dried at room temperature by allowing evaporation of uncovered centrifuge tubes. The pellets were suspended in an appropriate volume (20-30 µl) of T10E1 buffer. [10, 11]

2.3 Qualitative and Quantitative estimation of DNA

The quality and concentration of genomic DNA were checked by running the DNA sample on 1% agarose gel. The DNA concentrations were rechecked by visual assessment of band intensity under UV-trans-illuminator (Biorad, GelDoc Go, USA). The quantity of 1 µl of isolated DNA was checked using Nanodrop (Thermoscientific, Nanodrop One, USA). [11]

2.4 PCR Amplification

The DNA barcode candidate ITS and rbcl were used for PCR amplification as the same resulted in amplification. The isolated DNA was used as a template for PCR reaction and carried out in a thermocycler (Applied Biosystem,VeritiTM, USA). The PCR products were then loaded onto 1% agarose gel and the amplification was confirmed. [12]

2.5 Sequence analysis and submission in NCBI

FASTA format of the nucleotides were obtained using Finch TV from the chromatogram. The FASTA was fed into Basic Local Alignment Search Tool (BLAST) algorithm of NCBI to identify the closest matching sequence in the nucleotide database of GenBank. After confirmation of the species, the sequence was submitted to NCBI with the necessary details to obtain GenBank ID. The sequence was converted to Barcode using the software BioRad barcode generator. [12]

3. RESULTS AND DISCUSSION

3.1 Genomic DNA isolation, PCR and Sequencing

The macroscopic details of the species of *Athiyadhi kashayam* is given in Fig.1. The genomic DNA was isolated from the authenticated sample and its quality was assessed spectrophotometrically followed by agarose gel electrophoresis (Table 1). The genomic DNA concentration was 183.4 ng/µl for *Ficus racemosa,* 1160.7 ng/µl for *Senna auriculata*, 793.3 ng/µl for *Cassia fistula*, 785.4 ng/µl for *Szygium cumini*, 102.7 ng/µl for *Salacia oblanga*. An absorbance (A260/A280) ratio of 1.96, 1.96. 1.44, 1.8, 2.1 respectively indicated insignificant levels of contaminating proteins and polysaccharides. The genomic DNA isolation was high in *Senna auriculata*, *Cassia fistula, Szygium cumini* compared to the other two species. The PCR amplified product after electrophoresis was subjected to gel documentation together with a 100 bp DNA ladder (Fig.2). The sequence was obtained using ITS as the marker (Fig. 3), the sequence was converted to barcode (Table 2) and the sequences were submitted to GenBank (Table 3).

