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Molecular phylogenetic analysis among five species of *Spilanthes* Jacq

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Abstract
DNA isolation and barcoding of medicinal plants are getting a great previlege and demand in recent years. Authentification using molecular markers is necessary for identifying correct raw material for herbal market in the preparation of new formulation in ayurvedic industry. The molecular characterization of *Spilanthes* species has not been reported in the literature. In the present study, attempts were made to optimize DNA isolation using the CTAB method and characterize five species of *Spilanthes*. Matk and rbcL primers are universal primers used to distinguish five *Spilanthes* species and their inter relationship were analysed using Clustal omega tool to draw the phylogeny tree. Thus from the study *S. ciliata* and *S. calva* showed similar banding pattern, similarly *S. paniculata* showed similar sequence pattern from phylogeny tree analysis. The work can provide an effective tool for scientific identification of plants in pharmaceutical industry and also help in investigations relating to characterization of this genus.

Keywords: barcoding, CTAB (Cetyl trimethy lammonium bromide), phylogeny, primer, maturase kinase (Matk), *spilanthes*

1. Introduction
In Brazil *Spilanthes* is commonly referred to as the toothache plant for its ability to numb the gums and mouth and calm tooth pain when the flower buds and new leaves are chewed. *Spilanthes* belongs to the family of Asteraceae, and has been used in ayurveda (Sharma V, *et al.*, 2011) [1] Common terms used to refer plant include Jambu, toothache plant, or Paracress. It derives the name of 'toothache' plant from the compound 'spilanthol' which is sometimes used to reduce the pain associated with toothaches and can induce saliva secretions (Ramsewak,.1999; Erickson,1999; Nair 1999) [2, 5, 5] alongside the other compound Acmellonate (Ley, *et al.*,2006) [3]. Other traditional uses of this herb according to Indian medicine are the treatment of rheumatism and inflammation, asialagogue for stammering, tongue paralysis, stomatitis, toothache and a treatment for fever, sore throat, and gum infections beyond these health effects, it has been used as an aphrodisiac and sexual tonic. (Abascal and Yarnell).

Spilanthes has got antibacterial and antifungal properties making it an good purifying herb used for disinfecting wounds and curing ringworm infections (Rai, 2004; Varma, 2004; Pandey. 2004) [4, 4, 4]. On the otherhand *Spilanthes* is also used as an antiparasitc agent (Ramsewak, 1999; Erickson, 1999; Nair. 1999) [2, 5, 5]. The present study basically focussed on the authentification of herbal drug using molecular markers like Matk and rbcL, which will provide a data which may help the pharmaceutical industry in identification of raw materials.

2. Materials and Methods
2.1 Plant Material
In the present study, six species of the genus *Spilanthes- S. calva, S. ciliata, S.tetralobeta, S. paniculata, S. Uliginosa* and occurring in the peninsular India were selected for their characterization by using the CTAB technique.

2.2 Reagents used in DNA Isolation
DNA isolation using Nucleo Spin® Plant II Kit (Macherey-Nagel)
About 100 mg of the tissue is homogenized using liquid nitrogen and the powdered tissue is transferred to a microcentrifuge tube. Four hundred microlitres of buffer PL1 is added and vortexed for 1 minute. Ten microlitres of RNase A solution is added and inverted to mix. The homogenate is incubated at 65ºC for 10 minutes. The lysate is transferred to a Nucleospin filter and centrifuged at 11000 x g for 2 minutes. The flow through liquid is collected and the filter is discarded. Four hundred and fifty microlitres of buffer PC is added and mixed well.

