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# DNA Barcoding with Ribulose-1, 5-Bisphosphate Carboxylase Oxygenase in Indian Medicinal Plant- *Cassia Fistula L*

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**Abstract:** To develop a barcode for the medicinally important plant *Cassia fistula L*. To collect leaf samples of the Indian medicinal plant *Cassia fistula L* and to isolate total genomic DNA from the collected sample then the qualitatively analyse the DNA using Agarose gel electrophoresis and the quantitate DNA yield using UV Spectrophotometry was done. The aim of study is to amplify *rbcL* gene using Polymerase Chain Reaction. In this current study, the leaf samples of *Cassia fistula L* were collected from Chennai and the PCR amplicons were analysed using Agarose gel electrophoresis, purify the amplicons and understand the sequence of DNA by Sanger method then the sequence alignment using BLAST (NCBI) tool.

**Keywords:** DNA Barcoding, RuBisCo, PCR and *Cassia fistula L*

## I. INTRODUCTION

### A. DNA Barcoding

Taxonomy, the science of classifying living things according to shared features, has always been a part of human society. Classical taxonomy falls short in this race to catalog biological diversity before it disappears. The International Barcode of Life (iBOL) organizes collaborators from more than 150 countries to participate in a variety of “campaigns” to census diversity among plant barcoding relies on short, highly variably regions of the genome. With thousands of copies per cell, mitochondrial and chloroplast sequences are readily amplified by polymerase chain reaction (PCR), even from very small or degraded specimens. A region of the chloroplast gene *rbcL*—RuBisCo large subunit—is used for barcoding plants. The most abundant protein on earth, RuBisCo (Ribulose-1, 5-bisphosphate carboxylase oxygenase) catalyzes the first step of carbon fixation.

DNA is extracted from the tissue sample, and the barcode portion of the *rbcL*, COI, or ITS gene is amplified by PCR. The amplified sequence (amplicon) is submitted for sequencing in one or both directions. The sequencing results are then used to search a DNA database. A close match quickly identifies a species that is already represented in the database. However, some barcodes will be entirely new, and identification may rely on placing the unknown species in a phylogenetic tree with near relatives. Novel DNA barcodes can be submitted to GenBank® ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)).

In effect, barcoding in its modern form was popularized in a paper by [5], who proposed to use the mitochondrial gene COI as the standard barcode for all animals. This was readily adopted by the scientific community, and assessments have since shown that COI can be used to distinguish over 90% of species in most animal. In recent years the barcoding movement has grown substantially, and worldwide efforts coordinated by CBOL (the Consortium for the Barcode of Life) are now being put into retrieving barcode sequences from all organisms [2].

A single barcoding locus combining these two traits has not been found, and a combination of two or more, probably plastid, loci will almost certainly be required to approach the level of species discrimination and universality that COI confers for animals [7]. DNA barcoding has been applied to a broad range of subjects, including taxonomic studies of “cryptic” taxa or species complexes, e.g. skipper butterflies [1]. Barcoding has also been used in ecological studies to survey animal diets through the analysis of plant remains in feces [14]. The main aim of DNA barcoding is to establish a shared community resource of DNA sequences that can be used for organismal identification and taxonomic clarification. This approach was successfully pioneered in animals using a portion of the cytochrome oxidase 1 (COI) mitochondrial gene. In plants, establishing a standardized DNA barcoding system has been more challenging. DNA barcoding offers a possible solution to this problem and could greatly improve our understanding of fern gametophytes and their biology. Recently, unknown fern gametophytes were shown to be identifiable, often to species level, by using plastid DNA sequences [10], [13] and [3], suggesting that this DNA-based identification tool has the potential to be applied to large-scale ecological surveys [9]. The DNA barcoding approach has also been useful in distinguishing among fern species in the

horticultural trade [12] and in Chinese herbal medicine [15] and [11], two areas where species names are frequently confused. Despite these promising applications, ferns, with their critical phylogenetic position as sister to seed plants, have largely been neglected in choosing the standardized barcode for all land plants [8] and [6].

