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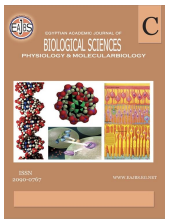

DNA Barcoding of Two Medicinal Plants Using Molecular Markers

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ABSTRACT

DNA barcoding is a novel method of species identification based on nucleotide diversity of conserved sequences. The establishment and refining of plant DNA barcoding systems are more challenging due to the high genetic diversity among different species. We, therefore, tested the potentiality of the ITS and *rbcL* markers for the identification of two medicinal species plants *Solanum nigrum* and *Solanum villosum*, which were collected from the Botanical garden of faculty of Science, Ain Shams Univ., Egypt. In this study, biological sequence homology and divergence of amplified sequences were studied using the Basic Local Alignment Tool (BLAST). Both DNA barcoding regions (ITS and *rbcL*) showed good universality amplification in the two species. The sequenced regions revealed conserved genome information for future identification of different medicinal plants belonging to these species. The amplified conserved barcodes revealed different levels of biological homology after sequence analysis. The results clearly showed that the use of these conserved DNA sequences as barcode primers would be an accurate way for species identification and discrimination. In this work, the ITS and *rbcl* markers were used to discriminate and confirm the identification of two medicinal plants, it was found that the viability and potentiality of ITS region in the identification process for the two plants used is more efficiency than *rbcl*, where *rbcl* confirm the identification of two plants at the generic level, while ITS at the species level. The findings of the study would be applicable in the medicinal industry to establish DNA based identification of different medicinal plant species to monitor adulteration.

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INTRODUCTION

DNA barcoding relies on finding different conserved regions in divergent species to produce a large scale reference genome library. DNA barcoding is a universally accepted and reliable method for species identification. The nuclear and mitochondrial sequences are mapped and sequenced in different species to design DNA based barcode primers. These barcodes are used for phylogenetic analysis, genetic diversity, and species discrimination in different organisms. This system not only helps to classify the organisms but also reveals genetic information for species ancestral inheritance and flagging of new species(Son *et al.*, 2003). In plants, finding effective and robust conserved regions are more challenging because of high genome diversity.

Chloroplasts are active metabolic machinery in green plants to convert light energy into carbohydrates. High-throughput sequencing technology has resulted in the sequencing of about 800 chloroplast genomes from different plants (Hebert *et al*., 2004). Two conserved regions from the plastid (chloroplast) genome (*rbcl*) were proposed as barcode primers to discriminate large groups of angiosperms. Internal Transcribed Spacer (ITS) is one of the most used polymorphic regions is, a space of noncoding RNA situated between structural ribosomal RNAs on a common precursor transcript. ITS spacer is known to be partitioned into ITS1 and ITS2 separated by 5.8S ribosomal cistron, in which the RNA poly-cistronic precursor transcript will be in this order 18S rRNA, ITS1, 5.8S rRNA, ITS2, 26S (Wheeler and Honeycutt, 1988). ITS1 and ITS2 are widely used for phylogeny reconstruction, due to the following reasons stated by many early studies (e.g. Baldwin *et al.,* 1995; Liston *et al.,* 1996 and Maggini *et al.,* 1998): 1- Biparental inheritance: in comparison to the maternally inherited chloroplast and mitochondrial markers. 2- Easy PCR amplification with several universal primers available for various kinds of organisms. 3- Multi-copy structure, which can be found in up to a few thousand copies *per* cell. 4- Moderate size, which allows reasonable sequencing. 5- Based on published studies the variation at the level that makes it suitable for evolutionary studies at the species or generic level (Poczai and Hyvonen, 2010).Generally, Extraction and purification of DNA of important medicinal plant species is a difficult task and it needs expert geneticist.

