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MOLECULAR IDENTIFICATION OF THE MEDICINAL PLANT *JUSTICIA GENDARUSSA* (BURM) F. USING ITS GENE

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ABSTRACT: The aim of the present study is to analyse the phytochemicals and to identify the plant at molecular level using ITS gene of the plant *Justicia gendarussa* (Burm) f. (family: Acanthaceae). Phytochemical analysis is employed to identify the chemical constituents present in the plant qualitatively. Molecular identification helps to identify the unknown to known samples or specimens and taxonomical clarification by sequentially. This method includes plant sample collection, isolation of DNA, PCR amplification and finally DNA Sequencing. In plant phylogenetic analysis ITS is the commonly used DNA marker. This is the first study to isolate DNA from *Justicia gendarussa* (Burm) f. plant leaf which is amplified by using ITS (FP & RP) primer. Plant leaf sample showed the result of amplified ITS2 gene present with high rates in the region of 750 -1000 base pairs congruently. This result shows the most reliable method for analysing ITS gene to identify the plant. The prime aim of this present study was to authenticate *J. gendarussa* plant by using molecular tools.

KEYWORDS: *Justicia gendarussa* (Burm) f, PCR, ITS2 and phylogenetic.

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1. INTRODUCTION

Herbal plants cover a broad range of plant taxa and closely related species. There is a growing international market for medicinal plants, which are used equally for herbal drug and for pharmaceutical products. Accurate and rapid authentication of plants and their respective adulterants is difficult to achieve at the scale of international trade in medicinal plants. As the natural medicines are reported to be much safer than synthetic drugs, they have gained popularity in recent years, leading to an incredible growth of phyto-pharmaceutical usage [1]. India is a rich source of medicinal plant. They growing naturally in different states. Herbal plants are used to cure most diseases in humans and animals for curatively and preventively [2]. The medicinal value of the plant could be attributed to the Phytochemicals such as primary and secondary metabolites. These phytoconstituents are being used as drugs in many pharmaceutical companies [3]. *Justicia gendarussa* (Burm) f. is a rare medicinal plant, which grows in Asian countries like India, Malaysia, Indonesia and Srilanka. Leafs, stem and roots of the plant are frequently used as traditional herbal medicine against some common diseases [4]. Such as rheumatism, fever, cough, jaundice, thrush, arthritis, cephalgia, hemiplegia, facial paralysis, otalgia, hemicrania, bronchitis, liver and kidney disorders [5]. Major active components of *J. gendarussa* are the flavonoids vitexin, apigenin and also sitosterols, alkaloids and reducing sugars [6]. Many authentication methods such as morphologic characteristics, chemical compositions, and anatomical procedures are available to identify the herbal plants. However, these methods faces manylimitations. So the method of species identification is closely convenient for the identification of medicinal plant in the form of phylogenetically [7]. The objective of the current study is to authenticate the plant employing DNA sequencing. Molecular analysis is cheaper and accurate than any other method. As DNA markers are unique, fixed, and are not affected mostly by any environment and physiological factors. A gene called internal transcribed spacers (ITS) is used to evaluate the medicinal plant effectively and to identify the information in molecular species [8]. The ITS region is a sequence of primary transcript of RNA and it has been removed by merging during RNA processing. Eukaryotes have two Internal transcribed spacers; ITS-1 located between 18S and 5.8S gene while ITS-2 is located between 5.8S and 28S gene [9, 10, 11].

2. MATERIALS AND METHODS

Plant Sample Collection

The medicinal plant *Justicia gendarussa* (Burm) f (Figure 1) was collected locally from Ichadi, Pudukkottai District, Tamilnadu, India which is grown in natural environment condition and the plant is authenticated (specimen - SJCBOT2183) by Dr .S. Soosairaj, Assistant Professor, Department of Botany, St. Joseph’s College, Tiruchirappalli District, Tamilnadu, India.

Figure 1: *Justicia gendarussa* (Burm) f. plant

Preparation of plant extract for phytochemical analysis

Justicia gendarussa (Burm) f. plant fresh leaves are washed in clean water, shade dried and was ground into fine powder. Using cold method this powder was soaked in 500ml ethanol and was kept in a shaker for 48h at room temperature. Then this mixture was filtered through a clean muslin cloth. Followed by filtration through Whatman no.1 filter paper. This extract was dried in a rotary evaporator at 37ºC. The dried extract was stored in a refrigerator for further use [12]. This ethanol extract of the plant was qualitatively analysed by various test procedures to detect the phytochemical compounds as follows [13].