#### *B. Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase (RBCL)*

Ribulose-1,5-bisphosphate carboxylase/oxygenase, commonly known by the abbreviations RuBisCO, RuBPCase, or RuBPco, is an enzyme involved in the first major step of carbon fixation, a process by which atmospheric carbon dioxide is converted by plants and other photosynthetic organisms to energy-rich molecules such as glucose. In chemical terms, it catalyzes the carboxylation of ribulose-1,5-bisphosphate (also known as RuBP). It is probably the most abundant enzyme on Earth. Among plastid regions, *rbcL* is the best characterized gene. Improvements in primer design make it easily retrievable across land plants and it is well suited for recovery of high-quality bidirectional sequences. Although not the most variable region, it is a frequent component of the best performing multi-locus combinations for species discrimination.

## II. MATERIALS AND METHODS

### A. Sample Collection

Fresh, disease free leaves of the Indian medicinal plant *Cassia fistula* L. were collected from the city of Chennai during December 2016. The samples were transferred to the laboratory within 24 hours of collection, washed and stored till further use.

### B. Genomic DNA Isolation [4]

Fresh-leaf tissue (0.5 g) was ground in a 1.5-mL centrifuge tube with a mortar and pestle and 4mL of preheated, freshly prepared CTAB extraction buffer (0.1 M Tris-Cl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, CTAB (3%, w/v),  $\beta$ -mercaptoethanol (0.2%, v/v), PVP (2% w/v) was immediately added to the tube. The tubes were incubated at 65°C for 60min, with inversion during incubation. An equal volume of chloroform: isoamyl alcohol (24:1, v/v) was added and then the tubes were inverted 8-10 times. The tubes were centrifuged at 10,000 rpm for 15 min. The supernatant was transferred to a new centrifuge tube. An equal volume of absolute ice-cold isopropanol was added. The tubes were centrifuged at 10,000 rpm for 10 min. The supernatant was discarded and the pellet was washed with 70% (v/v) ethanol. The pellet was air-dried at room temperature and then dissolved in 50  $\mu$ L TE buffer. The DNA samples were stored at -20°C until further use.

### C. Qualitative Analysis of Genomic DNA by Agarose Gel Electrophoresis

For the majority of DNA samples, electrophoretic separation is carried out in agarose gels. This is because DNA molecules and their fragments are considerably larger than proteins; therefore larger size agarose gels are required. Under an electric field, any given fragment of DNA should move towards the anode with the same mobility. This is due to the charge per unit length owing to the phosphate groups. Separation on agarose gels is achieved because of resistance to their movement caused by the gel matrix. Thus the largest molecules will have difficulty moving, whereas the smallest molecules will be relatively unhindered. Consequently the mobility of DNA molecules during gel electrophoresis will depend on size. Gel concentrations must be chosen based on the molecules to be separated such as for plasmid molecules – 1%; genomic DNA – 0.8% and RNA – 1.5%, mitochondrial DNA – 0.8% and amplified samples at 1.5%. 0.8 % agarose gel was prepared with 1X TAE buffer and stained with 2 $\mu$ l of ethidium bromide. Samples were loaded with loading dye (2 $\mu$ l of loading dye is used). Electrophoresis of DNA fragments was performed at 50volts. Visualization of DNA fragments was done in the UV trans-illuminator.