Solanum nigrum (Solanaceae) commonly known as Makoi or black nightshade It is an herbaceous plant with small rounded berry fruits used in traditional medicine for the treatment of various diseases including cancer, Polyphenolic extracts of this plant also induced apoptosis and arrests the cell cycle at G2/M phase in HepG2 carcinoma, providing basis behind complete inhibition of tumor progression in the experimental animal.

It was also shown that the total alkaloid isolated from *S. nigrum* interfered with the structure and function of the tumor cell membrane, disturbed the synthesis of DNA and RNA, changed the cell cycle distribution in tumor cells. Hence, total alkaloids could play a role in the inhibition of tumor cells, while a glycoprotein isolated from the plant exhibited anticancer abilities by blocking the antiapoptotic pathway of NF-kappaB via activation of caspase cascades reaction and increase nitric oxide production (Javed, *et al.*,2011)

S. nigrum seeds extract was found to exhibit potent antiviral activity against HCV 3a genotype, where Hepatitis C is a major health problem causes liver cirrhosis, hepatocellular carcinoma, and death. The plant has been extensively used in traditional medicine in different parts of the world to cure tuberculosis, diuresis (Chopra *et al*.,1956) various nerve disorders (Perez ,*et al*.,1998), ulcer healing (Jainu, M. and Devi, C.S.2006) , liver disorders (Lin *et al*.,.2008) effects of *S. nigrum* Linn extract against CCl(4)-induced oxidative damage in rats(Lin *et al*.,), antiseizure and inflammatory conditions (Wannang *et al*.,2008). The methanolic extract of *S. nigrum* contains important secondary metabolites including flavonoids, saponins, alkaloids, phytosterols (Harborne 1995). Methanolic extract of *S. nigrum* seeds resulted in a 37% reduction in HCV RNA of 3a genotype at non - toxic concentration (Javed, *et al*., 2011).

Solanum villosum is an economically important medicinal plant worldwide and represents an invaluable source of health-related compounds. It was found that this species is characterized by salinity tolerance and its ability to improve its antioxidant pool(Ben –Abdallah, *et al*.,2019).

At present 391000 vascular plant species are known to science, 94% of which are flowering plants (Shinwari *et al*., 2018).

However, a large number of plant species are yet to be identified. About 2000 species are being described every year. Therefore, the limited number of species has been identified through conventional identification and classification methods(Chase & Fay, 2009). DNA barcode is one of the advanced molecular marker-based methods that identify target plant species in a short duration. The purpose of DNA barcoding is nucleotide sequence-based identification of multiple plant species with accuracy and is one of the widely accepted technologies (Group *et al*., 2009). It is an efficient, quick, low-cost and standard method for evaluation and identification of different plant species (Khan *et al*., 2015). Also, this method can efficiently identify unknown species and/or species having complex morphometric behaviors. The DNA barcode is an improved and efficient method to differentiate among different plant species (Zahra *et al*., 2014, 2016), also,among different medicinal species (El-Atroush *et al*., 2015). These medicinal plants play key role in controlling and treatment of many diseases (Khan *et al*., 2017).

The present study aims to amplify and sequence the *rbcL* and ITS regions in the two plant species and study functional annotation and homology modelling of both sequences (ITS + *rbcl*) among these species using Basic Local Alignment Search Tool (BLAST), to confirm the identification of the two medicinal plants understudy and explore the evolutionary relationship between *Solanum nigrum* and *Solanum villosum*, also to detect the efficiency of ITS and *rbcl* markers in identification and discrimination of two medicinal species. A comprehensive study was designed to test *rbcL,* and ITS regions for authentication and phylogenetic investigation of two medicinal plant species from different regions of Egypt.

MATERAILS AND METHODS

DNA Barcode Analysis:

1. Extraction and Purification of DNA:

Solanum nigrum and *Solanum villosum* leaf specimens were collected and ground to a fine powder in liquid nitrogen using a sterile mortar and pestle. For DNA extraction and purification, DNeasy Plant Kit (Qiagen, Germany) was used. The concentration and quality of extracted DNA were estimated by running on 1% agarose gel electrophoresis, using a DNA size marker (100bp DNA Ladder from New England Bio-labs).