Test for carbohydrates: To about 1 ml ethanol extract of the plant about 5 ml of Benedict's reagent was added and was boiled for 5 minutes. Appearance of bluish green showed the presence of carbohydrates.

Test for glycosides: About1 ml concentrated sulphuric acid was added to 1 ml ethanol extract of the plant. Fehling's solution was added to this test solution. A black red precipitate was formed indicating the presence of glycosides.

Test for alkaloids: To 1 ml of the plant extract about 2 ml of 2N hydrochloric acid and Mayer's reagent (Potassium mercuric iodide solution) were added. Formation of turbid white precipitate shows the presence of alkaloids.

Test for flavonoid: Few drops of 1% NH_3 was added to 1 ml ethanol extract of the plant. Observation of intense yellow colour indicates the presence of flavonoid compounds.

Test for tannins:2 ml of 5% FeCl3 was added to 1 ml ethanol extract of the plant. Appearance of blue-black precipitate indicated the presence of tannins compounds.

Test for saponins: 5 ml ethanol extract and 5 ml de-ionize distilled water was placed in a test tube, and was shaken vigorously. Appearance of foam that lasted for 15 minutes indicates the presence of saponins.

Test for steroids: 2 ml of ethanol extract of the plant was dissolved in 2 ml of chloroform and concentrated sulphuric acid was added along the sides of the test tube. The upper layer turned red and sulphuric acid layer showed yellow colour with green fluorescence indicating the presence of

steroids.

Test for terpenoids:2 ml of ethanol extract of the plant was dissolved in 2 ml of chloroform and was evaporated to dryness. 2 ml of concentrated sulphuric acid was added to this mixture which form a layer of reddish brown colour indicating the presence of terpenoids.

Plant DNA extraction procedure

Justicia gendarussa (Burm) f. Plant DNA Extraction has done by modified CTAB procedure [21]. 100 mg of fresh leaves was ground to a fine powder in liquid nitrogen using a mortar and pestle [8]. About 2 ml of solution was added and (use as a frozen material) was ground further and grinding was continued to make a homogenous mixture. This ensures close contact of the tissue ingredients and the reagents that would help in instantaneous denaturation of protein. The mixture was allowed to thaw completely with intermittent grinding. Then 800 µl of nuclease free water was added and mixed with grinding. The contents were transferred to two, 2 ml micro-centrifuge tubes and were left for 5 min at room temperature. 200 µl of chloroform was added to each tube, and were vortexed briefly (< 10 s) and left for 10 min at room temperature. It was centrifuged at 13,000 rpm for 10 min at 4 °C and the upper aqueous phase was transferred into fresh tubes. 0.6 volumes of isopropanol was added, briefly (<10 s) vortexed and left for 10 min at room temperature. Again it was centrifuged at 13,000 rpm for 10 min at 4 °C and the supernatant was discarded. DNA pellet was washed with 70% ethanol, air dried and dissolved in 20 to 50 µl of DEPC-treated water. The Purified Plant DNA products were resolved by electrophoresis in a 1% agarose gel in 1×TAE buffer. The gels were pre-stained with 10 mg/ml ethidium bromide.

Solution I: phenol saturated with tris (hydroxyl methyl) amino methane buffer to a pH of 6.7 ± 0.2. To this sodium dodecyl sulphate [SDS; 0.1% (w/v)], sodium acetate [NaOAc; 0.32 M (w/v)] and ethylene di amine tetra acetic acid (EDTA; 0.01 M final concentration from a stock solution of 0.5 M, pH 8.0) were added.

PCR Protocol

Polymerase Chain Reaction (PCR) is a process that uses primers to amplify specific cloned or genomic DNA sequences with the help of a unique enzyme. PCR uses the enzyme DNA polymerase that directs the synthesis of DNA from deoxynucleotide substrates on a single-stranded DNA template. DNA polymerase adds nucleotides to the 3` end of a custom-designed oligonucleotide when it is annealed to a longer template DNA. Thus, if a synthetic oligonucleotide is annealed to a single-stranded template that contains a region complementary to the oligonucleotide, DNA polymerase can use the oligonucleotide as a primer and elongate its 3` end to generate an extended region of double stranded DNA.

Composition of the Taq Master Mix

1. Taq DNA polymerase supplied in 2 X Taq buffer
2. 0.4mM dNTPs,

3. 3.2mM MgCl2 and
4. 0.02% bromophenol blue.