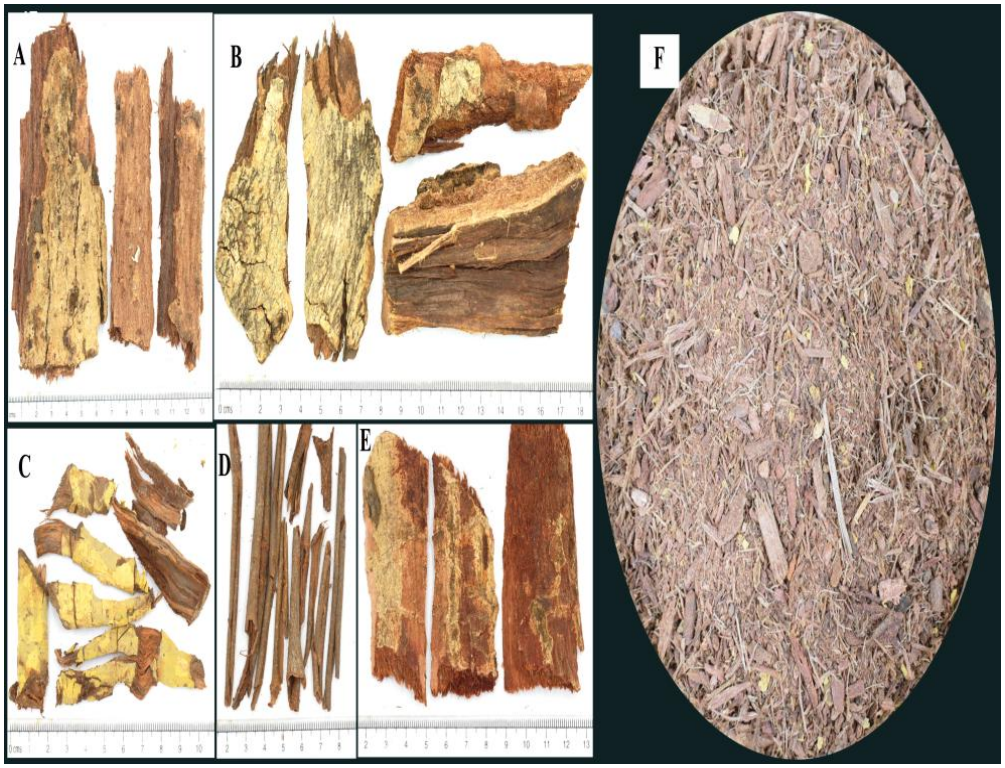
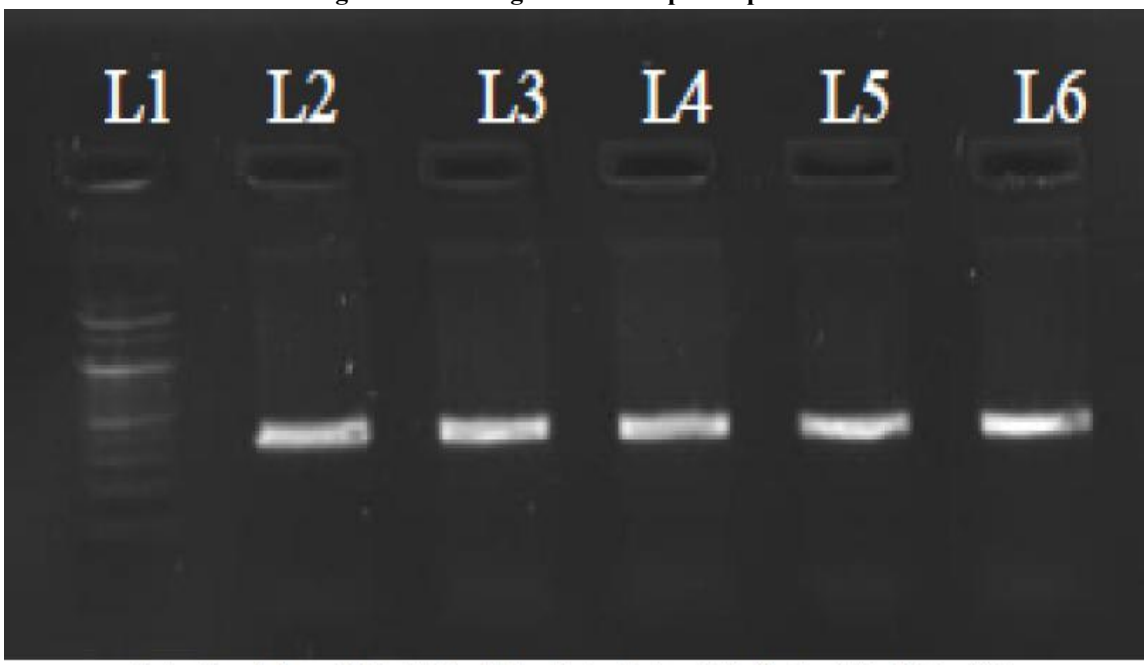


Fig. 1 Macroscopy of Athiyadhi kashayam A, Syzygium cumini; B, Ficus racemosa; C, Salacia reticulata; D, Cassia auriculata; E, Cassia fistula; F, formulation

Table 1. Quality check and quantification of DNA

Sample	Concentration in ng/µl	A260/280	A230/260
Ficus racemosa	183.4	1.96	1.44
Senna auriculata	1160.7	1.96	1.32
Cassia fistula	793.3	1.44	0.48
Szygium cumini	785.4	1.80	0.51
Salacia oblanga	102.7	2.10	0.39