2. PCR and Gene Sequencing:

The PCR reaction was carried out as reported by (Ibrahim *et al.,* 2016) in a total volume of 50 µL PCR master mixture consisted of the following: 1x buffer (Promega, USA), 15 mM $MgCl_2$, 0.2 mM dNTPs (Promega, USA), 20 pcoml of each primer (Invitrogen, USA), 1 u of Taq DNA polymerase (GoTaq, Promega, USA), 40 ng DNA and ultra-pure water to the final volume. DNA barcoding was performed with the ncDNA ITS gene and cpDNA *rbcL* gene. For PCR amplification and sequencing of *rbcL* and ITS, the following primer pairs were used: ITS-*F* (5'-CCT TAT CAT TTA GAG GAA GGA GC-3'), *ITS-R* (5'-TCC TCC GCT TAT TGA TAT GC-3') and *rbcL*-F (5'-ATG TCA CCA CAA ACA GAG ACT AAA GC-3'), *rbcL-R* (5'-TCG CAT GTA CCT GCA GTA GC-3'). The average amplification size/bp was 722, 693 and for ITS, *rbcL* respectively.

The PCR was carried out with a Perkin-Elmer/GeneAmp® PCR System 9700 (PE Applied Biosystems, USA) programmed to fulfill 40 cycles after an initial denaturation cycle for 5 min at 94ºC. Each cycle consisted of a denaturation step at 94ºC for 30 s, an annealing step at 50ºC for 30 s and an elongation step at 72ºC for 30 s. The primer extension segment was extended to 7 min at 72ºC in the final cycle.

The amplification products were determined by electrophoresis in a 1.5% agarose gel using ethidium bromide (0.5 ug/ml) in 1X Tris borate Edita (TBE) buffer at 95 volts. For PCR product size determination, a marker 100 bp DNA ladder (Promega, USA), was used as a molecular size standard. Gel images were visualized using UV transilluminator and photographed using a Gel Documentation System (BIO-

RAD 2000, USA).

Purification of PCR products was carried out by a QIA quick PCR Purification Kit (Qiagen, USA). The PCR product was sequenced using the dideoxynucleotide chain termination method with a DNA sequencer (ABI 3730XL, Applied Biosystems) (Microgen, Korea) and a BigDye Terminator version 3.1 Cycle Sequencing RR-100 Kit (Applied Biosystems, USA) according to the protocol supplied by the manufacturer.

Assignment of Species:

DNA barcoding of *Solanum nigrum* and *Solanum villosum* were carried out using the Basic Local Alignment Tool (BLAST) available on the National Centre of Biotechnology Information (NCBI) website. All sequences were submitted to GenBank, USA. It provided MN852575and MN852576 accession numbers for the nucleotide sequences *rbcl*, while it provided MN901225 and MN901226 accession numbers for the nucleotide sequences ITS.

BLAST searches were applied to all produced sequences using the online database (GenBank), analyzed using BLASTN 2.9.0 program (http://www.ncbi.nlm.nih.gov/BLAST) and aligned using Align Sequences Nucleotide BLAST. The identification of species was considered successful when the highest similarity percentage included a single species scored more than 97% (de Groot, et al., 2011). Phylogenetic analysis was conducted using MAFFTv6.864, http://www.genome.jp/tools-bin/mafft, and phylogenetic trees were generated.

RESULTS

PCR based amplification of conserved regions (ITS and *rbcl*) is primarily required to establish DNA barcodes for species identification. With both universal primers *rbcl* and ITS we observed good results of PCR amplification in two species (*Solanum nigrum* and *Solanum villosum*).

