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Research Paper

ISOLATION AND *IN-SILICO* ANALYSIS OF ELONGATION FACTOR-1ALPHA GENE IN *Piper longum* L.

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Abstract

Isolation and Insilco analysis of Elongation factor 1alpha gene isolated from Piper longum L., used for the current study. A modified protocol for DNA isolation from *Piper longum* is standardized and the electrophoretic and spectroscopic data of isolated DNA confirmed its quality and also proved the standardized protocol for DNA isolation gives good yield. The PCR product of 0.6 kb corresponding to -1alpha gene fragment was amplified from isolated genomic DNA using degenerate primers. The sequence of purified PCR product shows that, the isolated fragment belongs to elongation factor 1 alpha. The elongation factor 1 alpha isolated from this study is the first one *Piper longum.* On BLAST analysis it shows homology with that of *B. napus* and *A. thaliana* sequences. A boot-strap phylogenetic tree was constructed using Mega4 programme by NJ method, and this shows its evolutionary relationship with other sequences in the database.

Key words: BLAST, DNA, EF 1 alpha, PCR and *Piper longum* L..

INTRODUCTION

Elongation factors are a set of proteins that enable the events of translational elongation. Translation elongation in eukaryotes is carried out with two elongation factors: eEF-1 and eEF-2[1]. The first is eEF-1, and has two sub units, α and β. The α subunit acts as counterpart to prokaryotic EF-Tu, mediating the entry of the aminoacyl tRNA in to a free site of the ribosome and is almost ubiquitously expressed (Berg, 2002). Elongation factor 1-alpha protein in humans is encoded by the gene EF IAI [2] . Genes encoding EF-1α from several species have been cloned and sequenced. These include

yeast, the brine shrimp *Artemia salina*, the fungus *Mucor racemosus*, and man. These EF-1α genes all encode proteins that are highly homologous to each other [3]. Once a gene or a DNA fragment has been cloned, its further study revolves around its sequence for which good sequencing data is a must. DNA sequencing is a recent technology; the new sequencing technologies are already making a great influence in academic research on medically important plants and microbes may soon revolutionize diagnostics, epidemiology, and infection control.

Piper longum L. comes under the family Piperaceae, is widely used as an important traditional medicine in Asia and the Pacific islands and it has been used as a good remedy for treating tuberculosis and respiratory tract infections [4]. Although e EF1α genes in some plant species have been well characterized. In this regard the present study was done to ascertain the degree of conservation between EF1α *Piper longum* L., one of the important medicinal plants used in Ayurveda, with other plants of divergent groups.

MATERIALS AND METHODS

Plant material

Piper longum is a flowering wine in the family Piperaceae, cultivated for its medicinal purposes and as spices. Piper longum or long pepper is good for aromatic, stimulant, carminative, constipation, gonorrhea, paralysis of the tongue, advised in diarrhea, cholera, scarlatina, chronic malaria, viral hepatitis. Piper longum is most commonly used to treat respiratory infections such as bronchitis and cough, tumors. In ayurvedic medicine, it is said to be a good rejuvenator [5].

Young leaves of *Piper longum* plant selected from Kannur district were used for the present gene isolation study.

Plant genomic DNA extraction using CTAB method

Materials required

- ✓ CTAB buffer
- ✓ Microfuge tubes
- ✓ Mortar and pestle
- ✓ 75% Isopropyl alcohol
- ✓ Chloroform
- ✓ 65 ◦ c Water bath
- ✓ Dnase free water

CTAB BUFFER 50 ml

- ➢ 1.0g CTAB (Cetyl trimethyl-ammonium bromide)
- ➢ 5.0 ml 1 M Tris HCl pH8.0
- ➢ 2.0 ml 0.5M EDTA pH8.0(Ethylene diamine tetra Acetic acid Di-sodium salt)
- ➢ 14.0 ml 5M NaCl
- ➢ 28.0 ml distilled water

