JOURNAL OF NATURAL REMEDIES
DOI: 10.18311/jnr/2022/27721

SHORT COMMUNICATION

Designing and Recognition of Internal Transcribed Spacer Region in *Solanum* Species

Nibedita Dey[1], Shalini Devaraji[2] and C. Kamatchi[3*]

[1]Saveetha School of Engineering, Saveetha Institute of Medical and Technical Sciences, Thandalam – 600124, Tamil Nadu, India
[2]Dr. V. Seshiah Diabetes Care and Research Institute, Aminjikari, Chennai – 600030, Tamil Nadu, India
[3]The Oxford College of Science, Department of Biotechnology, HSR Layout, Bengaluru – 560102, Karnataka, India; ckamatchi@gmail.com

Abstract

Identifying the authenticity of any material has been of great importance to people for decades. The use of plant-based therapy is of great interest to people all around the world. Today 1000 of plant varieties are being investigated by many researchers for their potential pharmaceutical activities. Apart from home remedies, these plant-based therapies are drawing the attention of several western countries towards patenting them for their commercial use. Even before formulation of a drug from plants, it is very essential to the purity of the variety to ensure the quality of the final product. When it comes to genome identification the first guess is always DNA barcoding. But the redundancy of the plant species towards evolution is the main reason why the closely related plant species are very difficult to classify. The mitochondrial can be preferred for DNA barcoding but has a disadvantage when it comes to plant varieties. Hence, to find the genomic uniqueness of *Solanum* species from our local area we opted for the utilization of internal transcribed spacers and found to be a partial ITS2 segment. The plant species were found similar to *Solanum supinum* and *Solanum virginianum.* The size of the amplified product was found to be 314, 350 and 346 bp, with respect to Sx1, Sx2 and Sx3. Although the sequence we obtained was partial ITS, it still helped us to identify the *Solanum* species found in our local region with great efficiency. Proper complete ITS2 sequencing is the perspective of the future work to be done.

Keywords: Gene Sequencing, Partial Sequence, Plant Authenticity, *Solanum xanthocarpum,* ITS2

1. Introduction

Even before allopathy came into the market, plants were used to treat many aliments and discomforts. Its roots go back to many centuries in time. Almost all the parts of herbs are employed in creating medicines. They are either utilized as an individual portion or in combination of other herbs to enhance their productivity. As the need for herbal medicine is increasing in the market, the hybrid varieties are creeping in to address the demand. This introduction of hybrid strains has made many authentic species endangered. So, to produce genuine and pure herbal medications it is very essential to retain the original genetic makeup of the plant. Hybrids can give way too many undesired side effects in the future as their genome varies from the original herb. To assay and analyse the ethnicity of very closely related plant species for conventional purposes, the techniques available are very limited. Many adulterants or substitutes used in the market for herbal medications are generally derivatives

*Author for correspondence

Article Received on: 28.04.2021 Revised on: 10.12.2021 Accepted on: 01.17.2022

of the similar family[1]. Although they might belong to similar families or genus, their pharmaceutical activity would vary by a great extent[2]. Mitochondrial cytochrome oxidase gene is mostly preferred to known the genome of many animal and fungal species. But when it comes to plants, the number of mitochondrial cytochrome oxidase and other genes are very low in count in terms of variations. The usefulness of the same is very limited as the mitochondrial structures lack variable structures. Thus, for plant genome recognition and identification, their nuclear DNA and plastid DNA are opted for assay[3].