In order to identify and classify plant species for conservation, traditional taxonomic tools are inadequate. Recently, the alternative approach of DNA barcoding is successfully introduced for authentication of rare and endemic plant species as an important base for evolutionary and ecological studies as well as for determining conservation priorities (Ferreira, *et al*., 2011, Fouad, *et al.,* 2019). DNA barcoding provides proper identification of endemic plant species, which is very important to help in the conservation of natural plant genetic resources (de Groot, *et al.,* 2011). It is a reliable tool to identify a plant species and a short genetic sequence from a standard part of the genome that can be sufficient.

1-Identification of *Solanum nigrum* by DNA Barcode Analysis:

The result of BLAST matching and phylogenic tree analysis of *Solanum nigrum* is shown in Tables (1 and 2) and Figure (1 and 2). The plant species of the highest percentages of similarity are represented. Newly generated sequences of the two markers; *rbcL* and ITS were used as barcodes. The alignments of *rbcL* and ITS sequences against GenBank accessions yielded query coverage 89 to 90% and 90 to 91%, respectively. Sequencing for *rbcL* and ITS regions of *Solanum nigrum* resulted in 580bp and 731bp sequences (effected length of query), respectively. Sequence alignment analysis revealed 100% the genus *Solanum nigrum* of length for *rbcL* and *ITS* sequences, respectively.

The phylogenetic trees of the plant species with the highest similarity percentages have a fan shape (Figs. 1 and 2) showing clustering of closely related species together and scattering of relatively distantly related species. Phylogenetic analyses with a combination of nrDNA and cpDNA are one of the most effective methods to understand evolutionary relationships between and within species. The results of the present study show a successful identification of *Solanum nigrum* on the species and genus levels. The success of species identification using DNA barcoding is contributing to the availability of nucleotide data of the corresponding taxa in the DNA sequences database. However, additional experiments with other markers to entirely identify the

plant more accurately are required. The present study reflects the potential use of DNA barcode analysis in the documentation of medicinal species concerning assigning to the proper taxonomic position.

Table 1; DNA barcode of *rbcl* downloaded from GenBank database including plant species with a similarity percentage of more than 99%.

Plant species	Accession no.	E-value	Query coverage (%)	Similarity (%)
Solanum nigrum	MH588530.1	0.0	90	100
Solanum nigrum	MH588529.1	0.0	90	100
Solanum nigrum	MH311570.1	0.0	90	100
Solanum nigrum	KY293568.1	0.0	90	100
Solanum physalifolium	MF135320.1	0.0	89	100
Solanum physalifolium	MF135316.1	0.0	89	100
Solanum americanum	JN545017.1	0.0	90	99
Solanum anguivi	JX511989.1	0.0	90	99
Solanum chenopodioides	HM850361.1	0.0	90	99
Solanum sp.	AB586592.1	0.0	90	99

Table 2; DNA barcode of *ITS* downloaded from GenBank database including plant species with a similarity percentage of more than 99%.

Plant species	Accession no.	E-value	Query coverage(%)	Similarity(%)
Solanum nigrum	KC540785.1	0.0	91	99
Solanum nigrum	KC540792.	0.0	91	99
Solanum nigrum	KC540796.1	0.0	91	99
Solanum nigrum	GU323360.1	0.0	91	99
Solanum nigrum	FJ980391.1	0.0	90	99
Solanum physalifolium	KY968822.1	0.0	91	98
Solanum physalifolium	KY968826.1	0.0	91	98
Solanum villosum	KC540788.1	0.0	91	98
Solanum americanum	MH050304.1	0.0	90	98
Solanum americanum	MK412137.1	0.0	90	98