The solution is transferred to a Nucleospin Plant II column, centrifuged for 1 minute and the flow through liquid is discarded. Four hundred microlitre buffer PW1 is added to the column, centrifuged at 11000 x g for 1 minute and flow though liquid is discarded. Then 700 µl PW2 is added, centrifuged at 11000 x g and flow through liquid is discarded. Finally 200 µl of PW2 is Added and centrifuged at 11000 x g for 2 minutes to dry the silica membrane. The column is transferred to a new 1.7 ml tube and 50 µl of buffer PE is added and incubated at 65ºC for 5Minutes. The column is then centrifuged at 11000 x g for 1 minute to elute the DNA. The eluted DNA was stored at 4ºC.

2.3 Agarose gel electrophoresis for DNA quality check

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

2.4 PCR analysis

PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X Phire PCR buffer (contains 1.5 mM $MgCl_2$), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 1 µl DNA, 0.2 µl Phire Hotstart II DNA polymerase enzyme, 0.1 mg/ml BSA and 3% DMSO, 0.5M Betaine, 5pM of forward and reverse primers.

2.5 Primers used

Target	Primer Name	Direction	Sequence (5' → 3')
matK	390f	Forward	CGATCTATTCATTCAATATTTC
	1326r	Reverse	TCTAGCACACGAAAGTCGAAGT
*rbc*L	rbcLa_f	Forward	ATGTCACCACAAACAGAGACTAAAGC
	rbcL724_rev	Reverse	GTAAAATCAAGTCCACCRCG

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

2.6 PCR amplification profile

matK

98 ºC	-	30 sec		
98 ºC	-	5 sec	40	cycles
50 ºC	-	10 sec		
72 ºC	-	15 sec		
72 ºC	-	60 sec		
4 ºC	-	∞		

rbcL

98 ºC	-	30 sec		
98 ºC	-	5 sec	40	cycles
58 ºC	-	10 sec		
72 ºC	-	15 sec		
72 ºC	-	60 sec		
4 ºC	-	∞		

2.7 Agarose Gel electrophoresis of PCR products

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. 1 µl of 6X loading dye was mixed with 5 µl of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

2.8 ExoSAP-IT Treatment

ExoSAP-IT (GE Healthcare) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications.

Five micro litres of PCR product is mixed with 2 µl of ExoSAP-IT and incubated at 37ºC for 15 minutes followed by enzyme inactivation at 80ºC for 15 minutes.

2.9 Sequencing using BigDye Terminator v3.1

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems. USA) following manufactures protocol.

The PCR mix consisted of the following components:

PCR Product (ExoSAP treated) - 10-20 ng
Primer- 3.2 pM (either Forward or Reverse)
Sequencing Mix - 0.28 µl
5x Reaction buffer - 1.86 µl
Sterile distilled water - make up to 10µl

The sequencing PCR temperature profile consisted of a 1st cycle at 96ºC for 2 minutes followed by 30 cycles at 96ºC for 30 sec, 50ºC for 40 sec and 60ºC for 4 minutes for all the primers.

2.10 Post Sequencing PCR Clean up

1. Make master mix I of 10µl milli Q and 2 µl 125mM EDTA per reaction
2. Add 12µl of master mix I to each reaction containing 10µl of reaction contents and are properly mixed.
3. Make master mix II of 2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol per reaction.
4. Add 52 µl of master mix II to each reaction.
5. Contents are mixed by inverting.
6. Incubate at room temperature for 30 minutes
7. Spin at 14,000 rpm for 30 minutes
8. Decant the supernatant and add 100 µl of 70% ethanol
9. Spin at 14,000 rpm for 20 minutes.
10. Decant the supernatant and repeat 70% ethanol wash
11. Decant the supernatant and air dry the pellet.

The cleaned up air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems).