### D. Quantitative Determination of DNA by Spectrophotometric Method

A solution of nucleic acids strongly absorbs UV with an absorbance maximum of 260nm and proteins at 280nm which is linearly related with the concentration of DNA and RNA and the amount of contamination in the solution in the solution. The intense absorption is primarily due to the presence of aromatic rings in the purine, pyrimidine. The concentration of nucleic acid in a solution can be calculated if one knows the value of A<sub>260</sub> of the solution. A solution of double-stranded DNA at a concentration of 50ug/ml in a 1cm quartz cuvette will give A<sub>260</sub> reading of 1. A solution of single-stranded DNA/RNA that has A<sub>260</sub> of 1 in a cuvette with a 1cm path length has a concentration of 40ug/ml. Proteins are usually the major contaminants in nucleic acids extract and these have absorption maximum at 280nm. The ratio of absorbance at 260 and 280nm hence provides a clear idea about the extent of contamination in the preparation. A ratio between 1.7 and 1.9 is indicative of fairly pure DNA preparation. But values less

than 1.8 signify the presence of proteins as impurities. The values greater than 1.8 signify the presence of organic solvent in the DNA preparations. A ratio of 1.8 determines the pure DNA preparation.

The spectrophotometer and the UV lamp was switched on. The wavelength was set at 260nm and 280nm. The instrument is set at zero absorbance with T.E buffer or sterile water as blank. 5 or 7ul of the sample is taken in a quartz cuvette and made up to 3ml with TE buffer or sterile water. Absorbance of the solution with the sample was read. The concentration of DNA in the sample was calculated using the given formula:

Concentration of dsDNA =  $A_{260} * 50\mu\text{g} * \text{dilution factor}$

Purity of the DNA =  $A_{260} : A_{280} \text{ ratio} = A_{260} / A_{280}$

= 1.8; pure DNA

= 1.7 – 1.9; fairly pure DNA (acceptable ratio for PCR)

= less than 1.8; presence of proteins.

= greater than 1.8; presence of organic solvent

#### E. Polymerase Chain Reaction

PCR was carried out in Eppendorf Personnel Master Cycler (Germany). The PCR reaction constituents are the following

Optimized PCR condition: rbcL

|                          |  |   |                           |
|--------------------------|--|---|---------------------------|
| <input type="checkbox"/> | Milli Q water                          | – | 7.8 $\mu$ l               |
| <input type="checkbox"/> | 10x Buffer with 20mM Mgcl <sub>2</sub> | – | 2.0 $\mu$ l (1x)          |
| <input type="checkbox"/> | 2mM DNTP's                             | – | 2.0 $\mu$ l (0.2 $\mu$ M) |
| <input type="checkbox"/> | DNA                                    | – | 4.0 $\mu$ l               |
| <input type="checkbox"/> | 3 $\mu$ M Forward Primer               | – | 2.0 (0.3 $\mu$ M)         |
| <input type="checkbox"/> | 3 $\mu$ M Reverse Primer               | – | 2.0 $\mu$ l (0.3 $\mu$ M) |
| <input type="checkbox"/> | Taq polymerase (5U/ $\mu$ l)           | – | 0.2 $\mu$ l (1Unit)       |

The Total Volume of the reaction is 20 $\mu$ l. The concentration of DNA was varied from 0.5  $\mu$ l to 1  $\mu$ l for optimization. The Whole reaction setup was carried out at 4°C.

#### PCR PROGRAMME

|        |                              |                              |
|--------|------------------------------|------------------------------|
| Step 1 | Initial denaturation         | -94°C for 3minutes           |
| Step 2 | Denaturation                 | -94°C for 45 seconds         |
| Step 3 | Primer annealing             | -51°C for 1minute            |
| Step 4 | Extension                    | -72°C for 1minute 20 seconds |
| Step 5 | Go to step 2 repeat 35 times |                              |
| Step 6 | final extensions             | -72°C 7 minutes              |
| Step 7 | Hold                         | - 4°C                        |

#### F. Gel Electrophoresis of PCR Products

DNA quality was assessed on a 1.5% Agarose Gel (in Tris Acetate EDTA buffer) electrophoresis at 50 Volts. DNA was stained with Ethidium Bromide visualized on a UV transilluminator.

#### G. DNA Sequencing and Blast

The PCR products were subjected to sequencing by Sanger method in a AB Sequencer. The result obtained was analysed using BLAST.