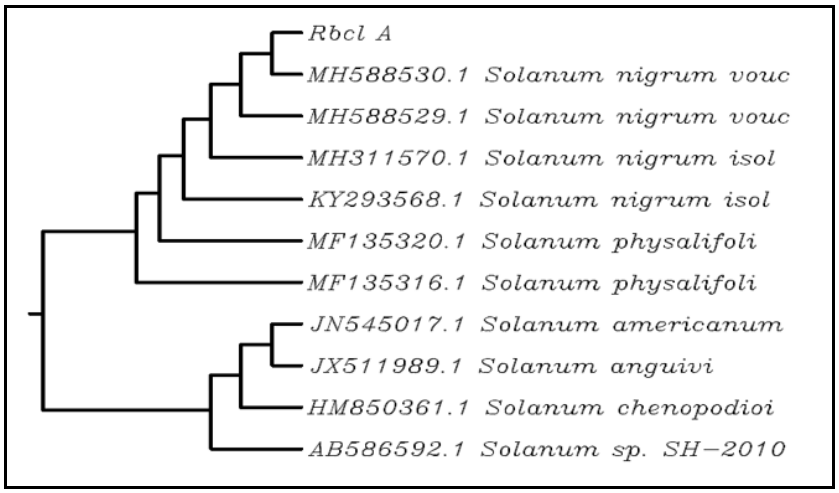

Fig. 1; Phylogenetic tree of *Solanum nigrum* using the cpDNA marker; *rbcL*

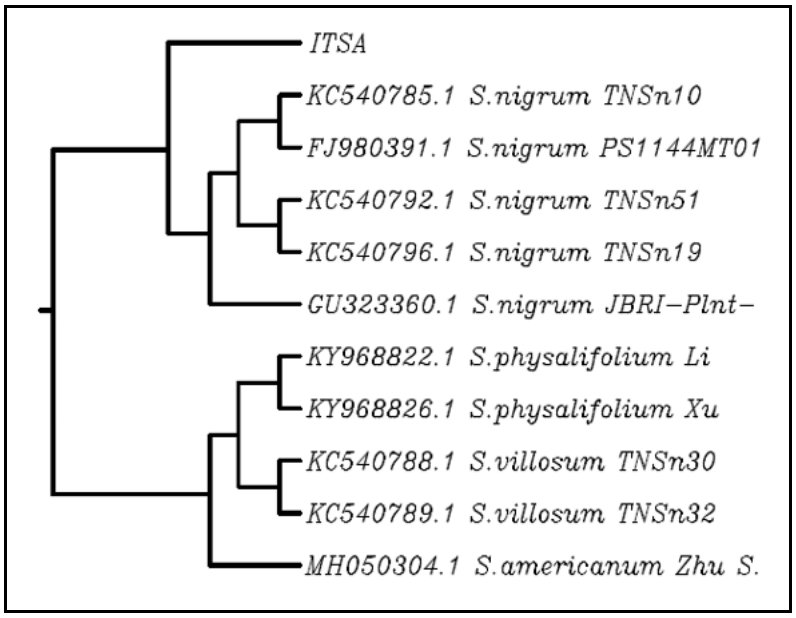

Fig. 2. Phylogenetic tree of *Solanum nigrum* nuclear marker; ITS.

1- Identification of *Solanum villosum* by DNA Barcode Analysis:

The result of BLAST matching and phylogenic tree analysis of *Solanum nigrum* is shown in Tables (3 and 4) and Figures (3 and 4).The plant species of the highest percentages of similarity are represented. Newly generated sequences of the two markers; *rbcL* and ITS were used as barcodes. The alignments of *rbcL* and ITS sequences against GenBank accessions yielded a query coverage between 93 to 94% and 89% respectively. Sequencing for *rbcL* and ITS regions of *Solanum villosum* resulted in 585bp and 738bp sequences (effected length of the query), respectively. Sequence alignment analysis revealed 100% the genus *Solanum villosum* of length for *rbcL*, while sequence alignment analysis revealed 99% the genus *Solanum villosum* of length for ITS.

The phylogenetic trees of the plant species with the highest similarity percentages have a fan shape (Fig. 3 and 4), showing clustering of closely related species together and scattering of relatively distantly related species.

Table 3:DNA barcode of *rbcl* downloaded from GenBank database including plant species with a similarity percentage of 99%.