Medicinal and herbal plants contain phenolic compounds and active ingredients in their cellular structure which need to be removed as they interfere with DNA sequencing and amplification processes. The recognition of their genome will require their DNA to be denatured by respective enzymes, followed by removal of the high-density polysaccharides. The RNA that tags along with the main genome also causes major nuisance in PCR amplification by interfering the primers. This would lead improper binding to the template[4]. DNA extraction and DNA barcoding using matK and rbcL have been performed previously with an intention to create a reliable database for plants. These markers worked best with fresh samples than the once that were from the herbarium. Although the universality and the sequencing provided by the rbcl marker were satisfactory, the recovery rate was low. The time taken by matK marker is found to be very time consuming[5,6]. Recently the numbers of plant species identified and sequenced are increasing. They are reported to the GENBank and stored in the database. But these data are found to have many errors due to contamination and the hybrid varieties available for the researcher. They were found to be independent to the procedure and protocol opted by the scientist. Sample mix up is also noticed to be a source of error[7]. When many herbal moieties are stored and sold as parts of the plant as roots, seeds, leaves, etc. it becomes tedious to recognize the parent herb. Endemic species are at utmost danger, as they show insufficient variation in their nuclear sequences[8]. One of the reasons why DNA barcoding has not been of much use to plant genome database for species identification is because of the lack or minimum of evolution in the plastid DNA. Based on the species of plant the DNA region for sequencing and identification varies. For example, in Epimedium, psbA-trnH DNA region is potentially a better marker for amplification[9]. Till date the techniques used to read and identify a genome are using specific and complementary markers, do complicated hybridization with the desired sequence and microarrays[10]. The most commonly recognized barcodes in plants are YCF5, rpoB, ndhJ, accD, trnH-psbA, rpoC1 and rbcL. These are found in the plastid genome[11]. To solve the issue related to plant genome and authenticity recognition internal transcribed spacers (ITS) are used in the sequencing[12]. They are found in the ribosomal unit of the plant cell. These form the DNA material present in the protein synthesizing unit. They are on structural component of the ribosome. They have higher mutation rates when compared to 18S, 28S and 5.8S sequences. Compared to 18S rDNA, ITS have larger variation between same species. Hence, minute variations in the same species can be reflected in their ITS sections. They can be used to chart out phylogenetic tree of many potential herbal families of plants[13]. A study in 2010 by chen and team, depicted the successful discrimination of ITS region in segregating plant varieties of close species. Up to 6600 varieties were easily identified and all had their own specific ITS2 region. ITS have been found very useful in charting out the citrus and algal family[13]. These species tend to undergo crossbreeding very easily due to bud mutation. Mutation rates are also found to be very elated for these plants. Since, there have different evolutionary patterns, they classified in multiple ways. These ITS has been of great aid in identifying each and every species in citrus variety[14]. ITS2 section of the ribosomal DNA is termed to be the most ideal candidate for sequencing and identifying any plant-based genome. Their barcodes have many different characteristics like ample number of variations, the tendency for amplification and the specific and unique conserved sections. These unique sections will aid in designing many DNA primers and markers of universal nature[15-17].

The target plant species of choice is *Solanum xanthocarpum*. They belong to the Solanaceae family. It is a plant of many traditional medicinal significance[18]. All the structural parts of this plant have been used in Ayurveda for the production of many traditional

medications. It has an efficacy to treat any form of aliment like asthma, tuberculosis, renal disorders, etc. They are the mixtures of may active ingredients in them. They harbour solamargin, alkaloids, solasonin, steroids, etc. This gives them the ability to be used as a precursor for hormone medications[19]. It has found to be efficient enough to be used as an alternative for diosgenins[20]. The parts above the ground for this plant are having special properties like anthelmintic with very bitter flavour. The roots are incorporated in drugs used to treat pains in the thoracic region due to asthma and tooth related ailments[21]. Leaves have been exclusively used as analgesics for several ailments. In the current market, the many species of *Solanum* are considered to be the same as there is not much genuine indicator or each *Solanum* species. Each and every species has its potency based on the respective part of the plant used in the treatment. Some physicians equate one *Solanum* species to other. This causes undesired or reduction in the pharmacological activity of the medicine[22]. Hence, it is very essential to come up with a marker-based study for *Solanum xanthocarpum*, which will be unique and universal for this species alone.

In our current study we have attempted to design an ITS marker for prickly *Solanum* species. Molecular spacer-based study on this specific species has not been attempted before. Their relation with other closely related solanum species will also be observed and inferred to get better classification and information of variation between these plant varieties. The sensitivity, reliability an accuracy of the measured will also be validated and verified.