### III. RESULTS AND DISCUSSION

Total genomic DNA isolated showed a purity of 1.83. Upon PCR amplification using 100ng of this DNA, an amplicon of size ~900bp was observed in the agarose gel, when compared with a 1KB DNA ladder. The gel was cut and purified followed by DNA sequencing. The sequence so obtained was about 1050 bp and when analysed using NCBI-BLAST Tool and the sample was found to be 100% congeneric to *Cassia fistula* L. The procedure was repeated for amplifying the rbcL marker gene. Upon PCR amplification using 100ng of the DNA, an amplicon of size ~1200bp was observed in the agarose gel, when compared with a 1KB DNA ladder. The gel was cut and purified followed by DNA sequencing. The sequence so obtained was about 770 bp and when analysed using NCBI-BLAST Tool and the sample was found to be 100% congeneric to *Cassia fistula* L

A. Genomic DNA

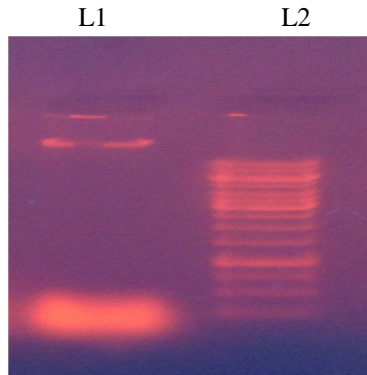


Fig. 1 Agarose gel (0.8 %) for genomic DNA of Plants  
Lane 1: Genomic DNA  
Lane 2: 1 Kb Ladder

B. Dna Quantiifcation by Spectrophotometric Method

1) DNA Quantification: The isolated DNA was quantified by spectrophotometer at two different wavelengths 260 nm and 280 nm. A260/A280 ratio gives purity of the DNA as proteins absorb at 280 nm due to tyrosine and tryptophan residues. The ratios were in the range of 1.83 which indicated a pure DNA. DNA concentrations are given in (Table 1).

Table 1: Concentration Blank and Sample

| Sample | OD at 260nm | OD at 280nm | Concentration (ng/μl) | Purity |
|--------|-------------|-------------|-----------------------|--------|
| Blank  | 0.000       | 0.000       | --                    | --     |
| Sample | 0.198       | 0.108       | 9900                  | 1.83   |

C. Polymerase Chain Reaction

1) PCR RBCL:

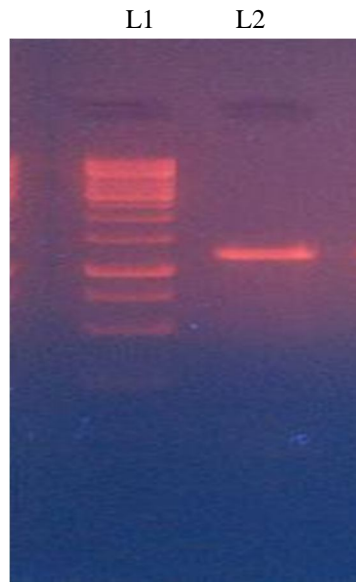


Fig. 2 PCR- rbcl  
Lane 1: 1 KB Ladder  
Lane 2: S1 ~1200bp

In plants, several DNA barcode the regions have been proposed. Among them, the matK and rbcL gene has been accepted as an important candidate barcode by many researchers. In addition, the CBOL (Consortium for the Barcode of Life) Plant Working Group [2] recommended the matK region as a plant barcode. The chloroplast gene matK is about 900 bp in length and is located within the trnK intron. It encodes a maturase-like protein that is involved in group II intron splicing. The gene exhibits a high rate of substitutions and is thus emerging as an important gene for the study of plant systematic and evolution. The PCR products were subjected to sequencing by Sanger method in the AB Sequencer. The obtained sequence from the purified PCR product was compared with nucleotide database and was found to have maximum identity to *Cassia fistula* L, a weed found mostly on in around Chennai and in agricultural farms. The sequence so obtained was about 900 bp and when analysed using NCBI-BLAST Tool and the sample was found to be 100% congeneric to *Cassia fistula* L. FASTA sequence thus obtained with Graphical Representation, Tabular representation (Table. 2) and Alignment representation Fig. 3- Fig. 5.