Figure 2. Gel image of PCR amplified product



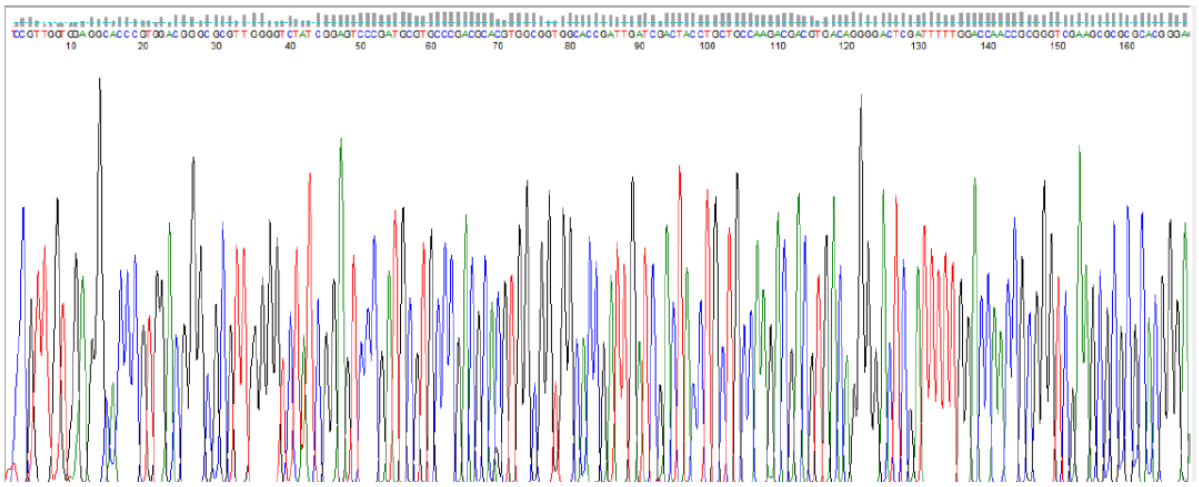
L1: Ladder, L2: FR, L3: SA, L4: CF, L5: SC, L6: SO

3.2 Chromatograms of *Athiyadhi kashayam*

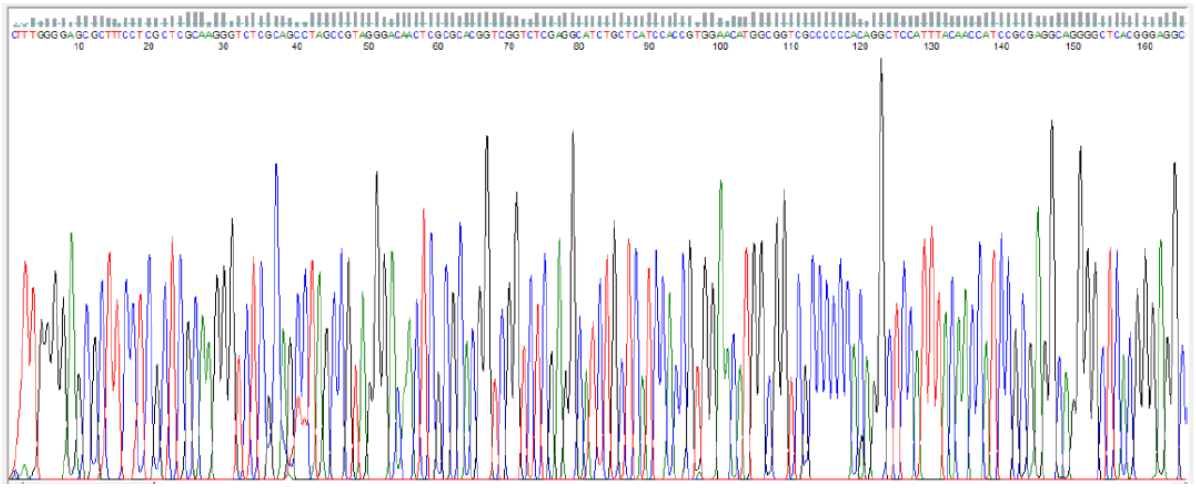
The DNA sequencing chromatograms for the barcode regions of *Ficus racemosa* (SCRIPCOGFR21), *Senna auriculata* (SCRIPCOGSA25), *Syzygium cumini* (SCRIPCOGSC28), and *Salacia oblonga* (SCRIPCOGSO22) were obtained using Sanger sequencing is given in Fig 3. The chromatograms showed clear and well-defined peaks for the first 80 to 100 bases. This indicated high-quality sequencing data. The base calls matched the expected nucleotides with minimal noise in the initial regions. However, the signal clarity gradually declined beyond the 100th base. This decline was characterized by overlapping peaks and background noise, likely due to reduced signal strength or sequence complexity. Despite some unclear areas, enough high-quality sequence data was generated for reliable species identification. Manual editing and verification of the sequences were done using chromatogram analysis software to fix ambiguous base calls. The final sequences were then used for further phylogenetic analysis and species authentication.

