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# Cross species amplification of potato (*Solanum tuberosum*) gene specific markers in *Withania somnifera* (L.) Dunal

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***Abstract:*** The CTAB method for DNA isolation was modified for Ashwagandha due to high levels of polysaccharides, polyphenols and secondary metabolites like alkaloids, flavonoids, phenols, terpenes etc., which interfere with the DNA isolation and purification procedure. Use of 3.5 % CTAB, 2% PVP, 1.4 M NaCl and 1 hour incubation time was found to be most appropriate. The isolated DNA was amplified by the primers from homologous species like potato for rbcl region and 18s ribosomal gene. The amplified region was sequenced as a marker for Ashwagandha.

***Key Words:*** *CTAB, DNA, primers, markers, cross species, Ashwagandha, Potato*

## 1. INTRODUCTION :

The advents of molecular markers have revolutionized the entire scenario of biological sciences. DNA based molecular markers have applications in various fields like taxonomy, DNA fingerprinting, genetic diversity analysis, gene tagging and marker assisted selection etc. They can be successfully employed to study the genetic diversity among naturally occurring populations and also in those populations where diversity could be conserved. The diversity evaluation has been tremendously empowered by invoking molecular techniques like polypeptide and DNA polymorphism profiling which facilitate direct and reliable measurement of genetic divergence.

Five polyphenolics (gallic, syringic, benzoic, p-coumaric and vanillic acids) and three flavonoids (catechin, kaempferol and naringenin) were found in high concentrations especially in the leaves, confirming the antioxidant potential and health benefits of *W. somnifera* (L.) Dunal (Alam et al., 2011); (Saidulu et al., 2014). The methods employed for extracting DNA from fresh and dried parts of medicinal plants are however time consuming and generally yield DNA in lesser quantity (Iqbal, 2013). Molecular ecologists increasingly require 'universal' genetic markers that can easily be transferred between species. Success in the cross-species amplification of any DNA sequence is inversely related to the evolutionary distance or the diversity between the two species (Steinkellner et al., 1997). The potential for successful cross-species transfer appears highest in species with long generation times, mixed or outcrossing breeding systems and where genome size in the target species is small compared to the source (Barbara et al., 2007). Zimmermann *et al.,* (2011) using DNA barcoding systematically tested the entire 18S gene sequence of diatoms and identified promising regions based on their variability. The rRNA gene sequences are easy to access due to highly conserved flanking regions allowing for the use of universal primers (Meyer et al., 2010). Because of its high and unrivaled amplification success and reasonable resolving power, 18S rRNA gene can be used as a potential marker. Lakshmi et al. (2002) reported that markers specific to ribosomal DNA and chloroplast genes have provided reliable information for the analysis of genomic relationships above the level of species owing to their highly conserved nature. Therefore, this experiment was framed to check the cross specific amplification of potato specific markers in Ashwagandha.

## 2. MATERIALS AND METHODS :

### Collection of plant material

In the present investigation, seed material of JA 134 genotypes of *W. somnifera* (L.) Dunal was procured from 'Botanical Garden, Department of Genetics and Plant Breeding, C. P. College of Agriculture', S.D. Agricultural University, S.K. Nagar, which were originally collected from different research stations.

### Reagents and chemicals for DNA extraction

| | | |
|---|---|---|
| 1 | **CTAB extraction buffer** | |
| | **Tris- HCl (pH 8.0)** | 200mM |
| | **EDTA (pH 8.0)** | 20mM |

| | | |
|---|---|---|
| | **Sodium chloride** | 1.4M |
| | **CTAB** | 3.5% |
| | **PVP** | 2% |
| | **β-mercaptoethanol (Added just before use)** | 2% |
| **2** | **Wash buffer** | |
| | **TrisHCl (pH 8.0)** | 100mM |
| | **PVP** | 1% |
| **3** | **Phenol- chloroform- isoamyl alcohol** | 25:24:1 |
| **4** | **Chloroform: Isoamyl alcohol** | 24:1 |
| **5** | **Isopropanal and ethanol** | 70% |
| **6** | **TE buffer** | |
| | **Tris (pH 8.0)** | 10 mM |
| | **EDTA (disodium, pH 8.0)** | 1 mM |
| **7** | **RNase** | 2mg/ml |