Plant species	Accession no.	E-value	Query coverage (%)	Similarity (%)
Solanum villosum	HE963683.1	0.0	94	99
Solanum chenopodioides	HM850361.1	0.0	94	99
Solanum sp.	AB586596.1	0.0	94	99
Solanum sp.	AB586592.1	0.0	94	99
Solanum nigrum	GQ436617.1	0.0	94	99
Solanum nigrum	EU677013.1	0.0	94	99
Solanum nigrum	MH588529.1	0.0	94	99
Solanum americanum	JN545017.1	0.0	94	99
Solanum nigrum	KU556630.1	0.0	94	99
Solanum americanum	MH050094.1	0.0	93	99

Table 4:DNA barcode of ITS downloaded from GenBank database including plant species with a similarity percentage of more than 98%.

Plant species	Accession no.	E-value	Query coverage (%)	Similarity (%)
Solanum villosum	KC540791.1	0.0	89	99
Solanum villosum	KC540789.1	0.0	89	99
Solanum villosum	KC540788.1	0.0	89	99
Solanum villosum	KC540795.1	0.0	89	99
Solanum villosum	GU323359.1	0.0	89	99
Solanum americanum	MH768325.1	0.0	89	98
Solanum nigrum	MH844596.1	0.0	89	98
Solanum physalifolium	MH844593.1	0.0	89	98
Solanum nigrum	FJ980391.1	0.0	89	98
Solanum americanum	MH050306.1	0.0	89	98