2.11 Sequence Analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond *et al.*, 2010) [8].

3. Results and Discussion

Recently, health foods, herbs as well as dietary supplements

enriched with medicinal ingredients such as antioxidants and bioactive metabolites have drawn considerable attention worldwide, especially herbs that are used as food and traditional medicine (Tyagi and Delanty, 2003) [10]. The concern centres around medicinal plants bearing bioactive compounds, which are employed as therapeutics and health care (Abascal and Yarnell, 2010) [9]. Thus concern for this medicinal plants are increasing in present day world, so correct identification need to be inorder to evaluate these different species of *Spilanthes* Jacq. From the present study *S.* ciliate and *S.* calva showed similar banding pattern and also their DNA sequence isolated were helpful in finding better relationship interspecifially. Similarly *S. paniculata* and *S. tetralobeta* showed similar sequence pattern from phylogeny tree analysis. So matK and rbcL primers are effective for the molecular clarification and taxonomy of *Spilanthes* species.

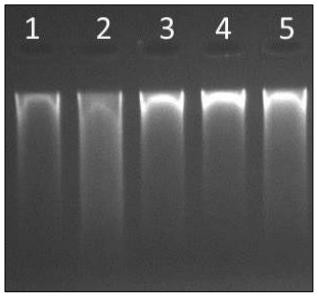
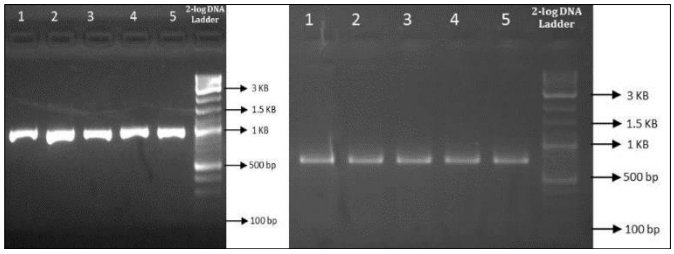


Fig 1: DNA



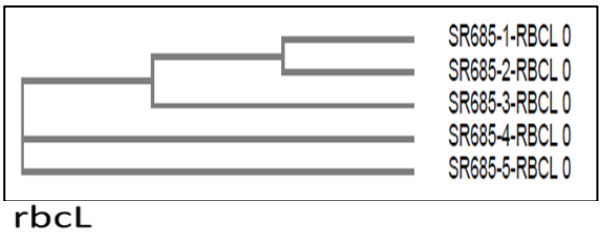
matK rbcL

Fig 2: PCR

Phylogeny tree

matK

Lane 1-5:
S. ciliata, S. calva, S. paniculata, S. tetralobeta* and *S. uliginosa, respectively.



rbcL
Lane 1-5:
S. ciliata, S. calva, S. paniculata, S. tetralobeta and *S. uliginosa, respectively.*

From the findings of rbcL phylogeny tree analysis more similarity was shown by *S. tetralobeta* and *S.uliginosa*. But there morphological analysis were totaly different characters are seen. This was because of the *S. tetralobeta*, florets are four lobed while in *S.uliginosa* were found to be small head and five ray florets are seen.

In the matK phylogeny tree analysis more similarity were shown by *S.paniculata* and *S.uliginosa* in the morphological view these are totaly different. *S.paniculata* had disc florets but *S.uliginosa* showed rayflorets.

The similar study reports (Lavanya and Devi, 2012) the simple DNA isolation protocol and optimized PCR-ISSR specific for *Spilanthes* species and could be useful in studies relating to genetic diversity of plants containing phenolics and other metabolites. More primers will be necessary for accurate preparation of phylogeny tree, then only we get a more clarification of the result for the comparison of the species of *Spilanthes* Jacq.

4. Conclusion

The present study reports the simple DNA isolation protocol specific for *Spilanthes* species and could be useful in studies relating to genetic diversity of plants containing phenolics and other metabolites. Molecular markers act as an effective tool in discriminating plant species that are closely related. A comprehensive database of barcodes for plants, and should prioritize the development of the traditional pharmacopeia because many locally produced herbal medicinal products are not indigenous. This could be useful in studies relating to genetic diversity of plants containing phenolics and other metabolites. Herbal products are of great demand in near future, such modern technique of authentification is required to identify the substitutes and adulterants in pharmaceutical industries.

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