**Isolation of genomic DNA**

Genomic DNA was extracted from medium size leaves from one month old seedlings of Ashwagandha using CTAB extraction method as reported by Doyle and Doyle (1990) with minor modification. This modification was essential because plant contains exceptionally high amount of polyphenols, polysaccharides, tannins and other secondary metabolites such as alkaloids, flavanoids, phenols, terpenes and quinines which could interfere in DNA isolation protocol. Fresh collected leaves were washed with D/W and surface sterilized with Triton X-100 and blotted with tissue paper to remove water. Midrib veins were removed and the leaves were chopped into 3-5mm small pieces. Around 1 gram chopped leaf was taken and ground (using prechilled morter and pestle) with 1.5 ml freshly prepared wash buffer and 60$\mu$l mercaptoethanol. The samples were transferred into 2ml eppendorf tubes and centrifuged at 12,000 RPM for 3 min at 4$^{o}$C. The upper aqueous phase was discarded and 1.5 ml wash buffer was added to the pellet, mixed well and centrifuged as above. The above process was repeated twice with wash buffer. In the next step, 1.5 ml extraction buffer (preheated at 65$^{o}$C) was added along with 0.06gm PVP and 7.5 $\mu$l mercaptoethanol to the remaining pellet and mixed well. The samples were kept for incubation into water bath at 65$^{o}$C for 1 hr. After incubation, tubes were kept at room temperature for 5 min to cool down. The incubated samples were centrifuged at 12,000 RPM for 10 min at room temperature and aqueous phase was transferred into fresh autoclaved tubes. Then equal amount of phenol-chloroform-isoamyl alcohol (25:24:1) solution was added to the transferred aqueous phase, gently mixed for 1 minute by inversion and centrifuged at 12,000 RPM for 10 min at 4$^{o}$C. Again the aqueous phase was transferred into a fresh autoclaved tube containing equal volume of chloroform- isoamyl alcohol (24:1) and mixed well for 1 min by inversion. The samples were centrifuged at 12,000 RPM for 10 min at 4$^{o}$C and the aqueous phase was transferred into a fresh autoclaved eppendorf tube having 5 $\mu$l of 5M NaCl and 0.6 volume of prechilled isopropanol. The samples were kept for overnight at -20$^{o}$C for better precipitation. These precipitated samples were centrifuged at 15,000 RPM for 10 min at 4$^{o}$C. The aqueous phase was discarded and the remaining pellets were air dried at room temperature for 3-4 hrs. To these pellet, 100 $\mu$l of high salt TE buffer and 10 $\mu$l of RNase were added and kept at 37$^{o}$C for 30 min followed by chloroform: isoamyl alcohol (24:1) extraction and ethanol precipitation in presence of 3M sodium acetate (pH 5.2). The samples were centrifuged at 12,000 RPM for 10 min at 4$^{o}$C. Extracted pellet was washed using 70% ethanol and centrifuged at 5,000 RPM for 5 min at 4$^{o}$C. The pellet was air dried for 3-4 hrs and 100 $\mu$l TE buffer was added and stored at -20 $^{o}$C for further use.

**Agarose gel electrophoresis**

DNA samples were resolved by submerged horizontal electrophoresis in 0.8% (w/v) agarose gels and visualized by staining with ethidium bromide.

**Reagents**

| **1)** | **1X TBE buffer** | **Amount** |
|---|---|---|
| | **Tris** | 10.8g |
| | **Boric Acid** | 5.5g |
| | **EDTA** | 0.75g |
| | **Final Volume** | 1L |
| **2)** | **6X Loading dye** | |
| | **Glycerol** | 30% |
| | **Bromophenol blue** | 0.25g |
| | **Distil Water** | 70ml |

| | | |
|---|---|---|
| | **Final volume** | 100ml |

All the chemicals and reagents used in the present investigation for DNA extraction and electrophoresis were of high purity analytical molecular biology grade of HiMedia. Loading dye solution was stored at 4°C in the refrigerator.