Figure 3. Chromatograms of samples

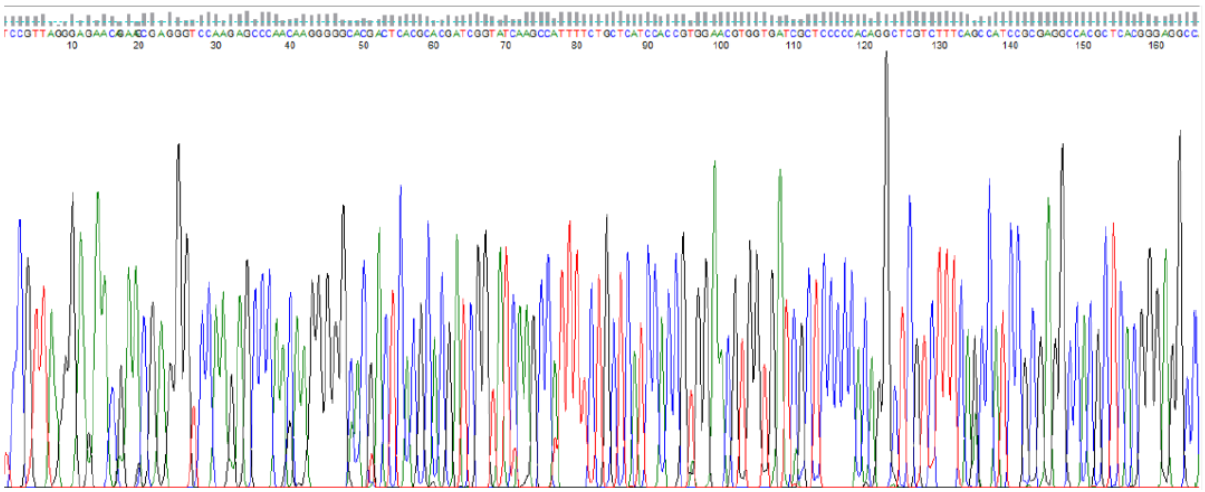
SCRIPCOGFR21 *Ficus racemosa*



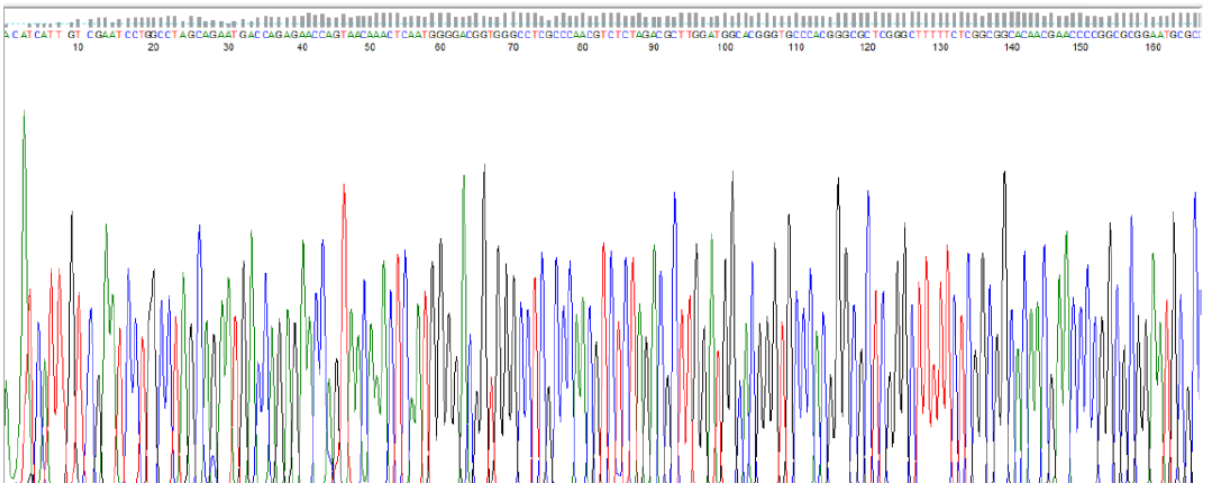
SCRIPCOGSA25 *Senna auriculata*



SCRIPCOGCF20 Cassia fistula



SCRIPCOGSC28 *Syzygium cumini*



SCRIPCOGSO22 *Salacia oblonga*