Methodology

Weigh accurately 1g of fresh leaf pieces and grind the leaf tissue to a fine paste in approximately 3ml of CTAB buffer. Then transfer plant mixture to a microfuge tube and keep for incubate in a water bath at 65^0c for at least 15 minutes. Then allow cooling. Add equal amount of chloroform and spin the plant extract mixture at 10000rpm for 5minute to spin down cell debris. Transfer the supernatant to clean microfuge tube. Add same amount of isopropanol mix the solution by inversion. After mixing spin the tubes at 4^oC, 12000 rpm for 10minutes.Transfer aqueous phase only (contains the DNA) to a clean microfuge tube and to each tube add equal amount of 75% isopropanol. Invert the tube slowly several times to precipitate the DNA. Generaly the DNA can be seen to precipitate out of solution. Spin the tubes at 4^oC, 12000rpm for 10 minutes. Then remove all supernatant and allow the DNA pellet to dry at 37^oC (approximately 15minutes). Do not allow the DNA to over dry or it will be hard to re-dissolve and finally resuspend the DNA in sterile 100 µl Dnase free water.

Quantification of DNA

Total DNA isolated was quantified spectrophotometrically at 260 nm. The concentration of DNA in 1µl of DNA sample was calculated using following equation,

$$\frac{50 \times \text{O. D at 260 nm} \times \text{dilution factor}}{1000}$$

DNA sample was electrophoresed on 0.8% agarose gel containing ethidium bromide (EtBr).

Designing of primer

Nucleic acid sequence coding for EF-1α were retrieved from the Gene Bank (http: //www.ncbi.nlm.nib.gov/). The nucleic acid sequence was aligned by ClustalW Sequence alignment program of BioEdit software [6] (Hall, 1999). The sequence at the most conservative region of the alignment was considered for primer designing.

Primer dilution

Primers were diluted to 100 pmol/µl working stock solution using sterile MilliQ water and were store at -20˚C. For PCR reaction, primers were diluted from the working stock to a concentration of 10-pmol/µl using nuclease free water.

Polymerase chain reaction

The PCR was carried out using the forward and reverse primers of EF-1α with good quality of genomic DNA as template. The reaction mixture contained the different components in the following composition

Template (genomic DNA)	-	1.0 µl (approximately 150 ng)
5X reaction buffer	-	10.0 µl
dNTPs (10mM)	-	1.0 µl
Forward primer	-	2.0 µl
Reverse primer	-	2.0 µl
Taq DNA polymerase	-	0.5µl
Nuclease free water	-	33.5 µl
Total	-	50.0 µl

The thermal conditions used for the amplification of partial length of EF-1α is as follows

Table : 1 Recommended PCR conditions

Cycles step	Temperature	Time	Cycles
Initial denaturation	94 ◦ C	2 minutes	
Cyclic denaturation	94 ◦ C	30 seconds	35
Cyclic annealing	50 ◦ C	30 seconds	35
Cyclic extension	72 ◦ C	40 seconds	35
Final extension	72 ◦ C	10 minutes	

Agarose gel electrophoresis of PCR product

After PCR, the samples were analyzed using a 1.2% agarose gel .The sample was mixed with the 6x loading dye prior to loading and the electrophoresis-run was done at 40-75 V for the required duration. The electrophoresis tank was attached to a DC power pack and resolved at 5 V/cm. The gel was removed when the bromophenol blue in the tracking dye reached 2/3 of the gel. DNA in the gel was observed on a UV Tran illuminator.

Elution of DNA from gel band

Methodology

The PCR products were purified with GFX PCR DNA purification kit (Amersham Biosciences). The eluted DNA sample was stored at -20^{o}C.

Sequencing PCR

The positive clones were sequenced by automated sequencing. Automated sequencing was carried out using Big Dye Terminator sequencing mix. The reaction was setup to a final volume of 10 µL as follows. The sequencing buffer and the sequencing reaction mix were taken based on a calculation:

2.5x+5y=10, where x is the reaction mix and y is the sequencing buffer.

Plasmid	-	1.0 µl (~25 ng)
5 X sequencing buffer	-	1.75 µl
Forward/Reverse primer	-	1.0 µl
Sequencing mix	-	0.5µl
Nuclease free water	-	5.75 µl
Total	-	10. 0 µl

PCR conditions

96°C	-	2 minutes	
96°C	-	30 seconds	25 cycles
50°C	-	30 seconds	
60°C	-	4 minutes	
4°C	-	α	

The Sequencing reaction was carried out in an Eppendorf cycler with 25% ramping.

