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DNA Barcode of Indian Medicinal Plant *Anethum* Graveolens L by Matk Gene

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Abstract*: The studies based on taxonomy, ethnobotany and DNA barcode of potential and imperative for plants with traditional medicinal value. Two important reasons for plant barcoding is to identity unknown specimens to known species and to share it with the community resource of sequences to establish its taxonomical clarification. MaturaseK gene (matK) of chloroplast is highly conserved in plants which is associated in Group II intron RNA splicing. This reputed gene product matK is the only maturase present in chloroplasts making its presence unique. DNA barcoding by plastid coding matK markers involves sequencing of a standard region of DNA as a tool for angiosperm identification. In this study, leaf samples of Anethum graveolens L were collected from Chennai and Polymerase chain reaction (PCR) was performed to amplify the matK gene and molecular characterization was performed.

Keywords*: DNA Barcoding, matK, PCR, Anethum graveolens L and MF426985.

I. INTRODUCTION

A. Traditional Herbal Medicine

Thousands of years ago, the medical knowledge of the Indian subcontinent was termed as Ayurveda. Today the pharmacologically active ingredients of many Ayurveda medicines are being identified and their usefulness in drug therapy being determined. Only a certain percentage of plants are used in traditional medicines. By definition, 'traditional' use of herbal medicines implies substantial historical use, and this is certainly true for many products that are available as 'traditional herbal medicines' the pharmacological treatment of disease began long ago with the use of herbs [22]. Methods of folk healing throughout the world commonly used herbs as part of their tradition. Dill (Anethum graveolens) is an annual herb in the celery family Apiaceae. It is the sole species of the genus Anethum.

B. DNA Barcoding

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 and in identifying smoked fish products sold under ambiguous product names [23]. In effect, barcoding in its modern form was popularized in a paper by [12], who proposed to use the mitochondrial gene CO1 as the standard barcode for all animals. This was readily adopted by the scientific community, and assessments have since shown that CO1 can be used to distinguish over 90% of species in most animal groups [17]. 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].

C. Megakaryocyte-Associated Tyrosine Kinase (MATK)

The rapidly evolving and highly variable gene maturase K [13] has been recommended as a locus for DNA barcoding by the Consortium for the Barcode of Life (CBOL) Plant Working Group [14]. Amplification and sequencing of the matK barcoding region is difficult due to high sequence variability in the primer binding sites [15]. Currently, there are three popular matK primer pairs available to amplify approximately the same region of the gene: 390F and 1326R [24] and [3], XF and 5R [10] and 1R_KIM and 3F_KIM [14], [16] and [19] used these three primer pairs to amplify DNA barcodes from 296 shrub and tree species. Disclosing evolutionary descents of different plant species with matK gene could be supreme for constructing a systematic phylogenetic tree. Comparing a molecular sequence data is indispensable to acquire knowledge of biodiversity and to provide insights into the selective force that occurred during evolution of different species [5] and [25].

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The trnK intron codes for the Chloroplast MaturaseK (matK) open reading frame. There are 16 group II introns shelled within 15 chloroplast genes [18] and [9], which requires a maturase for RNA splicing and proper protein translation. Out of the 15 chloroplast genes, only 6 genes have introns which fail to possess a nuclear imported maturase, making matK as the putative maturase domain [7] and [11]. Thus, matK is considered as a strong phylogenetic marker for classification of plants at various levels. The plastid coding region of matK (~1,500bp) and its flanking ribosome regions of a few hundred base pairs of DNA on each side together constitute a lariat-forming intron residing in the trnK gene [21]. So far only few studies have been conducted to develop unified barcodes suitable for making identifications within a family, within a genus, or between closely related sister species [26].

II. MATERIALS AND METHODS

A. Sample Collection

Fresh, disease free healthy leaves of the Indian medicinal plant Anethum graveolens L. Were collected from the city of Chennai during July 2017. The collected plant was authenticated by Plant Anatomy Research Centre (**PARC/2017/3468**). The samples were transferred to the laboratory within 24 hours of collection, washed and stored till further use.