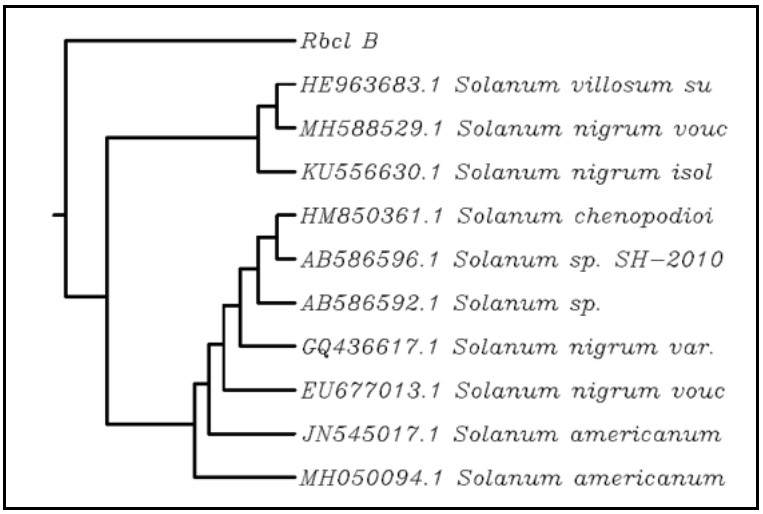

Fig. 3; Phylogenetic tree of *Solanum villosum* using the cpDNA marker; *rbcL*

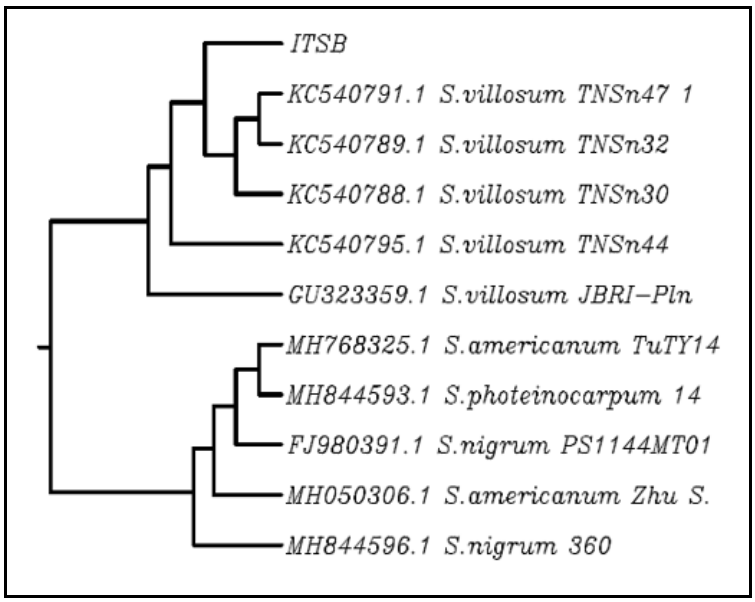

Fig. 4. Phylogenetic tree of *Solanum villosum* using the nuclear marker; ITS.

DISCUSSION

The genome sequence analysis of many species followed by the mapping of complex traits associated with divergent phenotypes resulted in the identification of many genes and their detailed inheritance patterns in many plant species. In PCR based amplification, the quality of DNA plays an important role. The presence of metabolites in medicinal plants affects DNA quality during isolation and even closely related species may require different DNA isolation protocols (Khanuja *et al*., 1999). The sequence variation from reference sequence and phylogenetic reconstruction is the basic principle for species identification in plants (Altschul *et al*., 1997). The use of DNA based markers (except RFLP) as universal primers have significant results in species identification as they result in good amplification across different genomic regions among divergent species(Kumar *et al*., 2009). Next-generation sequencing is another facility of advanced genomics era to have a more precise picture of species genome and to identify more orthologous and paralogous regions at different loci of different species. Sequencing of the entire plastid genome along with ITS & nrDNA is becoming a method of choice for species discriminations (Khan *et al*., 2017) In the current study, we amplified and sequenced the conserved regions in two different plant species. These medicinal species have an important role in the pharmaceutical industry. These DNA barcodes were first time applied in native species which would be a pioneering step to establish DNA based monitoring protocols for medicinal adulteration under national and international trade. However, there are still a lot of plant genomes lacking sequence information. A genus-based identification system would be a method of choice in species identification in indigenous land plants. Re-sequencing of additional loci for target-based improvements would relatively be helpful to detect more conserved genomic regions among different plant species. The findings of the current study suggest that the use of universal primers (*rbcL* and ITS) for DNA barcoding is successful for amplification, identification, and discrimination of above mentioned indigenous plant species. The amplification success rates were 100% for all two species. Both the primer pairs resulted in sharp bands that were required for reliable DNA sequencing. Thus, using DNA barcodes primers systems is a reliable, fast and cheap system for the identification of medicinal plants at the genus and species level of indigenous land species. Generation of extensive DNA database (using next-generation sequencing) while focusing on more conserved regions would be powerful for medicinal plant identification (Zahra *et al*., 2016).These records would further be helpful to study taxonomy, ecology, phylogeny, and morphology of different species(Hajibabaei *et al*., 2007). The markers chosen for this study represent the most commonly used plant molecular marker with a suitable length that will not incur extravagant costs in sequencing and also for its ease in amplification. *rbcL* produced high-quality sequences as compared to ITS. The reason for the difference in the quality of sequences generated lies with the nature of the markers themselves. *rbcL* belongs to the coding region of the plastid genome and is important in the synthesis of the enzyme *RuBisCo* (Kanevski & Maliga, 1994) This particular enzyme is necessary for carbon dioxide fixation for food production in plants.

Conclusion:

The results of the present study show a successful identification of *Solanum nigrum* on the species and genus levels. The success of species identification using DNA barcoding is contributing to the availability of nucleotide data of the corresponding taxa in the DNA sequences database. However, additional experiments with other markers to entirely identify the plant more accurately are required. In this work, the ITS and *rbcl* markers were used to discriminate and confirm the identification of two medicinal

plants, it was found that the viability and potentiality of ITS region in the identification process for the two plants used is more efficiency than *rbcl*, where *rbcl* confirm the identification of two plants at a generic level, while ITS at the species level.

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