*D. Sequence 1 (RBCL)*

```
>BRT_CF_rbcLGTTGGGTTCAAAGCTGGTGTAAAGATTATAAATTGACTTATTATACTCCTGACTATGAAACCAAAGA
TACTGATATCTTGGCAGCATTCCGAGTAACTCCTCAACCTGGAGTTCGCGCTGAAGAAGCAGGTGCCGCGGTAGCTGC
TGAATCTTCTACTGGTACATGGACAACCTGTGTGGACCGATGGGCTACCAGTCTTGATCGTTACAAAGGACGATGCTAC
CACATCGAGCCCCTTGCTGGAGAAGAAAATCAATATATTGCTTATGTAGCTTATCCCTTAGACCTTTTTGAAGAAGGT
TCTGTTACTAACATGTTTACTTCCATTGTGGGTAATGTATTTGGGTTCAAGGCCCTGCGCGCTCTACGTCTGGAGGATT
TGCGAATCCCTATTTCTTATATTAATAAACTTTCCAAGGTCCGCCTCACGGCATCCAAGTTGAGAGAGATAAATTGAACA
AGTACGGCCGTCCCCTATTGGGATGTACTATTAACCTAAATTGGGGTTATCCGCTAAGAATTACGGTAGAGCTGTTT
ATGAATGTCTCCGCGGTGGACTTGATTTTACCAAAGATGATGAGAATGTGAATTCCCAACCATTTATGCGTTGGAGAG
ACCGTTTCTGCTTTTGTGCCGAAGCACTTTATAAAGCACAGGCCGAAACAGGTGAAATCAAAGGGCATTACTTGAAT
GCTACGGCAGGTACATGCGAAGAAATGATCAAAGAGCTGTATTTGCCCGAGAATTGGGCGTTTCTATCGTAATGCA
```