B. Genomic DNA Isolation [8]

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), b-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 µL 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 2µl of ethidium bromide. Samples were loaded with loading dye (2µl 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 Spectrophotomeric 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 I n 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 A260 of the solution. A solution of double-stranded DNA at a concentration of 50ug/ml in a 1cm quartz cuvette will give A260 reading of 1.A solution of single-stranded DNA/RNA that has A260 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 ration 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

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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 = A260 * 50µg * dilution factor

Purity of the DNA = A260 : A280 ratio = A260 / A280

= 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: matK

1) Milli Q water _ 8.8µl
2) 10x Buffer with 20mM Mgcl2 _ 2.0µl (1x
3) 2mM DNTP's _ 2.0µl (0.2µM)
4) DNA _ 3.0 µ
5) 3µM Forward Primer _ 2.0 (0.3µM)
6) 3µM Reverse Primer _ 2.0µl (0.3µM)
7) Taq polymerase (5U/µl) _ 0.2µl (1Unit)

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

F. PCR Programme

1) Initial denaturation -94°C for 3minutes
2) Denaturation -94^{0}C for 45 seconds
3) Primer annealing -47^{0}C for 1minute
4) Extension -72^{0}C for 1minute 20 seconds
5) Go to step 2 repeat 35 times
6) final extensions -72^{0}C 7 minutes
7) Hold - 4^{0}C

G. 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.

H. 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

DNA barcoding is a method of recognizing an organism based on sequence data from one to several gene regions. Barcoding has several applications and has been used for ecological surveys [6], enigmatic taxon identification, and authentication of medicinal plant samples [27]. A number of chloroplast gene regions are characteristically used as plant barcodes, with maturase K (matK) considered core barcodes.

An ideal DNA barcode should be routinely retrievable with a single primer pair and amenable to bidirectional sequencing [4]. matK gene is one of the most rapidly evolving plastid coding regions consistently illustrating high levels of discrimination among angiosperm species. Studies have proved wholeness of matK primers assorting from successful identities to erratic resurgence with no impact on its constant usage [4]. Total genomic DNA was isolated from the collected plant samples and its purity (1.73) was found using a Spectrophotometer. Amplicons obtained after PCR were of 900bp visualized on a 0.8% agarose gel as shown in (Fig.1). Majority of Indian tribes still adhere to herbal medicines for treating infections and various outrageous diseases. This

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knowledge of potential use of medicinal plants are in a verge of extinction as they are of oral forms and also most of them are not taxonomically identified [20].

A. DNA Barcoding

Total genomic DNA isolated showed a purity of 1.73. 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 Anethum graveolens L.

B. Total Genomic DNA Isolation

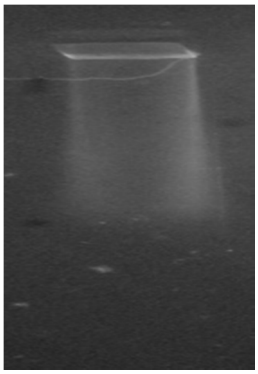

Fig. 1 Genomic DNA from *A.graveolens* leaves

C. Estimation of DNA

D. DNA Quantiifcation by Spectrophotometric Method

E. 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 1.73 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	0.130	0.075	9900	1.73

PCR – matK L1 L4

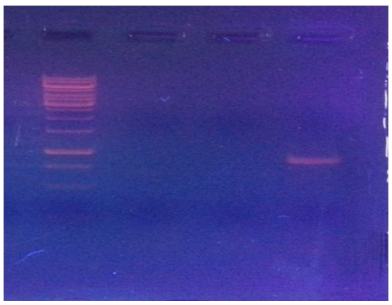

Fig. 2 PCR- matK

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Lane 1: 1 Kb Ladder
Lane 4: PCR Amplicon Sample: ~ 900bp
Agarose gel (0.8 %) for genomic DNA of Plants

In plants, several candidate DNA barcode regions have been proposed. Among them, the matK 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. Sequence was submitted to NCBI Genbank and its accession number generated MF426985. 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 Anethum graveolens L. FASTA sequence thus obtained with Graphical Representation, Tabular representation and Alignment representation Fig. 3- Fig. 6.

FASTA Sequence 1 (matK)