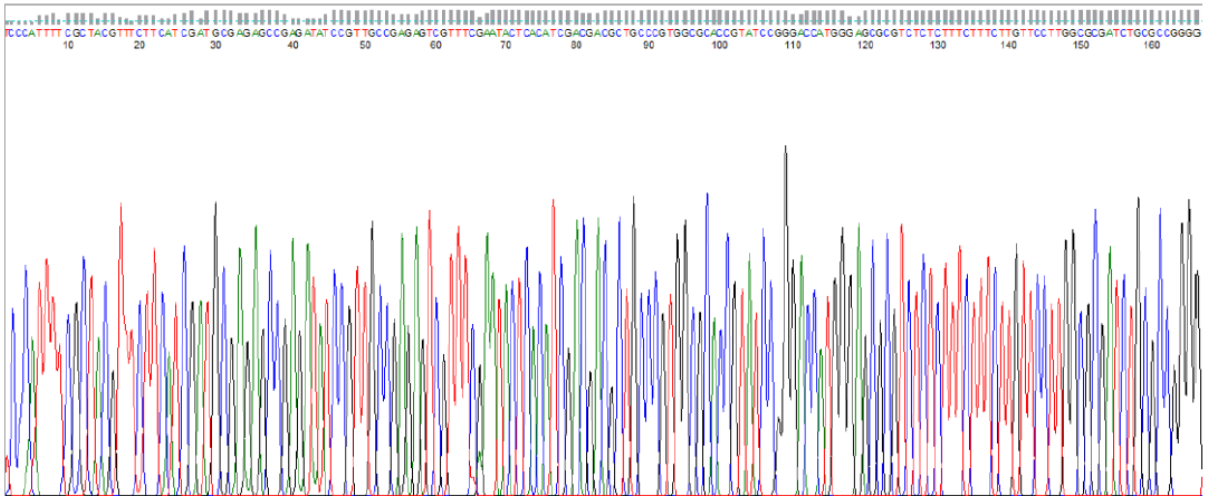


Table 2. Sequence obtained

Voucher No.	Sequence with Barcode and BLAST Hit
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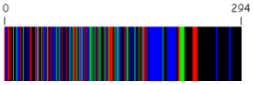

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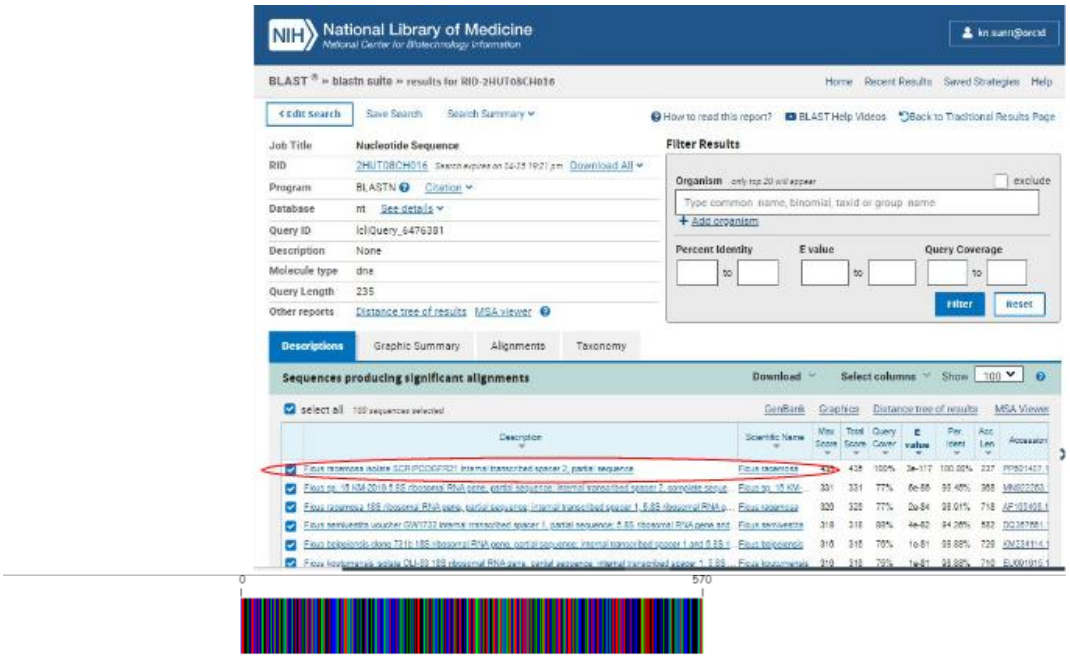