Fig. 3 FASTA Sequence

*E. Graphical Representation*

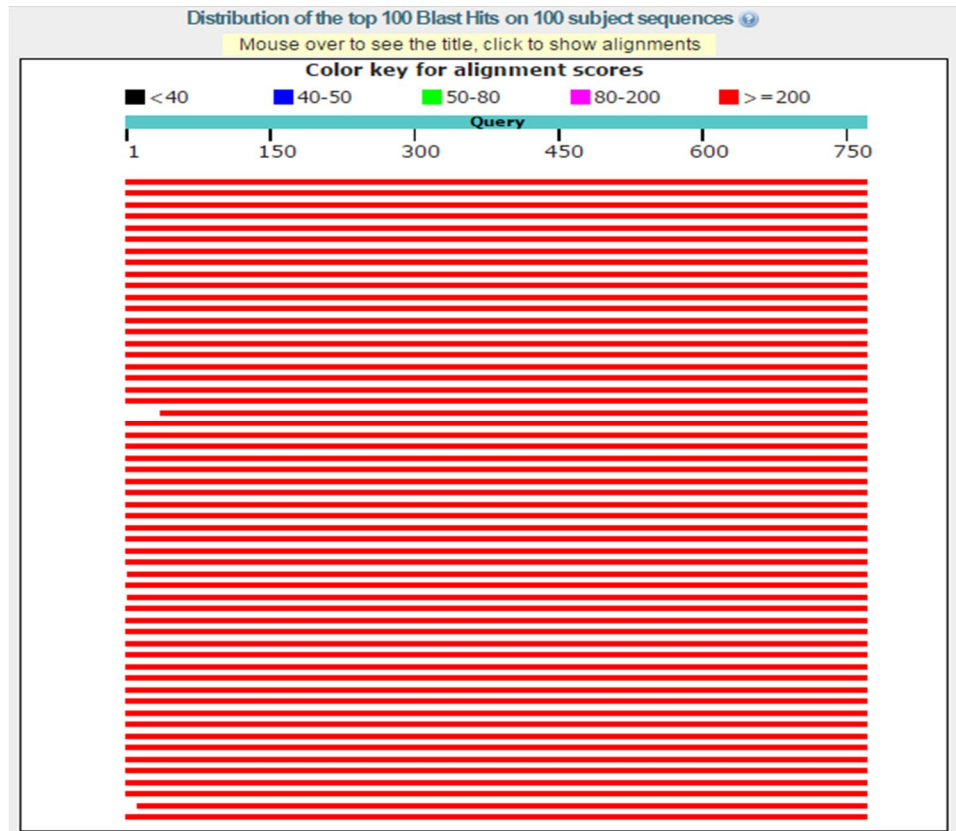


Fig. 4 Graphical Representation

Table. 2 Tabular Representation

Sequences producing significant alignments:

Select All None Selected:0

| Alignments  |           |             |             |         |       |                            |
|---|-----------|-------------|-------------|---------|-------|----------------------------|
| Download GenBank Graphics Distance tree of results  |           |             |             |         |       |                            |
| Description   | Max score | Total score | Query cover | E value | Ident | Accession                  |
| <a href="#">Cassia fistula ribulose 1,5-bisphosphate carboxylase-oxygenase large subunit (rbcL) gene, chloroplast gene encoding chloroplast protein, partial cds</a>          | 1423      | 1423        | 100%        | 0.0     | 100%  | <a href="#">U74195.1</a>   |
| <a href="#">Cassia grandis partial rbcL gene for ribulose bisphosphate carboxylase large chain, specimen voucher Smith 2061 (MT)</a>  | 1406      | 1406        | 100%        | 0.0     | 99%   | <a href="#">AM234244.1</a> |
| <a href="#">Haematoxylum brasiletto chloroplast, complete genome</a>  | 1373      | 1373        | 100%        | 0.0     | 99%   | <a href="#">KJ468097.1</a> |
| <a href="#">Erythrophloeum ivorense ribulose 1,5-bisphosphate carboxylase-oxygenase large subunit (rbcL) gene, chloroplast gene encoding chloroplast protein, partial cds</a> | 1367      | 1367        | 100%        | 0.0     | 99%   | <a href="#">U74205.1</a>   |
| <a href="#">Mezoneuron cucullatum chloroplast, complete genome</a>  | 1362      | 1362        | 100%        | 0.0     | 99%   | <a href="#">KU569489.1</a> |
| <a href="#">Senna tora chloroplast, complete genome</a>   | 1362      | 1362        | 100%        | 0.0     | 99%   | <a href="#">KR136271.1</a> |
| <a href="#">Senna tora voucher JKTU-1-000064 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds, chloroplast</a>                          | 1362      | 1362        | 100%        | 0.0     | 99%   | <a href="#">KP058311.1</a> |
| <a href="#">Sclerobolium sp. Kiloqaard 687 partial rbcL gene for ribulose bisphosphate carboxylase large chain, specimen voucher Kiloqaard B. B. 687 (K)</a>                  | 1362      | 1362        | 100%        | 0.0     | 99%   | <a href="#">AM234242.1</a> |
| <a href="#">Caesalpinia calycina partial rbcL gene for ribulose bisphosphate carboxylase large chain, specimen voucher Lewis 1865 (K)</a>                                     | 1362      | 1362        | 100%        | 0.0     | 99%   | <a href="#">AM234236.1</a> |
| <a href="#">Parkinsonia microphylla voucher Hawkins 127 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds, chloroplast</a>               | 1362      | 1362        | 100%        | 0.0     | 99%   | <a href="#">AY904404.1</a> |
| <a href="#">Jacqueshuberia kretensis voucher Rimachi 9050 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds, chloroplast</a>             | 1362      | 1362        | 100%        | 0.0     | 99%   | <a href="#">AY904391.1</a> |
| <a href="#">Balsamocarpon brevifolium isolate 54 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds, chloroplast</a>                      | 1362      | 1362        | 100%        | 0.0     | 99%   | <a href="#">AY308524.1</a> |
| <a href="#">D.regia chloroplast rbcL gene</a>   | 1362      | 1362        | 100%        | 0.0     | 99%   | <a href="#">Z70156.1</a>   |
| <a href="#">C.senna chloroplast rbcL gene</a>   | 1362      | 1362        | 100%        | 0.0     | 99%   | <a href="#">Z70155.1</a>   |
| <a href="#">C.pulcherrima chloroplast rbcL gene</a>   | 1362      | 1362        | 100%        | 0.0     | 99%   | <a href="#">Z70153.1</a>   |
| <a href="#">C.didymobotrya chloroplast rbcL gene</a>  | 1362      | 1362        | 100%        | 0.0     | 99%   | <a href="#">Z70154.1</a>   |
| <a href="#">Tachiquil paniculata ribulose 1,5-bisphosphate carboxylase-oxygenase large subunit (rbcL) gene, chloroplast gene encoding chloroplast protein, partial cds</a>    | 1362      | 1362        | 100%        | 0.0     | 99%   | <a href="#">U74240.1</a>   |
| <a href="#">Cercidium andicola voucher Hughes 2313 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds, chloroplast</a>                    | 1358      | 1358        | 100%        | 0.0     | 98%   | <a href="#">AY904410.1</a> |
| <a href="#">Caesalpinia eriostachys voucher Hughes 1832 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds, chloroplast</a>               | 1358      | 1358        | 100%        | 0.0     | 98%   | <a href="#">AY904381.1</a> |
| <a href="#">Libidibia coriaria chloroplast, complete genome</a>   | 1356      | 1356        | 100%        | 0.0     | 98%   | <a href="#">KJ468095.1</a> |
| <a href="#">Cassia sp. SH-2010 chloroplast gene for ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial cds, isolate: A123</a>                             | 1356      | 1356        | 95%         | 0.0     | 100%  | <a href="#">AB596310.1</a> |
| <a href="#">Parkinsonia africana voucher Kolberg sn ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds, chloroplast</a>                   | 1356      | 1356        | 100%        | 0.0     | 98%   | <a href="#">AY904411.1</a> |
| <a href="#">S.japonica chloroplast rbcL gene</a>  | 1356      | 1356        | 100%        | 0.0     | 98%   | <a href="#">Z70142.1</a>   |
| <a href="#">Xanthocercis zambesiaca ribulose 1,5-bisphosphate carboxylase-oxygenase large subunit (rbcL) gene, chloroplast gene encoding chloroplast protein, partial cds</a> | 1356      | 1356        | 100%        | 0.0     | 98%   | <a href="#">U74189.1</a>   |
| <a href="#">Pentactethra macrophylla partial rbcL gene for ribulose bisphosphate carboxylase large chain, specimen voucher DeWilde 11496 (WAG)</a>                            | 1351      | 1351        | 100%        | 0.0     | 98%   | <a href="#">AM234250.1</a> |
| <a href="#">Caesalpinia velutina voucher Hughes 255 ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcL) gene, partial cds, chloroplast</a>                   | 1351      | 1351        | 100%        | 0.0     | 98%   | <a href="#">AY904382.1</a> |