Clean up of PCR products

In order to remove the unused dNTPs and other contaminants that could interfere with the automated sequencing run, the sequencing PCR products were subjected to a clean up protocol.

Electrophoresis of the sequencing sample

20 µl of the template suppression reagent (TRS) was denatured at 95^0C for 10 minutes and loaded into the ABI PRISM 310 genetic analyzer. The sample was resolved through POP 6 polymer and the sequencing data was normalized using standard. The electrophorogram was analyzed for the sequence.

Bioinformatic analysis

The nucleotide sequences obtained from the study are subjected to detailed bioinformatics analysis using both online and offline programmes. The nucleotide sequences were analysed by BLASTN software of NCBI (http://www.ncbi.nlm.nih.gov/BLAST) [7]. The nucleotide sequences of other EF-1α

from other plants and showing homology with the querry sequence were retrieved from NCBI database was used for phylogenetic analysis. For phylogentic analysis the sequence was aligned by CLUSTALW multiple alignment tool. Aligned sequences were used to construct a boot-strap phylogenetic tree by Neighbour-joining method of Mega4 software.

RESULT AND DISCUSSIONS

Significant molecular studies in this plant are still lacking. Hence in the present study, a method is standardized for the DNA isolation; where the method is essentially a modification of CTAB buffer protocol; [8] and further a partial fragment of elongation factor-1α gene was amplified from this genomic DNA by polymerase chain reaction using degenerate primers designed from the conserved sequences of reported EF-1α gene sequences. The nucleotide sequence obtained, were analyzed for the presence of conserved sequences and also to know its homology with other elongation factor gene sequences deposited in the database.

DNA ISOLATION

Most of the *Piper longum* conserved naturally in India are rarely exploited commercially as there is a dearth of references for its genetic variability. Total DNA was isolated from young leaves of *Piper longum* by using a modified CTAB buffer protocol and quantified spectrophotometrically at 260nm. The quality and integrity of the isolated DNA was checked by running through 0.8 % agarose gel (Figure. 1). The DNA sample was further diluted by mixing 1 µl of DNA with 99 µl of nuclease free water and the concentration of DNA was

The absorbance obtained is 0.073

So the concentration of DNA is = 50 × 0.073100

= 365µg of DNA /ml

= **0.365 µg** of DNA / µl

The agarose gel electrophoresis separated high molecular weight *Piper* genomic DNA and ~365 ng/µl quantity of the genomic DNA was obtained. The electrophoresis also showed high molecular weight band without smearing and fragmentation.

POLYMERASE CHAIN REACTION

A polymerase chain reaction was carried out to amplify the required gene fragment. The reaction was carried out using good quality genomic DNA as template, clear bands of approximately 0.65 kb size was obtained. The bands are clearly viewed when run on 1.2 % agarose gel (Figure. 2). Similar type of PCR amplification was done from cDNA to amplify conserved L gene sequence of Strawberry crinkle virus in plants [9]. The PCR product was further eluted from the gel using GFX gel-band elution kit. The gel elution of DNA bands using GFX column was found to be working well with minimum sample loss in the experiment [10].

SEQUENCING

For molecular analysis of elongation factor - 1α gene from *Piper longum,* the eluted fragment was quantified and then sequenced in both directions using the forward and reverse primers, which were used for the initial PCR amplification from genomic DNA. The sequencing results from both the directions were aligned using Bio Edit software. This gave a 636 bp nucleotide sequence (Figure. 3).

BIOINFORMATIC ANALYSIS

The nucleotide sequence was used for the BLAST (Basic Local Alignment and local Search Tool) analysis. This was done using BLASTN programme. The BLAST results showed that the obtained sequence aligning well with reported elongation factor 1 alpha gene sequences of other plants reported in the database. The sequence obtained from the study showed maximum homology with the corresponding gene from *Brassica napus*, *Arabidopsis thaliana*, *Chrysanthemum seticuspe* etc. It showed 82% homology with *Brassica napus* elongation factor-1α with an E-value of 3e-96, 78% homology with *Arabidopsis thaliana* EF-1α with an E-value of 1e-93. The full length gene is having 1396 nucleotides in *Brassica napus* and 1783 nucleotides in *Arabidopsis thaliana*. The sequence has (G+C) content of 42.14% and (A+T) content of 57.86%. The nucleotide content is presented in figure 4. The multiple alignment of *P. longum* EF-1α sequence with that of *B. napus* and *A. thaliana* sequences are given in figure 5. The elongation factor 1 alpha reported in this study is the first such a gene from this plant.