2. Material and Methods

Solanum species were collected and sampled from different areas of Tamil Nadu. *Solanum nigrum* was kept as a positive control for this test. Three samples of prickly *Solanum* species were procured from areas of Thiruvannamalai and Kanchipuram District. They were labelled as Sx1 (shown in Figure 1), Sx2 from Thiruvannamalai and Sx3 from Kanchipuram District. DNA isolation was performed using the SDS method as reported previously by Nalini *et al.*

The DNA obtained from extraction were characterised and quantified using UV Spectrophotometer (Shimadzu). Amplification by Polymerase chain reaction (PCR) was performed to enhance the quantity of the desired ITS sequence. The primers for this experiment were designed by us using the blast software. The positive control used in this study was *Solanum nigrum,* whose accession number in NCBI is JF978793.1. The designed ITS primer for *Solanum species* (Assish associates) is depicted below. A 100bp ladder was used to identify the size of the amplicon. The ladder was commercially purchased from Biosource and Surgicals, Chennai. The amplicon was later sequenced. The sequencing was performed at Scigenome Labs Pvt. Ltd (India). The sequencing was performed for ITS1, 5.8S and ITS2. The closely related sites were recognized by using the BLASTn program. The primer designed specifically for the amplification of ITS was done by referring the Primer BLAST database. The sequence unique for ITS in PCR was 5'-GGAAAGCGCCAAGGAATACT-3' and 5'-CGCGACACAAAGAGAGTTGA-3' (Table 1). It is a 20 bp long segment with 50 percent of GC regions.

Figure 1. Picture of *Solanum* species.

Table 1. DNA sequence for ITS

Sequence (5'-3')	Template strand	Length	Start	Stop	Tm	GC%
Forward primer GGAAA GCGCCA AGGAA TACT	Plus	20	142	161	58.25	50.00
Reverse primer CGCGA CACAAA GAGAGT TGA	Minus	20	543	524	58.51	50.00

3. Results

The three samples of prickly *solanum species* were collected and their leaves were utilized for extraction of the DNA. The isolated DNA was quantified. It was found to be around 200 ng- 400 ng, this was measured by comparing the bands to the 1 Kb marker ladder used in lane 1 represented as M. The three samples were represented as Sx1, Sx2, Sx3 and positive control represented as Sn in Figure 2.

PCR was performed for the ITS region from isolated samples. All the 4 samples of *solanum*, including the positive control showed amplification for the ITS region. The base pair of the amplified sequence was compared with commercially purchased 1Kb ladder was used as marker in the lane 1. Water was kept as negative control in line 6. The size of the amplified product was found to be 402 bp, represented in Figure 3.

Further, amplified sequence was sent for sequencing and the obtained sequence was analysed by BLAST to check the ethnicity of the ITS sequences from the samples. The trimmed amplified sequences of three samples Sx1, Sx2 and Sx3 were 314, 350 and 346 bp respectively are shown in Figures 4, 5, 6 (supplementary pictures).

From the phylogenetic analysis, shown in Figure 7 cladogram represents the Sx1 and Sx2 are Sx3 of the

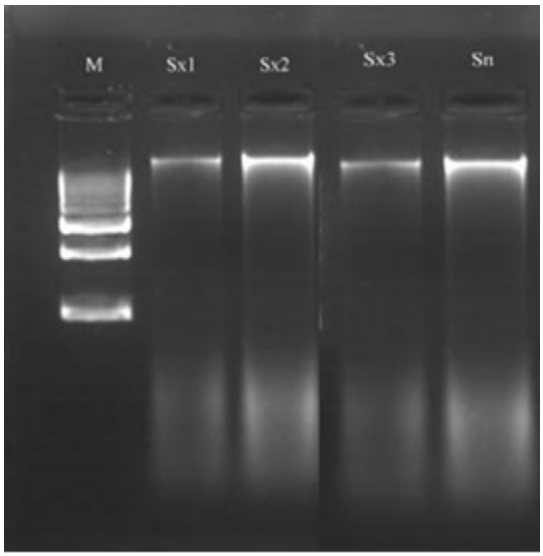

Figure 2. Agarose gel electrophoresis (0.6%) shows the genomic DNA Isolation of *Solanum* species. Lane 1-M 1 Kb, lane 2,3,4 represents the Genomic DNA from *Solanum* species (Sx1, Sx2, Sx3) and lane 5 - *S.nigrum* (Sn).