**Procedure**

**Estimation of quantity and quality of genomic DNA of *Withania somnifera* (L) Dunal**

**Quantity of DNA by UV-spectrophotometric estimation**

An aliquot of isolated DNA sample was properly diluted and absorbance (A) was determined at 260 nm and 280 nm wavelength in U.V. spectrophotometer. Using the relationship of 1.0 O.D. at 260 nm equivalent to 50 $\mu$g DNA per ml, the quantity of DNA was estimated from the following formula:
Concentration of DNA ($\mu$g/ml) = A260 x 50 x dilution factor.

**Quality of DNA by agarose gel electrophoresis**

Quality of DNA sample was checked both by UV-spectrophotometer and on agarose gel electrophoresis. Using spectrophotometer the ratio of the absorbance at 260 nm and 280 nm was noted. Sample with a ratio of 1.8 to 2.0 were considered of good quality. The quality of DNA preparation was also tested by submerged horizontal agarose (0.8%) gel electrophoresis. Appearance of high molecular weight band near the wells and no smear on gels assured a quite good quality DNA. DNA quantity was also determined on the basis of band intensity as compared with the lambda DNA marker (used to determine the concentration) on agarose gels.

**PCR Amplification of DNA and sequencing**

Different parameters were tested to determine optimal concentrations of template DNA, $MgCl_2$, dNTPs, Taq DNA polymerase, primer and different temperatures and time intervals during denaturation, annealing and elongation steps which affect amplification, banding pattern and reproducibility. For this, varying concentrations of template DNA (500 ng, 1000 ng, 2000 ng), primers (0.5 µl, 1.0 µl, 1.5 µl, 2.0 µl), dNTPs (0.5 µl, 1.0 µl, 1.5 µl, 2.0 µl) and $MgCl_2$ (1mM, 1.6 mM, 2 mM and 2.5 mM) were used in a reaction volume of 20 µl in different combinations at different annealing temperatures (36°C, 40°C, 45°C, 50°C and 55°C).

| **Reagent** | **Quantity** |
|---|---|
| **Template** | 1 $\mu$l |
| **Forward Primer (1or 3)** | 1$\mu$l |
| **Reverse Primer (2 or 4)** | 1$\mu$l |
| **dNTPs (10mM)** | 1$\mu$l |
| **10X Taq DNA polymerase Assay Buffer** | 2.5$\mu$l |
| **Taq DNA Polymerase (3U/ $\mu$l)** | 0.5$\mu$l |
| **Water** | 18$\mu$l |
| **Total reaction volume** | 25$\mu$l |

**Selection of primers**

| | | | |
|---|---|---|---|
| **1)** | **Ribulose-1, 5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene** | Hein et al., 2007 | 5’CTGCAGGTACATGCGAAGAA 3’(f)<br>5’TTGCTAATACCCGGAAGTGG 3’(r) |
| **2)** | **18S ribosomal RNA gene** | Nicot et al., 2005 | 5’GGGCATTCGTATTTCATAGTCAGAG 3’(f)<br>5’CGGTTCTTGATTAATGAAAACATCCT 3’(r) |