>BRT_AG_matK

AATCATTTGATTCTTTTTGCTAATGATTTTAACCAAAATCCATTTTTTGGGCGCAACAAGAATTTTAATTTTCAAATGA
TATCAGAAGGATTTGCAGTCATTGTAGAAATTCCGTTTTATCTCCGATTATTATCTTCGCTAGAAAGGAAAGGAATAG
TTAAATCTCATAATTTACGATCAATTCATTCAATATTCCCTTTTGTAGAGGACAAATTTTCACATTTAATTTATGTGTT
AGAGATACTAATACCCTACCCAGCCCATCTGGAAATATTGGTTCAAACTCTTCGCTATTGGGTAAAAGACGCTTCTTC
TTTACATTTATTAAGATTCTTTCTCCACGAGTATCGTAGTTGGAATACTCCAAATAAAGCCAGTTCTTGTTTTTCAAAA
AGAAATCAAAGGTTTTTCTTCGTCCTATATAATTCTCATCTATGTGAATACGAATCCATCTTCGTCTTTTTTCGTAACC
AATCTTCTCATTTATGCTCAACGTCTTCTGGAACCCTTCTTGAACGAATCTTTTTCTATGGAAAAATAGAACATCTTGG
ACTTGTAGAAGCTTTTGATAAGGCCTTTCAGGACAATCTATGGTTGTTTAAGGACCCTTTCATGCATTATATTAGGTAT
CAAGGAAAATCAATTCTCGCTTCAAAAGGGACGCCCCTTTTGATGAAAAAATGGACATATTTTTTTGTCAATTTATGG
AAATGTCATTTTTACCTATGGTCTCAGCCGGGACGGATCTGTATAAACCAATTATATAATAATTCCCTAGCTCTTCTGG
GCTATCTATCAAGTGCGCGACTAAATCCTTCAATGGTACGCAGTCAAATGCTAGAAAATGCATTTATAATTGATAATC
CTATTAAAAAGTTCGATACTCTTGTTCCAATTGTTCCTCTGATTGGATCATTGGCTAAGGCGAGATTTTGTAACGTATT
AGGGCACCCTATTAGTAAGGCGGTTTGGGCTGATTTATCAGATTCTGATATTGTTGTCCGATTTAGGAGTATCTGCAG
AAATCTTTCTCATTATTATAGTGGATCC

Fig. 3 FASTA Sequence

BLAST-Graphical Representation

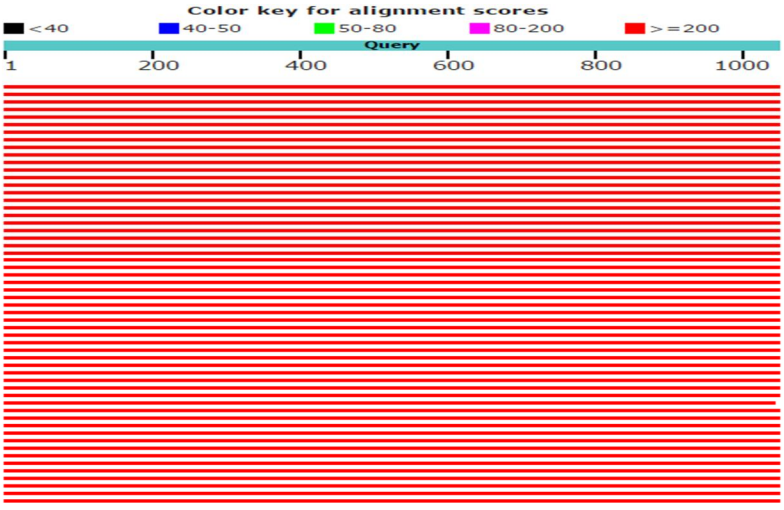

Fig. 4 Graphical Representation

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BLAST-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
Anethum graveolens chloroplast, complete genome	1940	1940	100%	0.0	100%	KR011055.1
Anethum graveolens maturase K (matK) gene, partial cds; chloroplast	1940	1940	100%	0.0	100%	EU016725.1
Foeniculum vulgare chloroplast, complete genome	1912	1912	100%	0.0	99%	KR011054.1
Petroselinum crispum chloroplast, complete genome	1879	1879	100%	0.0	99%	HM596073.1
Apium graveolens chloroplast partial matK gene for maturase	1857	1857	100%	0.0	99%	AJ429370.1
Apium graveolens maturase K-like (matK) gene, partial sequence; chloroplast	1845	1845	100%	0.0	98%	FJ986058.1
Ligusticum tenuissimum voucher 13I-08 chloroplast, complete genome	1823	1823	100%	0.0	98%	KT963039.1
Conium maculatum voucher Aust 166 maturase K (matK) gene, complete cds; plastid	1818	1818	100%	0.0	98%	KT176597.1
Ligusticum sinense chloroplast trnK, matK genes for tRNA-Lysine, maturase, partial and complete cds	1801	1801	100%	0.0	98%	AB262552.1
Cnidium officinale chloroplast trnK, matK genes for tRNA-Lysine, maturase, partial and complete cds	1801	1801	100%	0.0	98%	AB262551.1
Pimpinella niitakayamensis voucher CPG09832 maturase K (matK) gene, partial cds; chloroplast	1796	1796	100%	0.0	98%	KX526497.1
Dickinsia hydrocotyloides voucher CPG15073 maturase K (matK) gene, partial cds; chloroplast	1796	1796	100%	0.0	98%	KX526490.1
Heracleum candicans maturase K (matK) gene, partial cds; chloroplast	1796	1796	100%	0.0	98%	FJ986061.1
Crithmum maritimum chloroplast, complete genome	1796	1796	100%	0.0	98%	HM596072.1
Pimpinella candolleana maturase K-like (matK) gene, partial sequence; chloroplast	1794	1794	99%	0.0	98%	FJ986080.1
Glehnia littoralis chloroplast, complete genome	1790	1790	100%	0.0	97%	KT153022.1
Angelica dahurica voucher 13T-05 chloroplast, complete genome	1790	1790	100%	0.0	97%	KT963037.1
Tetrataenium canescens maturase K (matK) gene, partial cds; chloroplast	1790	1790	100%	0.0	97%	FJ986062.1
Angelica sylvestris maturase K (matK) gene, partial cds; chloroplast	1790	1790	100%	0.0	97%	DQ133783.1
Glehnia littoralis voucher CPG14835 maturase K (matK) gene, partial cds; chloroplast	1784	1784	100%	0.0	97%	KX526492.1