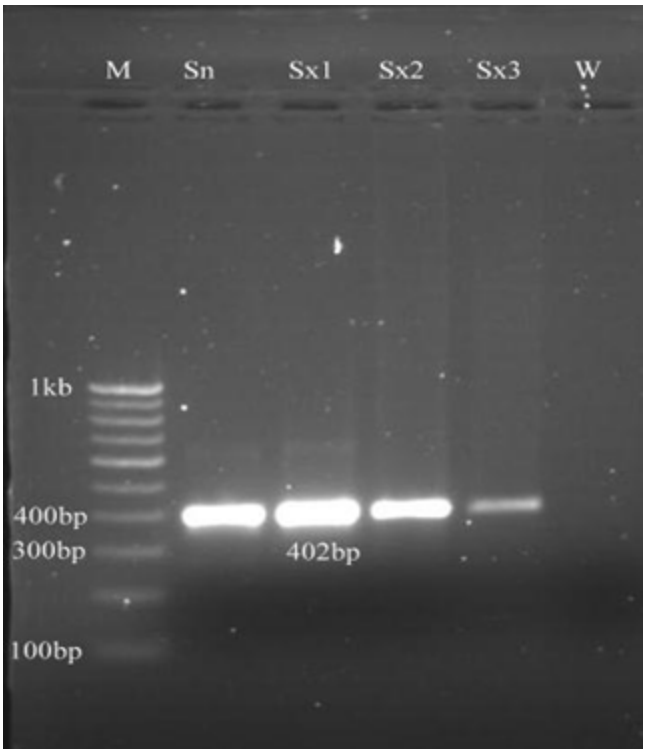

Figure 3. Agarose gel electrophoresis (1.5%) shows amplified ITS region. Lane 1- M 100bp ladder, Lane 2 – amplified product of ITS region of *Solanum nigrum* as a positive control and lane 3, 4 and 5 –amplified product of ITS region of *Solanum* species. W- Water control.

GCGTGCTTCTTTCGAAACCAAAACGACTCTCGGCAACGGATATCTCGGCTCTCGCATCGA
TGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAACCATCGAG
TCTTTGAACGCAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTCTGCCTGGGCGTCA
CGCATCGCGTCGCCCCCCGCACGCCGCTCGGCGTCGTGGGGGCGGATACTGGCCTCCCGT
GCGCCTCGCGCCCGCGGCCGGCCTAAATGCGAGTCCACGTCGACGGACGTCGCGGCAAGT
GGTGGTTGTAACTC

Figure 4. Sample SX1, the sequence was found to be of 314bp.

CGTGCGGGTGGATGCGTGCTTCTTTCGAAACCAAAACGACTCTCGGCAACGGATATCTCG
GCTCTCGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCC
GTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCGTCAGGCCGAGGGCACGTC
TGCCTGGGCGTCACGCATCGCGTCGCCCCCCGCACGCCGCTCGGCGTCGTGGGGGCGGAT
ACTGGCCTCCCGTGCGCCTCGCGCCCGCGGCCGGCCTAAATGCGAGTCCACGTCGACGGA
CGTCGCGGCAAGTGGTGGTTGTAACTCAACTCTCTTTGTGTCGCGAACAC

Figure 5. Sample SX2, the sequence was found to be of 350bp.