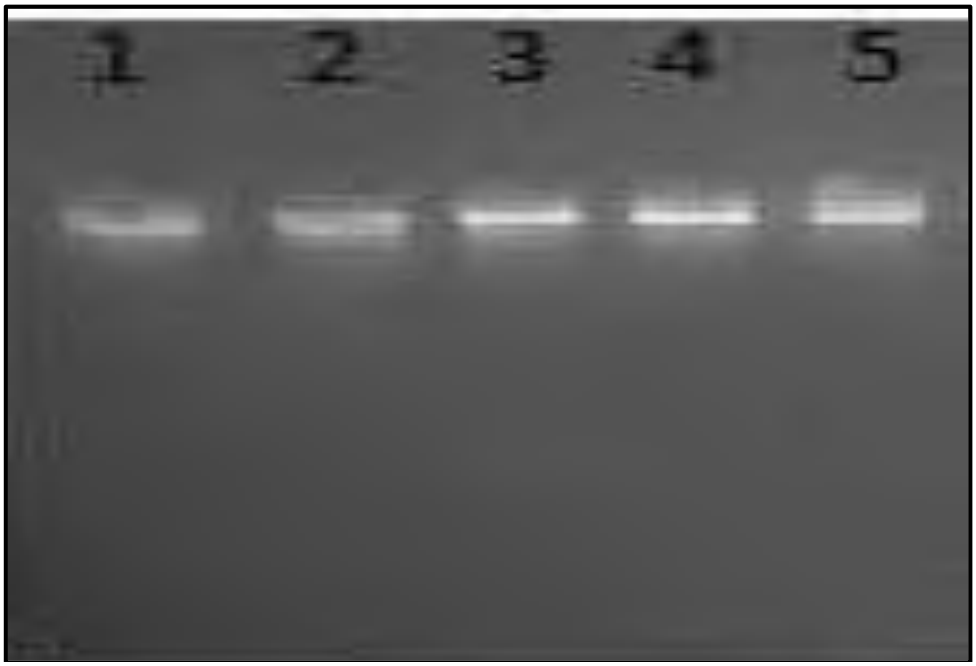


**Fig. a: Gel picture of isolated DNA samples using CTAB modified method**

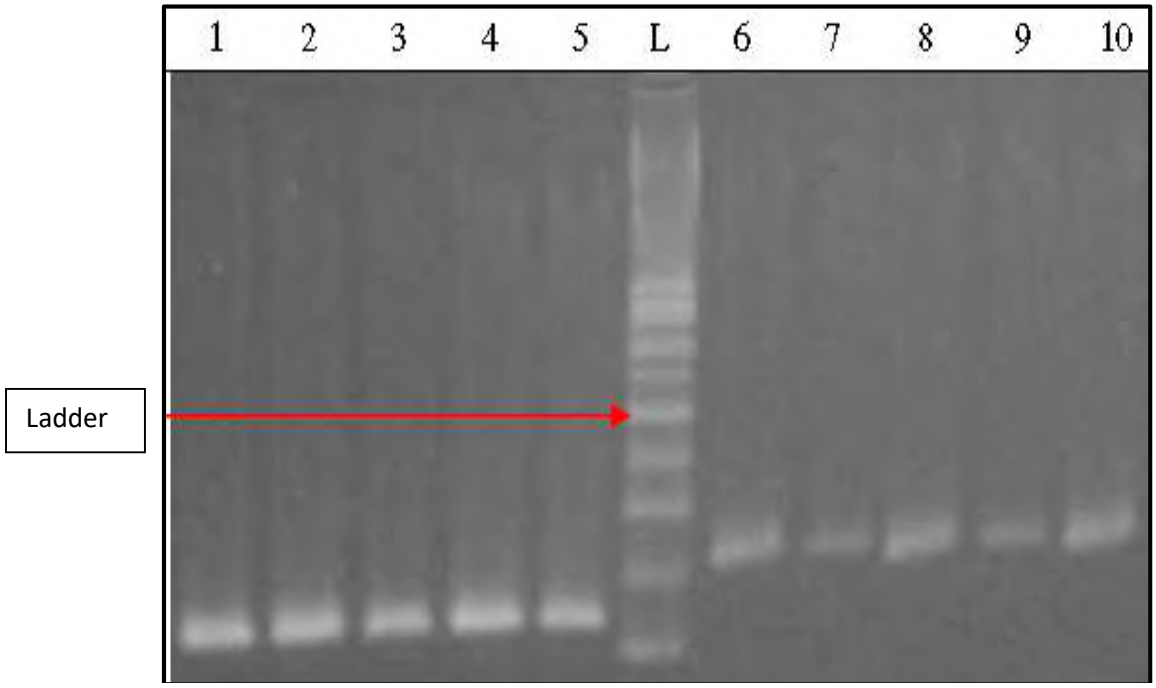


**Fig. b: Cross species PCR amplification of rbcL gene & 18s ribosomal RNA gene**

**Lane Description**: **Lane 1-5 (rbcL gene) & 6-10 (18s ribosomal RNA gene), L-1000 bp ladder**

**PCR Cycle conditions used**

| Initial denaturation | Denaturation | Annealing | Extension | Final extention |
|---|---|---|---|---|
| **94°c** | 94°c | 50°c | 72°c | 72°c |
| **5 min** | 30 sec | 30 sec | 30 sec | 7 min |
| | 35 cycles | | | |