Fig. 5 Tabular representation

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BLAST-Alignment representation

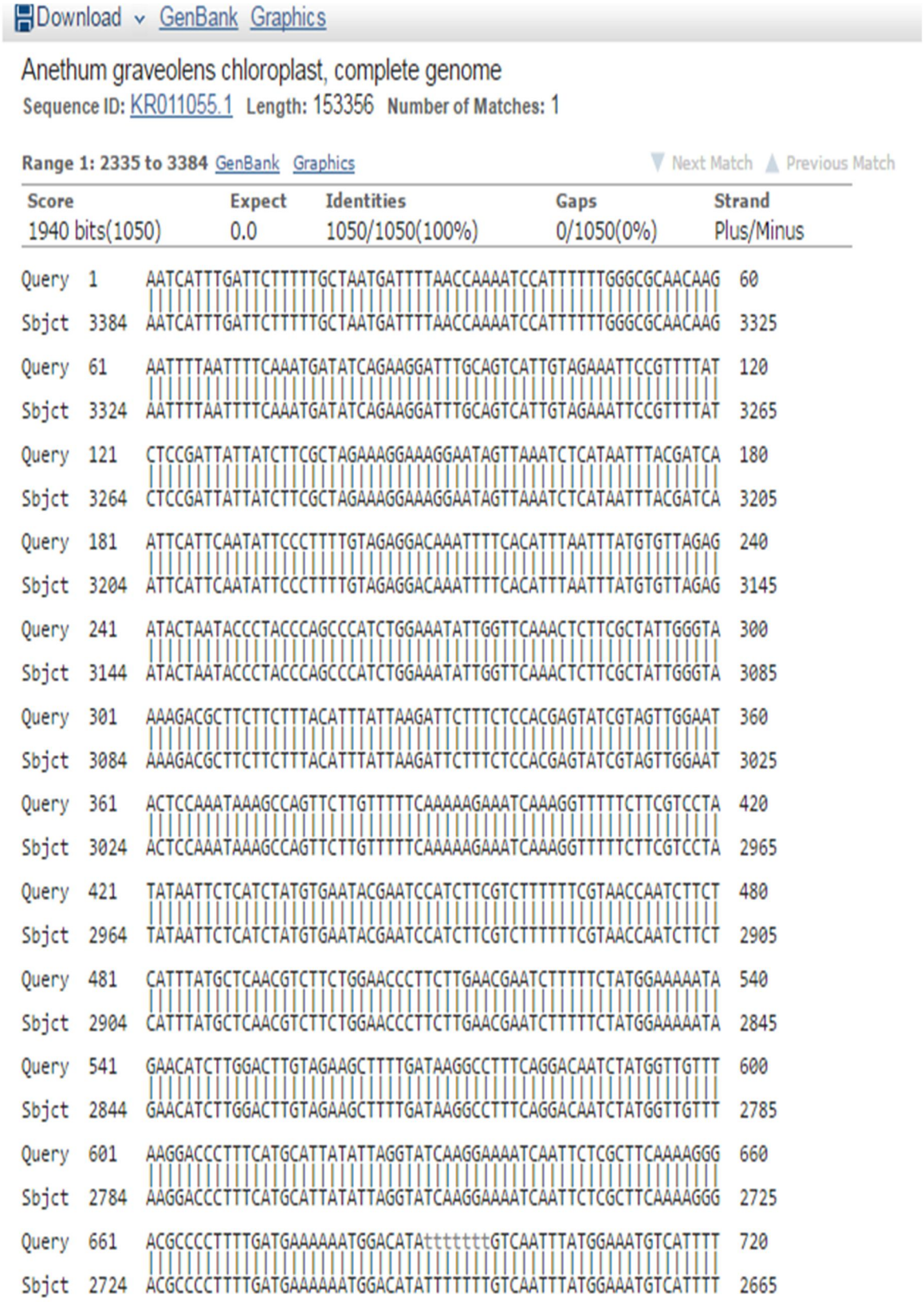

Fig. 6 Alignment representation

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IV. CONCLUSION

In this current study, the 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 enhancing ecological and the identification of the plant specimen. The