Add 5 µl of isolated DNA in 25 µl of PCR reaction solution (1.5 µl of Forward Primer and Reverse Primer, 5 µl of deionized water, and 12 µl of Taq Master Mix). Perform PCR using the following thermal cycling conditions.

Table 1: Primer Details

Primer Name	Sequence Details	Number of Base
Plant ITS2 FP	5'ATGCGATACTTGGTGTGAAT 3'	20
Plant ITS2 RP	5'GACGCTTCTCCAGACTACAAT 3'	21

PCR Amplification

PCR was performed in a total volume of 20 µl containing10 µl master mixture, 1 µl of µM each of Plant ITS2 FP and Plant ITS2 RP (table 1) and 40 ng template DNA. PCR conditions were as follows: denaturation at 94°C for 5 min; 40 cycles of 94°C for 30 sec, primer-specific annealing temperature at 56°C for 30 sec and extension at 72°C for 45 sec, and a final extension at 72°C for 10 min. The PCR products were resolved by electrophoresis in a 1% agarose gel in 1 × TAE buffer. The gels were pre-stained with 10 mg/ml ethidium bromide [14].

DNA Sequencing and species identification

Amplified PCR product was purified using Qiaquick PCR purification kit (QIAGEN, USA). Sequencing reactions were carried out in both directions using same forward and reverse primers used for amplification with Big Dye Version 3.1 kit (Applied Bio-systems) on an ABI-PRISM 3730 DNA Sequencer (Applied Bio-systems). Ambiguous sequences from the base called sequences were corrected with Chromas (Version 2.01) and the sequences were assembled with Bio-Edit (Version 7.0.9.0). The search for sequence homolog of potential isolate for species identification was made using the BLAST program (NCBI) and the nucleotide sequence has been submitted to GenBank for reference.

3. RESULTS AND DISCUSSION

The phytochemical analyses were done qualitatively using ethanolic extract of the plant *Justicia gendarussa* (Burm) f in table 2. Our results shows that the plant leaf contains Carbohydrates, Glycosides, Alkaloids, Flavonoids, Tannins, Saponins and Steroids were present and Terpenoids compound were absent (Table 2). Among these phytochemical compounds Alkaloids, Flavonoids, Tannins are therapeutically important [15, 16, 17, 18].

Table 2: Phytochemical analysis of the Ethanol extract of *Justicia gendarussa* (Burm) f. plant

S.No	Phytoconstituents	Ethanolic plant extract
1	Carbohydrates	+
2	Glycosides	+
3	Alkaloids	+
4	Flavonoids	+
5	Tannins	+
6	Saponins	+
7	Steroids	+
8	Terpenoids	-

This phytochemical screening is useful to identify the source of pharmacologically active chemical compounds. This result confirms the presence of the phyto constituents which could cure some diseases like fever, hemiplegia, rheumatism, arthritis, headache, earache, muscle pain, respiratory disorders, and digestive trouble [19]. Based on the previous study presence of alkaloids, steroids and saponins compounds to cause significant anti-inflammatory and analgesic effect was confirmed [20].

Plant DNA sequence of ITS

Result of this study was focused to identify the gene and to perform molecular characterisation of the plant *Justicia gendarussa* (Burm) f. The leaf of the plants genome was characterised by using ITS gene. This ITS region was commonly used as a phylogenetic marker of the plant sequencing. ITS region of the plant were amplified by using ITS2 FP & ITS2 RP primers. Figure 2 and 3 shows the results of the PCR amplification of nuclear ITS2 region in plant *Justicia gendarussa* (Burm) f. The ITS gene shows a high rate of substitutions and important gene for the study of plant systematic and evolution [21, 22, 23].

Figure 2: DNA extracted from the *J. gendarussa* (Burm) f. Plant

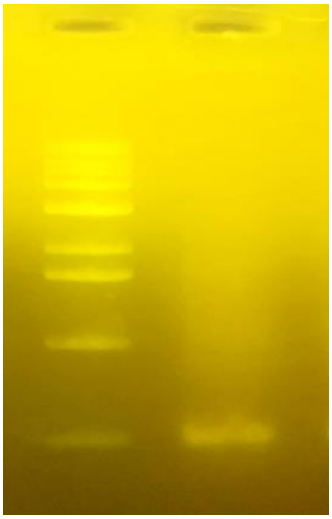

Figure 3: PCR amplification of Plant ITS gene

Sanger dideoxy sequencing technology contains 5.8S ribosomal RNA, internal transcribed spacer 2, and large subunit ribosomal RNA Sequence was submitted to NCBI GenBank and its accession number generated MH591755.1. These sequence so obtained was about 493 bp and when using NCBI-BLAST Tool analysed and the sample was found to be 99% congeneric to *Justicia gendarussa* (Burm) f. The spectrophotometer methods analysed by quantitative procedure between 260 to 280 nm absorbance shows the purity of the DNA to be 1.2 kb. According to the previously mentioned by GenBank deposition is not only a substitution ‘‘barcode library,’’ which will be a high quality DNA sequence building from verified voucher specimens and the sequences currently available to provide an independent data to test for the discriminatory powers of various loci [24, 25].