## 3. RESULTS AND DISCUSSION :

Published methods of DNA isolation including those of Doyle and Doyle (1990), Rogers and Benedict (1985) and Dellaporta et al., (1983) proved to be relatively less successful and reliable for *Withania somnifera* (L.) Dunal. The DNA acquired was dirty yellow in appearance and with high viscosity. This is due to high levels of polysaccharides, polyphenols and secondary metabolites like alkaloids, flavonoids, phenols, terpenes etc., present in the medicinal plants

which meddle with the DNA isolation and purification procedure. Secondary metabolites and polysaccharides interfere with total DNA isolation procedures and PCR based downstream applications. The removal of such contaminants needs complicated and time-consuming protocols (Devi, 2013). Polysaccharide contaminations are particularly problematic (Scott and Playford, 1996) as they can inhibit the activity of many commonly used DNA modifying enzymes, such as polymerases (Fang et al. 1992), ligases and restriction endonucleases. This is because nucleic acids form tight complexes with polysaccharides creating a gelatinous pellet and the embedded DNA is inaccessible to the enzymes (Sharma et al., 2002). During homogenization, polyphenols are released from vacuoles and react rapidly with cytoplasmic enzymes. DNA isolation protocols generally use CTAB to avoid co-purifying polysaccharides from plant tissues. Therefore, DNA extraction procedure was standardized by using different concentrations of CTAB (2%, 3%, 3.5% & 4%) and PVP (1% and 2%). Use of 3.5 % CTAB, 2% PVP, 1.4 M NaCl and 1 hour incubation time was found to be most appropriate. Highly purified genomic DNA was obtained when the optimized protocol described in "Materials and Methods" was used. A sufficient amount of clean genomic DNA was obtained with this method **(Figure a)**.

Present protocol involves initial repeated washing steps with wash buffer containing PVP, which has been reported to remove polyphenols from mature, damaged and improperly stored leaf tissues (Rogers and Bendich, 1985; Doyle and Doyle, 1987, Howland et al., 1991). PVP forms complex hydrogen bonds with polyphenolic compounds which can be separated from DNA by centrifugation (Maliyakal, 1992). Complete digestion with restriction endonucleases and amplification by PCR indicates the absence of polysaccharides. Polysaccharides are difficult to separate from DNA (Murray and Thompson, 1980). The problem arising from the presence of high levels of polysaccharides was overcome by using NaCl at a higher concentration (1.4 M). These compounds are easily identifiable in the DNA preparations as they impart a sticky, viscous consistency to the DNA preparations dissolved in TE buffer. The polysaccharides interfere with several biological enzymes such as polymerases, ligases and restriction endonucleases. This problem was overcome by using 5M NaCI and high salt TE buffer.

**Cross species PCR amplification**

An Amplicon size of 219bp and 300bpwas obtained for rbcL gene and 18s ribosomal RNA gene, respectively **(Figure b).**

**Sequence Data Results:**

**rbcL gene**

GTTGAGCTATCATGCTAGAGCATTGGGCGTTCCGATCGTAATGCATGACTACTTAACGGGGGGATTCAC
CGCAAATACTACCTTGGCTCATTATTGCCGAGATAATGGTCTACTTCTTCACATCCACCGTGCAATGCAT
GCGGTTATTGATAGACAGAAGAATCATGGTATCCACTTCCGGGTATTAGCAAAGA

**18s ribosomal RNA gene**

AAGGAAGCTAGCTATATGTTAACACATGCAAGTCGAACGTTGTTTTCGGGGAGCTGGGCAGAAGGAAA
AGAGGCTCCTAGCTAAAGGTAGTTTTTGTCTCGCCCAGGAGGTGAGAAGAGTTGAGAAACAAAGTGGCG
AACGGGTGCGTAACGCGTGGAATCTTTTGCCGAACAGTTCGGGCCAAATCCTGAAAAGCTAAAAAGCGC
TGTTTGATGAGCCTGCGAGTATTAGGTAGTTGGT

Sequence data of the sample was aligned using the nucleotide Basic Local Alignment Search Tool (BLASTn) (https://blast.ncbi.nlm.nih.gov/Blast).

BLAST result showed that rbcL gene sequence was highly homologous with the *Solanum melongena* (also known as Egg plant**)**, ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, present in chloroplast genome with sequence ID KJ773910.1 having score 327 bits with E value 3e-86 and 98% identity **(Figure c)**.