CGACGTGGACTCGCATTTAGGCCGGCCGCGGGCGCGAGGCGCACGGGAGGCCAGTATCCG
CCCCCACGACGCCGAGCGGCGTGCGGGGGGCGACGCGATGCGTGACGCCCAGGCAGACGT
GCCCTCGGCCTGACGGCTTCGGGCGCAACTTGCGTTCAAAGACTCGATGGTTCACGGGAT
TCTGCAATTCACACCAAGTATCGCATTTCGCTACGTTCTTCATCGATGCGAGAGCCGAGA
TATCCGTTGCCGAGAGTCGTTTTGGTTTCGAAAGAAGCACGCATCCACCCGCACGCTCCG
CGGACGGGGCGCGGGTGGAGGGCTCTCGTTTGAGTATTCCTTGGCC

Figure 6. Sample SX3, the sequence was found to be of 346bp.

Figure 7. Represents the phylogenetic analysis.

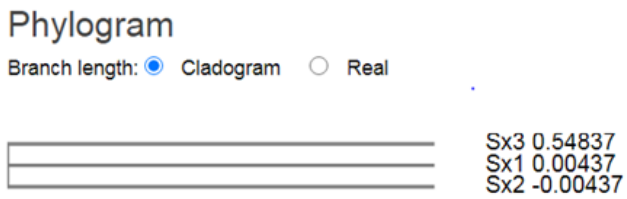

Figure 8. Picture represents spiny *Solanum* species plant and seed, which was used for the study.

Solanum species. The closely related *Solanum* species, shows 0.00437 for Sx1 and Sx2 another sample, Sx3 shows 0.54837.

4. BLAST Analysis

The ITS sequences included 18S, ITS1, 5.8S and ITS2. The identity of one of the samples was found to have the maximum recognition when compared with BLASTn. The two amplified sequence products SX1 and SX2 shows homologous to *Solanum supinum,* and SX3 shows homologous to *Solanum virginianum.* It is previously reported that ITS2 has been extensively used in making universal primers for the species of the plant kingdom. Generally, the ideal and reported length of an ITS sequence is seen to be around 1500 bp. In dicots it is inferred that the length may vary up to 100-700 bp. This shows that the sequence that we got was a partial ITS2 region as the size smaller than the reported data. In order to employ our ITS marker into gene encoding the whole ITS2 region needs to be amplified[23,24]. The primer used in the current study was specifically designed by us for amplifying ITS region during polymerase chain reaction. The primer was designed using data available in the bioinformatics search tools. This aided us in amplification of the desired region that we needed for sequencing.

5. Morphological Assay

We conducted the morphological assay of the samples. All the samples were found to be very similar to *Solanum xanthocarpum*. They were inferred to have close resemblance to *Solanum virginianum* too. The seeds of each species varied a little when seen for the phenotypical aspect of the plant. The dimension of the seeds was globus for *Solanum xanthocarpum* while it was bean shaped for *Solanum virginianum.* All prickly species were inferred to be from the subgenus of Leptostemonum. The prickles were ideally found on the stems of the plant. They extended to the branches, twigs as well as the petioles and the leaves of the plant. These whole range of plants are found to be having very close similarities when it comes to appearance. Based on the habitat conditions where they thrive, the phenotype of the same species will vary[25,26]. The sample was used in the study and the plant sample, thorny leaves and stem with flower and seeds were represented in Figure 8.

However, the partial ITS2 sequenced in our study when compared with our BLASTn were found to homologous to *Solanum supinum* and *Solanum virginianum,* with the samples Sx1, Sx2 and Sx3 respectively. The sequenced ITS was found to have many conserved regions, so the sequence was found to be a partial sequence. The presence of more GC regions gives the originality of the sequence. Even partial sequence ITS2 of the *Solanum* species, yielded us in finding out the correct species and subgenus of the locally available *Solanum* plant. Hence, we are able to infer that partial ITS2 can also be used as tool to identify any species of close origin and features. Further amplification of the entire ITS2 sequence needs to be done to check the feasibility and validation of the

results were have reported. The sequences we got were for both samples were *Solanum supinum* and *Solanum virginianum*. The cladogram represents the Sx1 and Sx2 are closely related or similar species of the *Solanum*, another sample, Sx3 shows distinctly related species that supports the BLASTn of our result