Phylogenetic tree analysis of the *Justicia gendarussa* (Burm) f

In molecular identification phylogenetic tree studies are commonly used to analyse, classify the species names and here we have used tree is maximum likelihood. This ML tree construct includes accession number followed by species name. The ITS2 phylogenetic trees are clearly yield one tree with an optimal likelihood as shown in Figure 4. The maximum likelihood (ML) of the tree construct for 40 hits and the targeted plant *J. gendarussa* using MEGA6 software [21].

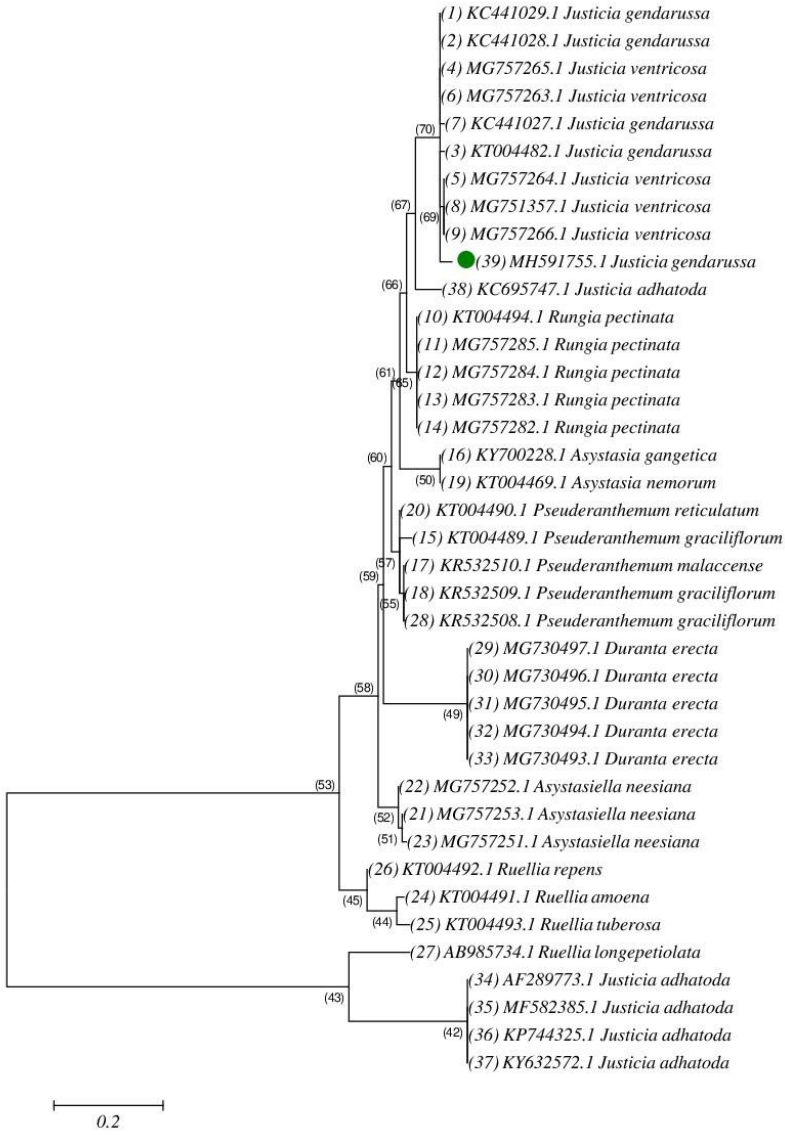

Figure 4: The ML-based tree concluded from nuclear ITS2 sequences of the *Justicia gendarussa* (Burm) f. Plant

The phylogenetic trees show significant difference in the branch lengths of taxa with in *JusticiaDuranta, Rungia, Asystasia, Asystasiella, Pseuderanthemum*and*Ruellia* relative to those other tribes. Figure 5 shows the evolutionary relationships of taxa history using the Neighbor-Joining method [26]. The optimal tree with the sum of branch length 2.8 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura 3-parameter method [27] and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter - 1). The analysis involved 39 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 143 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [28, 29, 30].