While 18s ribosomal RNA gene was found homologous with *Solanum tuberosum* mitochondrial gene for 18S ribosomal RNA with Sequence ID AB971541.1 having score 377 bits with E value 4e-101 and 95% identity **(Figure d)**.

Robertson et al., (2010) recognized high self-compatibility and polyploidy in different members of solanaceae family like *Solanum tuberosum, Solanum melongena and Withania somnifera* (L.) Dunal. Rana et al., (2014) identified that P450 gene of *Withania somnifera* (L.) Dunal showed 94% homology with *Solanum tuberosumand 83% similarity with Solanum melongena*. Haq et al., (2014) evaluated cross transferability and polymorphism among 11 plants belonging to five different solanaceae members and found highest in case of *Withania somnifera* (L.) Dunal with 98.06%.

Parmar et al., (2015) identified cross species transferability between Ashwagandha and egg plant and identified EST SSR markers for it.

***Solanum melongena* ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds; chloroplast**

**Sequence ID: gb|JX675575.1**

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 327 bits(177) | 3e-86 | 179/180(99%) | 0/180(0%) | Plus/Plus |

```
Query  13   TGCTAGAGCATTGGGCGTTCCGATCGTAATGCATGACTACTTAACGGGGGGATTCACCGC  72
            |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  714  TGCTAGAGAATTGGGCGTTCCGATCGTAATGCATGACTACTTAACGGGGGGATTCACCGC  773

Query  73   AAATACTACCTTGGCTCATTATTGCCGAGATAATGGTCTACTTCTTCACATCCACCGTGC  132
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  774  AAATACTACCTTGGCTCATTATTGCCGAGATAATGGTCTACTTCTTCACATCCACCGTGC  833

Query  133  AATGCATGCGGTTATTGATAGACAGAAGAATCATGGTATCCACTTCCGGGTATTAGCAAA  192
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  834  AATGCATGCGGTTATTGATAGACAGAAGAATCATGGTATCCACTTCCGGGTATTAGCAAA  893
```

**Fig. c: BLAST n result- rbcL gene sequence showing homology with *Solanum melongena***

***Solanum tuberosum* mitochondrial gene for 18S ribosomal RNA, partial sequence**
**Sequence ID: AB971541.1|Length: 1896Number of Matches: 1**

| Score | Expect | Identities | Gaps | Strand |
|---|---|---|---|---|
| 377 bits(204) | 4e-101 | 234/246(95%) | 11/246(4%) | Plus/Plus |

```
Query  1    AAGGAA-GCTAGCTATATG-TTAACACATGCAAGTCGAACGTTGTTTTCGGGGAGCTGGG  58
            |||||| |||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  1    AAGGAACGCTAGCTATATGCTTAACACATGCAAGTCGAACGTTGTTTTCGGGGAGCTGGG  60

Query  59   CAGAAGGAAAAGAGGCTCCTAGCTAAAGGTAGTTTTTGTCTCGCCCAGGAGGTGAGAAGA  118
            |||||||||||||||||||||||||||||||   |||||||||||||||||||||||||
Sbjct  61   CAGAAGGAAAAGAGGCTCCTAGCTAAAGGTAGC--TTGTCTCGCCCAGGAGGTGAGAAGA  118

Query  119  GTTGAGAAACAAAGTGGCGAACGGGTGCGTAACGCGTGGGAATCTTTTGCCGAACAGTTC  178
            |||||| |||||||||||||||||||||||||||||||||   ||||||||||||
Sbjct  119  GTTGAG-AACAAAGTGGCGAACGGGTGCGTAACGCGTGGGAATC---TGCCGAACAGTTC  174

Query  179  GGGCCAAATCCTGA--AAAGCTAAAAAGCGCTGTTTGATGAGCCTGCG-AGTATTAGGTA  235
            |||||||||||||  |||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  175  GGGCCAAATCCTGAAGAAAGCTAAAAAGCGCTGTTTGATGAGCCTGCGTAGTATTAGGTA  234

Query  236  GTTGGT  241
            ||||||
Sbjct  235  GTTGGT  240
```

**Fig. d: BLASTn result-18s ribosomal RNA gene showing homology with *Solanum tuberosum* mitochondrial gene**

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