6. Conclusion

The present study was designed with an intention to find a better alternate marker that will be specific for even closely related plant species. Even closely related plat species have shown to have different pharmacological features. Hence authenticity is very essential for the proper formulation of medicines. The utilization of DNA barcoding works for animal models. But for plants an even more specific ITS sequence in the genome to find the originality of the plant sample. The samples were collected from different regions of Tamil Nadu. They were phenotypically very similar. Based on morphology they were inferred as *Solanum xanthocarpum* or *Solanum virginianum*. However, from results the sequence (partial ITS2 region) threw a light on the fact that the plant sample under our study was actually *Solanum supinum* and *Solanum virginianum*. It was of great help for us to infer the exact species of *Solanum* we had in our locality. The complete sequencing of the ITS2 region is very much needed to have even more specific marker for the identification of plant varieties.

7. References

1. Kool A, de Boer HJ, Kruger A, Rydberg A, Abbad A, Bjork L, *et al*. Molecular identification of commercialized medicinal plants in Southern Morocco. PLosONE, 2012; 7(6).https://doi.org/10.1371/journal.pone.0039459. PMid:22761800. PMCid:PMC3384669
2. Animesh DK, Rita P. An updated overview on *Solanum xanthocarpum* Schrad and Wendal. Int. J. Ayurveda Res. 2011; 2(3):730–5.
3. Jayanthy A. Sulaiman CT, Shree ABR. Pharmacognostical and phytochemical comparison of roots of solanum species used in ayurvedic formulations, Int J Pharmacogn Phytochem. 2012; 4(1);28–37.
4. Bohs L, Myers NR, Levin RA. Phylogenetic relationships among the "spiny solanums" (Solanum subgenus leptostemonum, solanaceae). Am J Bot. 2006; 93(1):157–69. https://doi.org/10.3732/ajb.93.1.157
5. Eswaran N, Jawali N, Jawali N, Bhagwat SG. A simple method for isolation of DNA from plants suitable for long-termstorage and DNA marker analysis. BARC Newsletter. 2003.
6. Moura CCd, Brambach F, Bado KJH, Konstantin VK, *et al.* Integrating DNA barcoding and traditional taxonomy for the identification of dipterocarps in remnant lowland forests of Sumatra. Plants (Basel). 2019; 8(11):461. https://doi.org/10.3390/plants8110461. PMid:31671512. PMCid:PMC6918277
7. Venkatasubramanian P, Balasubramani SP, Goraya GS. Development of ITS sequence-based markers to distinguish *Berberis aristata* DC. from *B. lyceum* Royle and *B. asiatica* Roxb. 3 Biotech. 2011; 1:11–19. https://doi.org/10.1007/s13205-010-0001-5. PMid:22558531. PMCid:PMC3339604
8. Rawat S, Joshi G, Annapurna D, Arunkumar AN, Karaba NN. Standardization of DNA extraction method from mature dried leaves and ISSR-PCR conditions for Melia dubia Cav. – A fast growing multipurpose tree species. Am J Plant Sci. 2016; 7:437–45. https://doi.org/10.4236/ajps.2016.73037
9. Pikaart MJ, Villeponteau B. Suppression of PCR amplification by high levels of RNA. Biotechniques. 1993; 14:24–7.
10. Ragupathy S, Newmaster SG, Fazekas A. DNA barcoding in land plants: Evaluation of rbcL in a multigene tiered approach. Can J Bot. 2006; 84:335–41. https://doi.org/10.1139/b06-047
11. Sheth Bp, Thaker VS. DNA barcoding and traditional taxonomy: An integrated approach for biodiversity conservation. Epub. 2017; 618–28. https://doi.org/10.1139/gen-2015-0167. PMid:28431212
12. Mishra S, Sharma G, Das MK, Pande V, Singh OP. Intragenomic sequence variations in the second internal transcribed spacer (ITS2) ribosomal DNA of the malaria vector Anopheles stephensi. PLoS One. 2021; 16(6):025317. https://doi.org/10.1371/journal.pone.0253173. PMid:34125861 PMCid:PMC8202910
13. Dixit D, Balar N, Trivedi M, Suthar P, Reddy CRK, Gadhavi D. Internal Transcribed Spacer (ITS) region targeted molecular characterization of macroalgal diversity along the overlooked expanse of Gulf of Kachchh, India. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences; 2021. https://doi.org/10.1007/s40011-021-01273-8
14. The evaluation of biological activity of methanolic extracts of *Solanum nigrum* and molecular docking analysis of selected phytoconstituents against vimentin'. J Intercult Ethnopharmacol. 2017; 6(4):391. https://doi.org/10.5455/jice.20170701065821