F. Alignment Representation

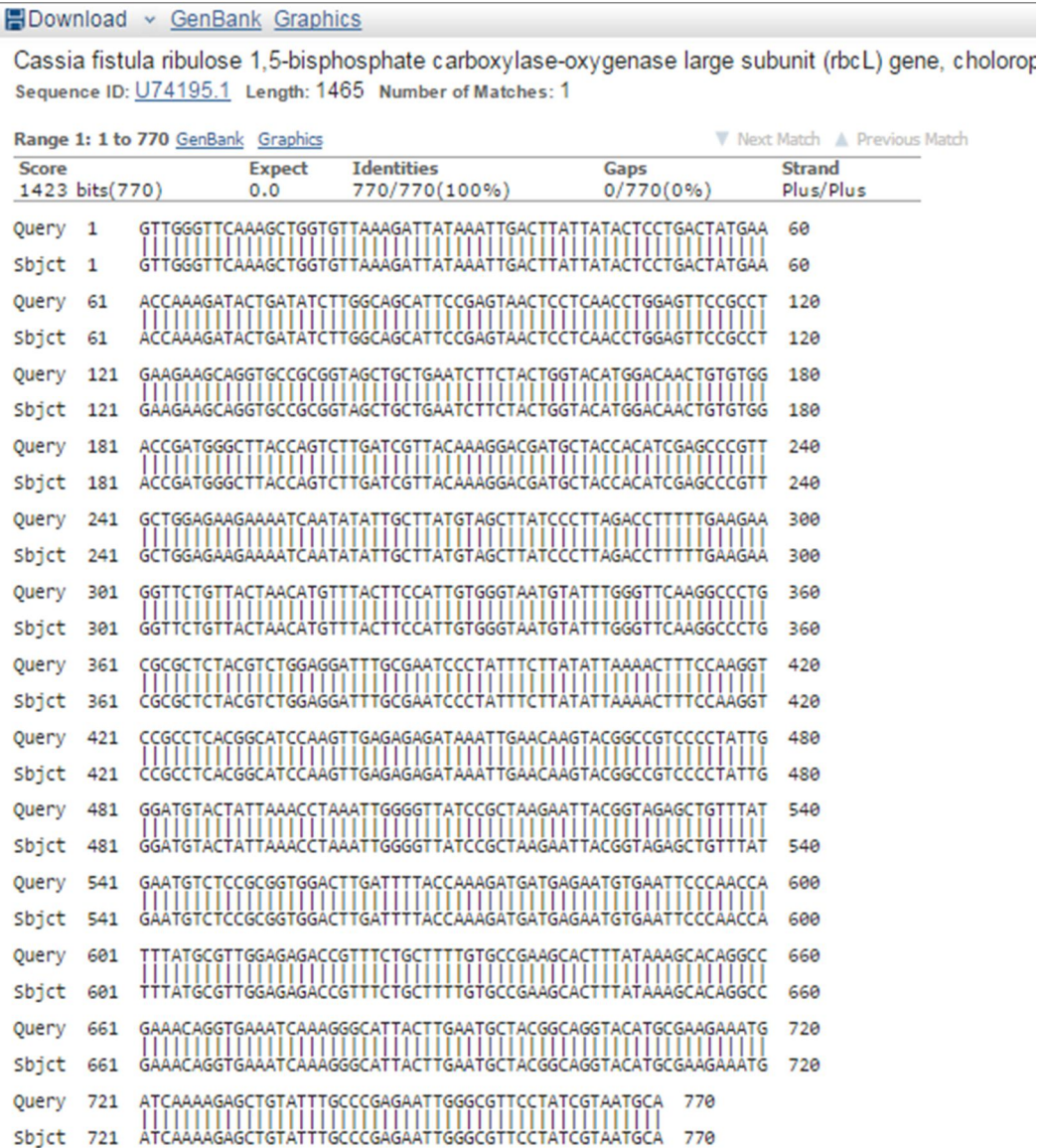


Fig. 5 Alignment representation

IV. CONCLUSION

It is clear that the DNA barcoding has great potential for enhancing ecological and evolutionary investigations if the right genetic markers are selected. In this study, molecular characterization of the collected plant was carefully studied and its evolutionary relationship was constructed. It is clear that the DNA barcoding has great potential for the identification of the plant specimen.

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