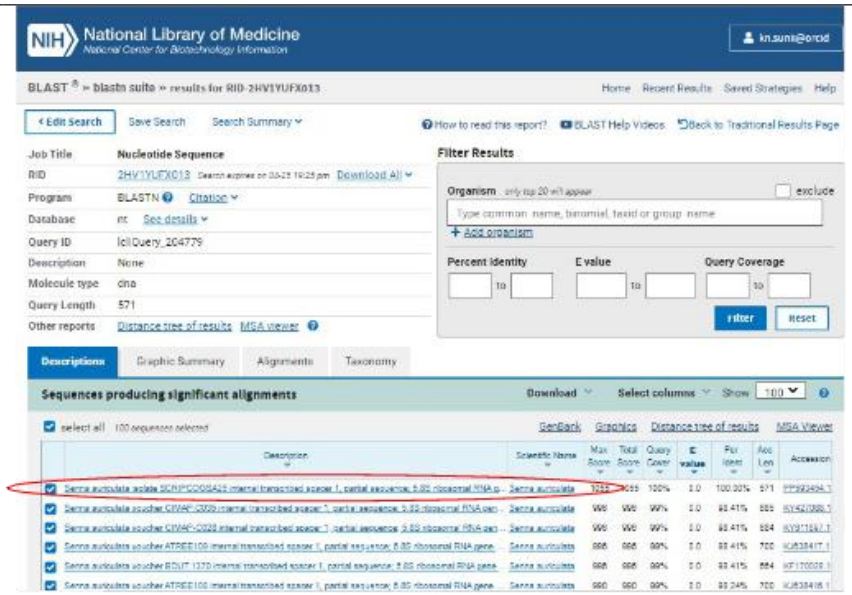

SCRIPCOGCF20tccgttagggagaacagaagcgagggtccaagagcccaacaaggggcacgactcacgcacgatcggtatc
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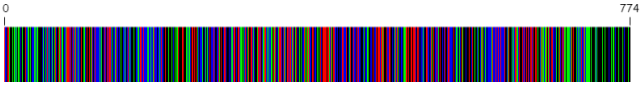

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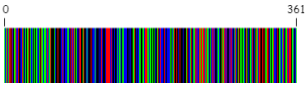