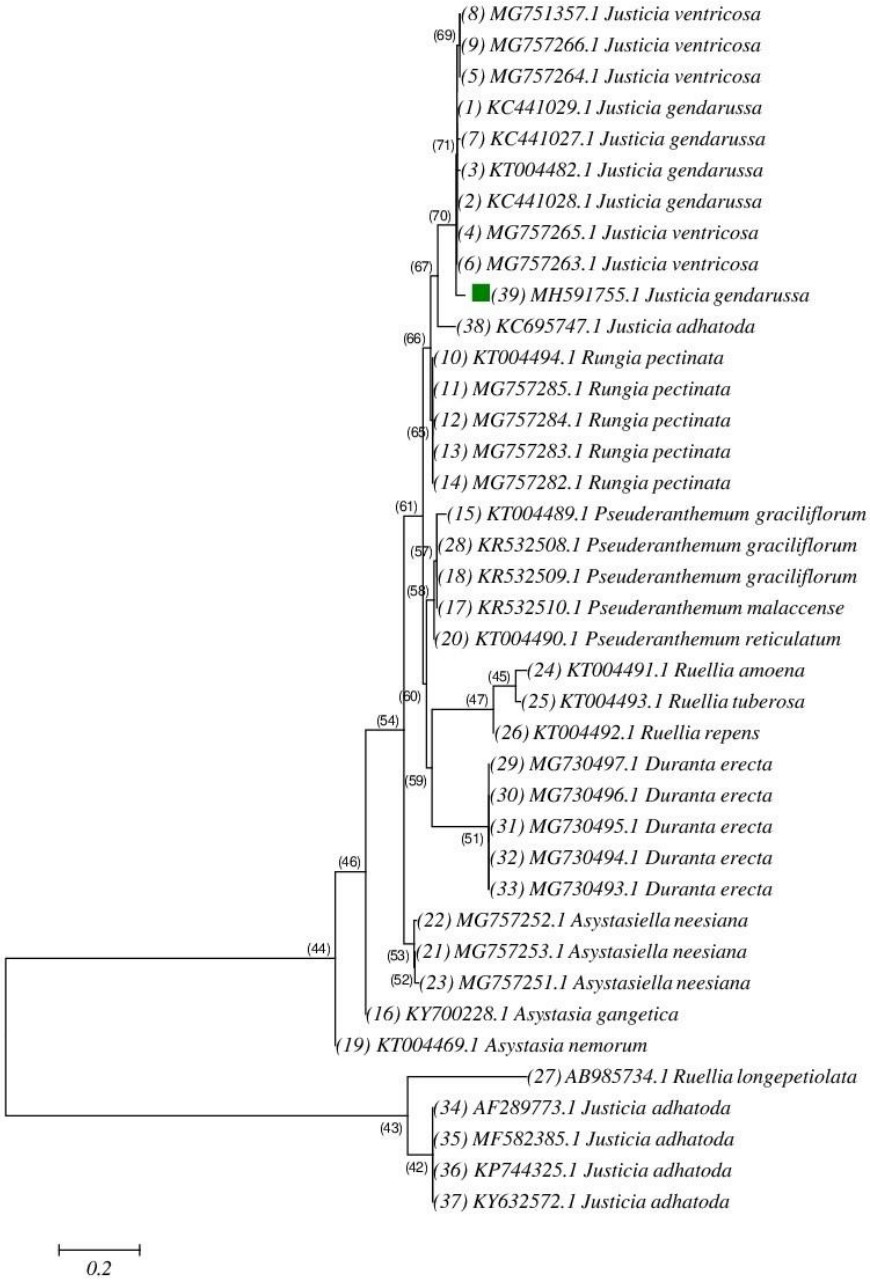

Figure 5: The Neighbour joining -based tree concluded from nuclear ITS2 sequences of the *Justicia gendarussa* (Burm) f. Plant.

4. CONCLUSION

In this study, molecular identification of the plant *Justicia Gendarussa* (Burm) f. is achieved and the evolutionary relations was completed. There were no documented reports of using ITS gene of the *J. gendarussa* species in the GenBank. Overall, the results of the present study demonstrate that the sequencing of ITS gene is a useful molecular tool for rapid identification and DNA bar coding of this plant. According to our results the use of sequencing techniques is a powerful method for the identification and isolation of plant samples for further researches.

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CONFLICT OF INTEREST

The authors have declared there is no conflict of interest.

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