15. Man SM, Kaakoush NO, Octavia S, Mitchell H. The internal transcribed spacer region, a new tool for use in species differentiation and delineation of systematic relationships within the campylobacter genus. Appl Environ Microbiol. 2010; 76(10).
16. Espada CR, Ferreira BA, Ortiz PA, Ulianaa SRB, Coelho SC. Full nucleotide sequencing of ribosomal DNA internal transcribed spacer of Leishmania species causing cutaneous leishmaniasis in Brazil and its potential for species typing. Acta Tropica. 2021; 223:106093. https://doi.org/10.1016/j.actatropica.2021.106093. PMid:34389323
17. Prah RE, Khan S, Deo RC. The role of internal transcribed spacer 2 secondary structures in classifying mycoparasitic Ampelomyces. PLoS One. 2021; 16(6):e0253772. https://doi.org/10.1371/journal.pone.0253772. PMid:34191835. PMCid:PMC8244850
18. Pang X, Shi L, Song J, Chen X, Chen S. Use of the potential DNA barcode ITS2 to identify herbal Materials. J Nat Med. 2013; 67:571–5. https://doi.org/10.1007/s11418-012-0715-2. PMid:23179313
19. Zou X, Dai Z, Ding C, Yang R. Relationships among six medicinal species of Curcuma assessed by RAPD markers. J Med Plant Res. 2011; 5(8).
20. Tong Y, Zhang Y-B, Shaw P-C, Sze C-W, Wang Z-T. Molecular authentication of Chinese herbal materials. J Food Drug Anal. 2007; 15:1–9.
21. Yip PY, Chau CF, Mak CY, Kwan HS. DNA methods for identification of Chinese medicinal materials. Chin Med. 2007; 2:9. https://doi.org/10.1186/1749-8546-2-9. PMid:17803808. PMCid:PMC2042497
22. Joseph CR, Rangasamy I, Patgiri BJ. Therapeutic potentials of Kantakari (*Solanum xanthocarpum* Schrad. and Wendl), Ayurpharm. Int J Ayur Alli Sci. 2012; 1(2):46–53.
23. Jayanthy A, Sulaiman CT, Shree ABR. Pharmacognostical and phytochemical comparison of roots of solanum species used in ayurvedic formulations. Int J Pharmacogn Phytochem Res. 2012; 4(1):28–37.
24. Eswaran N, Jawali N, Bhagwat SG. A simple method for isolation of DNA from plants suitable for long-term storage and DNA marker analysis, BARC Newsletter. 2008; (249):208–14.
25. Chen S, Yao H, Han J, Liu C, Song J, Shi L, *et al*. Validation of the ITS2 region as a novel DNA barcode for identifying medicinal plant species. PLoS One. 2010; 5(1):e8613 https://doi.org/10.1371/journal.pone.0008613. PMid:20062805. PMCid:PMC2799520
26. Chase MW, Christenhusz MJM, Palsson RL, Fay MF, Dodsworth S, Conran JG, *et al*. Species delimitation in nicotiana sect. Suaveolentes (Solanaceae): Reciprocal illumination leads to recognition of many new species. Curtis's Bot Mag. 2021; 38(3):266–86. https://doi.org/10.1111/curt.12410