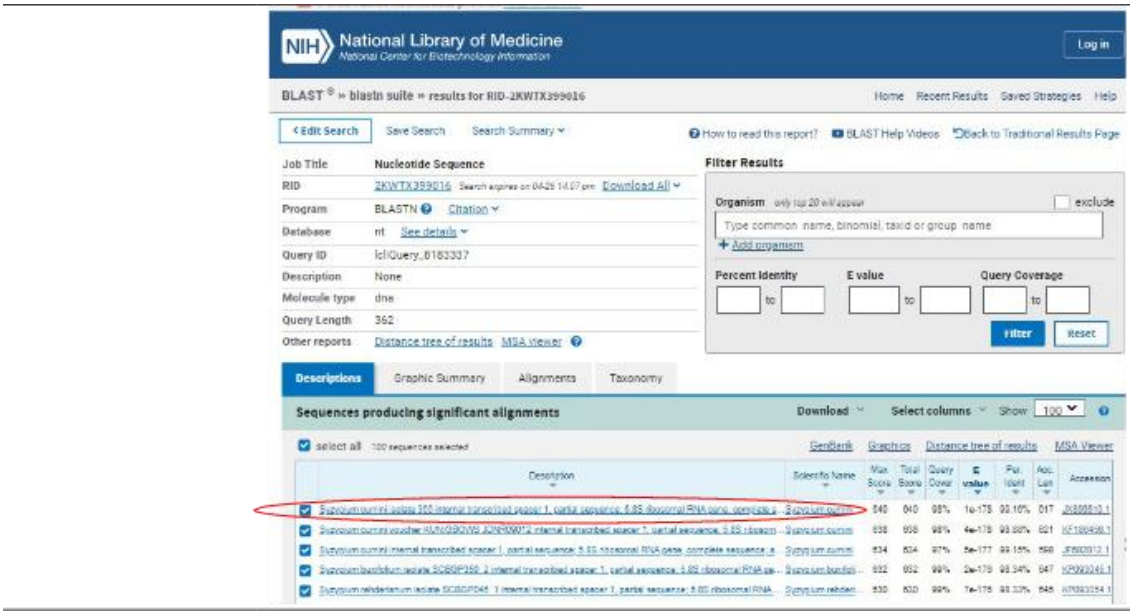

SCRIPCOGSO22 tcccattttcgctacgtttcttcatcgatgcgagagccgagatatccgttgccgagagtcgtttcgaatactcacatc
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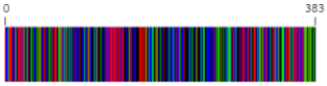

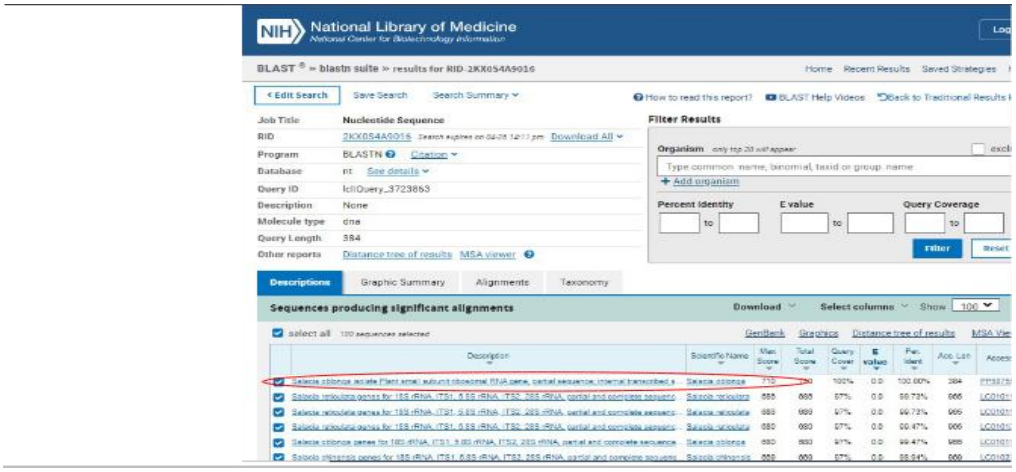

Table 3. GenBank submission details

Code	Voucher no.	Submission Id	Accession No	Authors	Gen Bank Link
07072301	SCRIPCOGFR21	SUB14359918	PP601407	Meenakshi,C BharatKumar,G. Remya,A. Divya,K.G. and Sunil Kumar,K.N.	https://www.ncbi.nlm.nih.gov/nuccore/PP601407.1?report=GenBank
07072302	SCRIPCOGSA25	SUB14359925	PP593454	Meenakshi,C. BharatKumar,G., Remya,A., Divya,K.G. and Sunil Kumar,K.N.	https://www.ncbi.nlm.nih.gov/nuccore/PP593454.1?report=GenBank
07072303	SCRIPCOGCF20	SUB14359905	PP590623	Meenakshi,C., BharatKumar,G. Remya,A. Divya,K.G. and Sunil Kumar,K.N, Pushkar K	https://www.ncbi.nlm.nih.gov/nuccore/PP590623.1?report=GenBank
07072304	SCRIPCOGSC28	SUB14399698	PP716805	Meenakshi,C., Bharat Kumar,Sunil Kumar K.N., Remya,A. and Divya,K.G,	https://www.ncbi.nlm.nih.gov/nuccore/PP716805
07072305	SCRIPCOGSO22	SUB14359922	PP587558	Meenakshi,C., Bharat Kumar,G., Divya,K.G., Remya,A., Sunil Kumar,K.N. and Pushkar K	https://www.ncbi.nlm.nih.gov/nuccore/PP587558.1?report=GenBank