PHYLOGENETIC ANALYSIS

The phylogenetic study of the isolated sequence of elongation factor 1 alpha with other sequences present in the database was conducted using Mega4 software. For this study, the required sequences were retrieved from the database. All these sequences were aligned, including the one obtained in the current study at the nucleotide level using Clustal W multiple alignment programme. Identical regions of all the sequence were used and phylogenetic tree was created using Mega 4 software(Figure 6). The phylogenetic analysis showed that the EF-1alphaSequence from *Piper* was found to form a separate cluster and shows closeness with *Chrysanthemum seticuspe* the EF-1alpha gene sequence. Further studies to be done for the elucidation of full-length information of this gene and its significance in *Piper.*

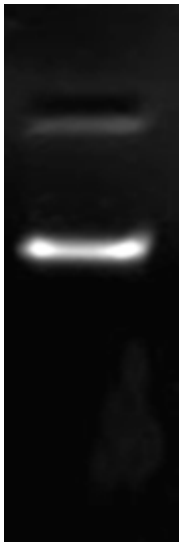

Fig. 1: Genomic DNA isolated from *Piper longum*

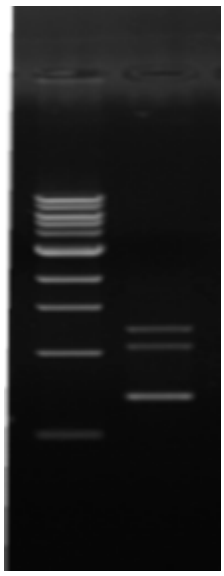

Fig. 2: PCR amplification of partial fragment of EF-1A from Piper

Lane1: 1 kb DNA ladder, Lane 2: PCR product

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                    10        20        30        40        50        60        70
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Piper longum        CAATCTGGGTCTGCAGCTGACGCTGAGCGTGAACGTGGTATTACCATTGATATTGCACTCTGGAAATTCG

                    80        90        100       110       120       130       140
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Piper longum        AGACTACCAAGTACTACTGCACGGTCATTGATGCCCCTGGACATCGTGACTTCATCAAGAATATGATTAC

                    150       160       170       180       190       200       210
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Piper longum        CGGTACCTCTCAGGCAGATTGTGCTGTTCTGATTATTGACTCAACCACTGGTGGTTTTGAGGCTGGCATC

                    220       230       240       250       260       270       280
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Piper longum        TCAAAGGATGGCCAGACACGTGAGCATGCTCTACTGGCCTTCACCCTTGGTGTGAAGCAAATGATCTGTT

                    290       300       310       320       330       340       350
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Piper longum        GTTGTAATAAGGTATCCTCTGGTTCTATTAGTCGGGTTTGTGCAGAATAGCTTTTGGTTTTCTAAAGGAA

                    360       370       380       390       400       410       420
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Piper longum        GGCGGTTTGAATAAGGCTGTTAATTTTTCTGTGCTGTCTTCTTTAGTGGAATGAATGGTCTTAAATGGAA

                    430       440       450       460       470       480       490
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Piper longum        AGGATTTGTTTTTTACAGATTATTTTTGACATATAAGGAAAAGCATATTGATCTTATGTGTTTTTTGTTT

                    500       510       520       530       540       550       560
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Piper longum        TCAGATGGATGCAACAACCCCAAAATACTCCAAGGCTAGGTACGAAGAAATTGTGAAGGAAGTTGCTTCC

                    570       580       590       600       610       620       630
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Piper longum        TACATCAAGAAGGTTGGCTACAACCCAGACAAGATTCCCTTTGTTCCCATCTCAGGTTGATGTTACAGCT