evolutionary investigations can be done if the right genetic markers are selected. Sequence was submitted to NCBI Genbank and its accession number generated MF426985. Nucleotide BLAST (blastn) shows 100% congeneric to Anethum graveolens L. FASTA sequence thus obtained.

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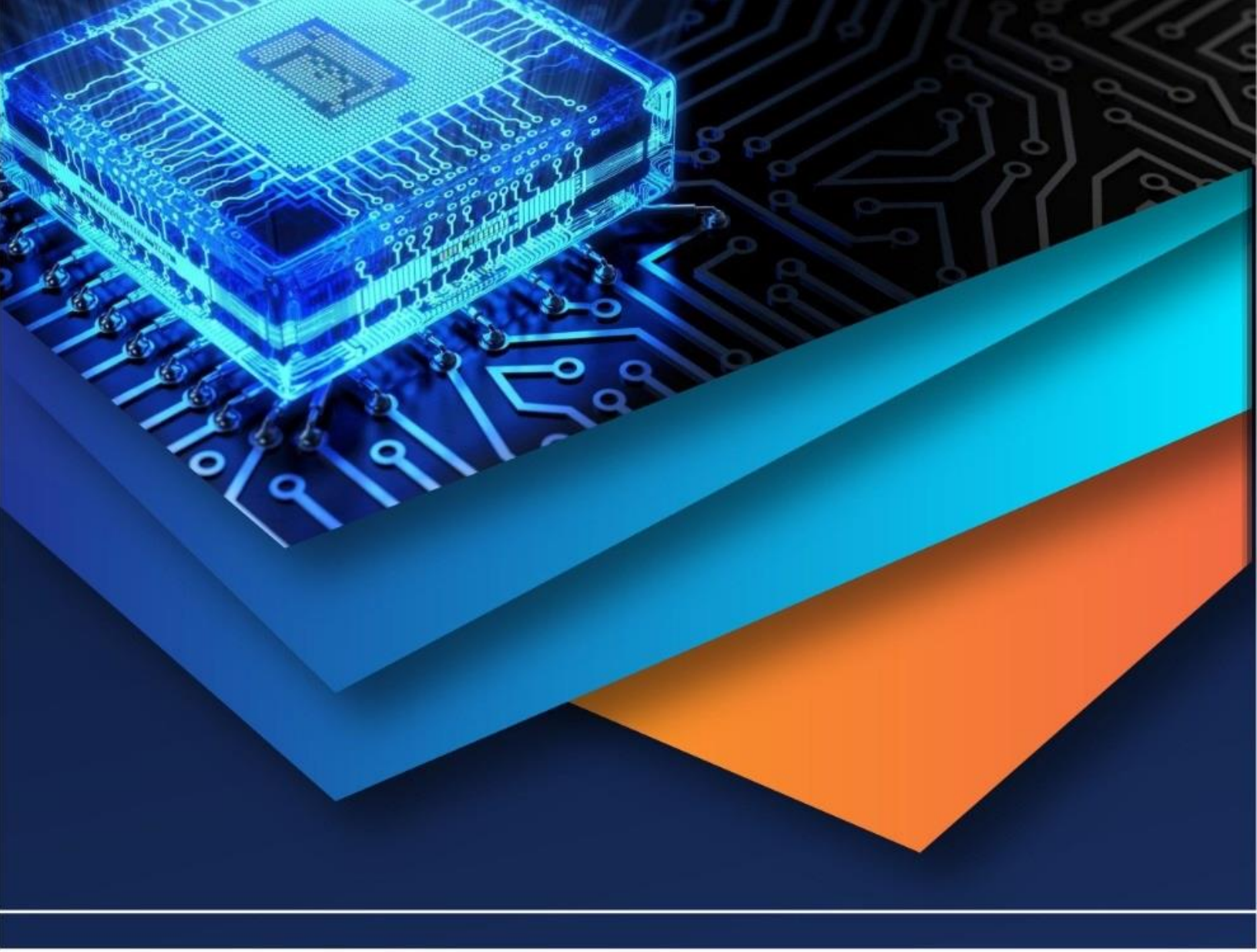

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