4. DISCUSSION

Variable concentrations were obtained from the genomic DNA isolation of the individual *Athiyadhi kashayam* plant species. This could be explained by variations in the species' levels of polysaccharides, secondary metabolite content, and leaf texture. *Senna auriculata, Cassia fistula,* and *Syzygium cumini* generally yielded high concentrations of DNA, while *Ficus racemosa* and *Salacia oblonga* yielded comparatively lower amounts. Due to the presence of polyphenols, tannins, and complex carbohydrates, which frequently impede the effectiveness and purity of DNA extraction, such variability in DNA yield is typical in medicinal plants. While *Cassia fistula* displayed a slightly lower ratio (1.44), indicating partial contamination with polysaccharides or phenolic compounds, the majority of the DNA samples were found to be of acceptable purity based on the A260/A280 ratios, which ranged between 1.44 and 2.1. However, the distinct bands that were obtained after agarose gel electrophoresis confirmed that the DNA was sufficiently pure for PCR amplification. [13]

All of the tested species had amplicons produced by amplification using the ITS (Internal Transcribed Spacer) marker. Because of its high copy number, ease of amplification, and species-level discriminatory power, ITS have been used extensively as a universal DNA barcode region for plants. *Athiyadhi Kashayam's* ability to successfully generate barcodes for every species attests to its value in authenticating intricate polyherbal formulations, where species-level identification may require more than just morphological and microscopic traits.[14]

The acquired sequences were added to the global database of DNA barcodes for medicinal plants by being submitted to GenBank. In addition to guaranteeing transparency and reproducibility, these submissions enable comparisons with other herbal formulations. With its ability to prevent species adulteration and guarantee pharmacological reliability, DNA barcoding has become a potent tool in the authentication of herbal drugs. This molecular approach offers a dependable way to verify raw materials used in preparation and to identify substitution or contamination in the herbal drug market for *Athiyadhi Kashayam*, which is made up of several plant species with therapeutic relevance. [15, 16]

All things considered, the findings demonstrate how crucial molecular authentication techniques are to standardizing conventional formulations. In order to ensure the safety and effectiveness of Siddha preparations, this work supports the incorporation of DNA barcoding into standard quality control procedures.

5. CONCLUSION

The results demonstrated successful genomic DNA isolation from the five species present in *Athiyadhi Kashayam*, with *Senna auriculata* showing the highest DNA yield, followed by *Cassia fistula* and *Syzygium cumini*. The A260/A280 absorbance ratios indicated good purity of DNA, suitable for downstream applications such as PCR and sequencing. The use of the ITS region as a barcode marker proved effective for species discrimination, consistent with its high variability and utility in plant identification studies. The Sanger sequencing chromatograms showed high-quality data in the initial regions, which is typical for this method, although signal degradation occurred toward the latter part of the sequences. These limitations are commonly encountered due to reduced fluorescent signal intensity or polymerase errors in long-read sequences. Nevertheless, the manual editing step ensured accurate base calling, allowing the generation of reliable barcode sequences. Submission of these sequences to GenBank provides a valuable reference for future molecular authentication of herbal formulations. Overall, this molecular approach provides a robust tool to detect potential adulteration and confirm the authenticity of complex herbal mixtures like *Athiyadhi Kashayam*.

6. ACKNOWLEDGEMENT

The authors would like to thank ‘Department of Pharmacognosy, Siddha Central Research Institute (CCRS), Ministry of Ayush, Govt. of India, Chennai 600106’ for helping in carrying out this DNA barcoding study.

Author’s contribution

Conceptualization of the study and formal analysis were performed by Dr. G. Bharath Kumar, The original draft was prepared by Dr. C. Meenakshi, Writing, Editing and Methodology were handled by Dr. S. Chithra, and Dr. M. Ramani, Visualization and Validation were carried out by Dr. S. Balamani, Review and Publication were conducted by Dr. S. Selvakumar. All authors have reviewed and approved the final version of the manuscript for publication.

Conflict of interest

The authors declare that there are no conflicts of interest

Funding

Self-funded research work.

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