                    ....|.
Piper longum        TTGGGT
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Fig. 3: Nucleotide sequence of partial fragment of EF-1A from *Piper*

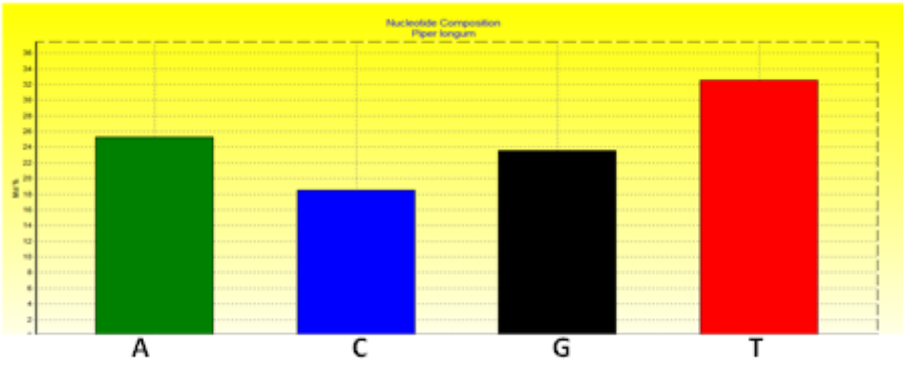

Fig. 4: Nucleotide composition chart of EF-1A sequence from *Piper*

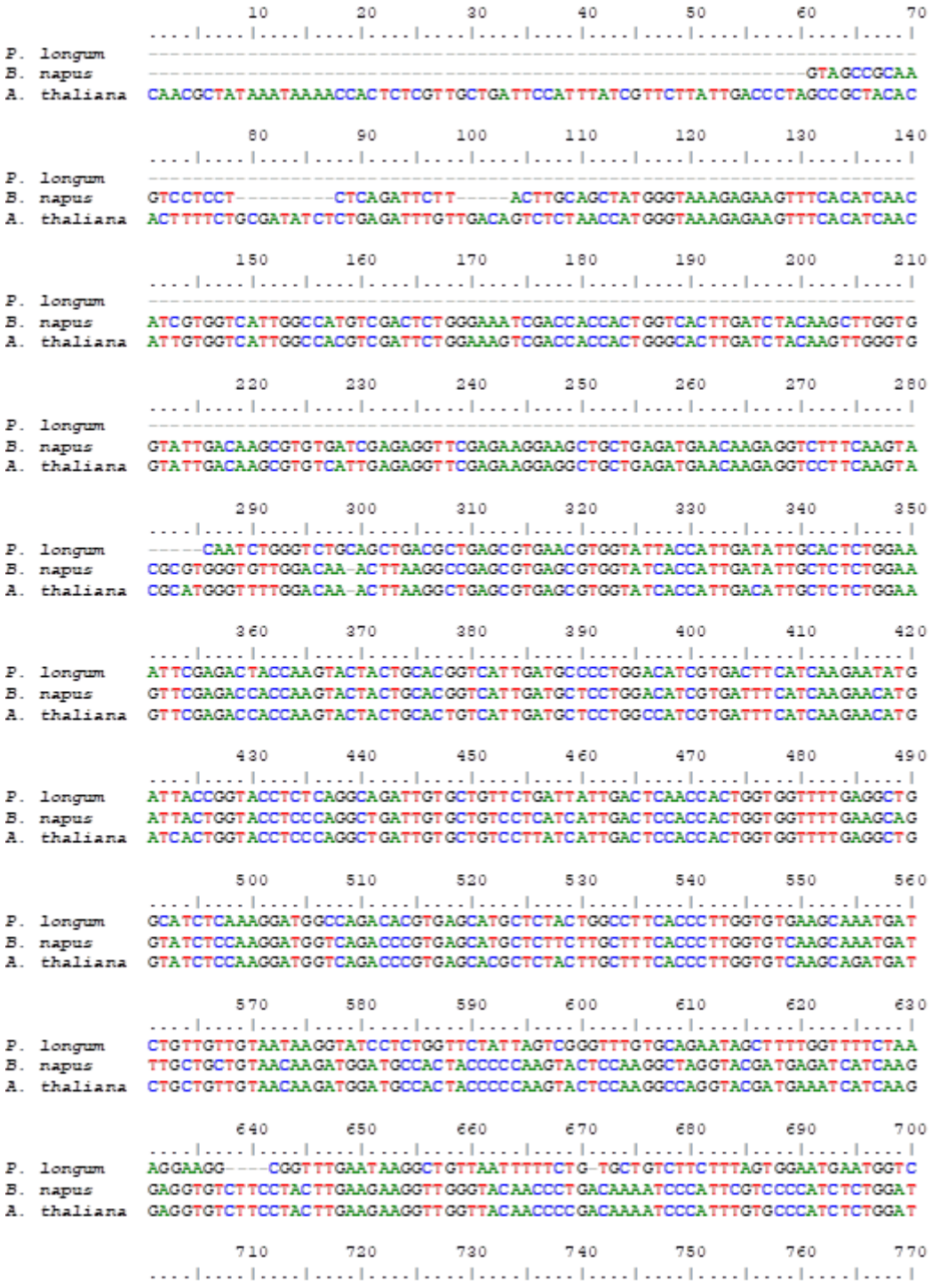

Fig. 5: Multiple sequence alignment of EF-1A sequence of *Piper longum with B. napus* and *A. thaliana* sequences

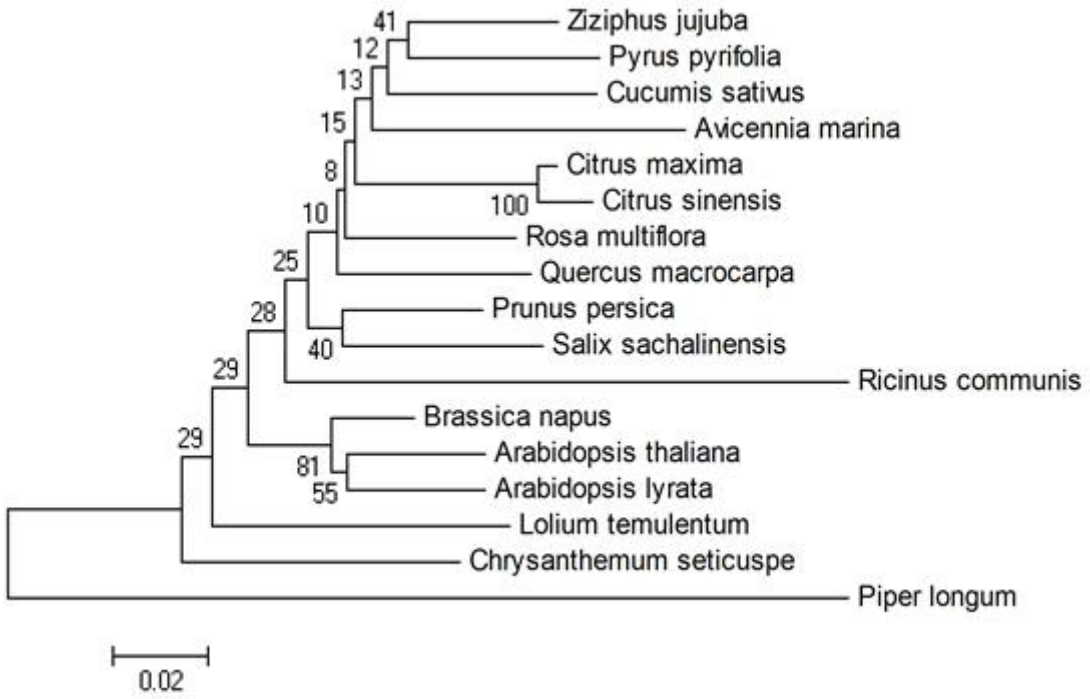

Fig. 6: Phylogenetic analysis of EF-1A sequence from *Piper* with known sequences in the database

CONCLUSION

A modified protocol for DNA isolation from *Piper longum* is standardized and the electrophoretic and spectroscopic data of isolated DNA confirmed its quality and also proved the standardized protocol for DNA isolation gives good yield. The agarose gel electrophoresis separated high molecular weight *Piper* genomic DNA and ~365 ng/µl quantity of the genomic DNA was obtained. The PCR product of 0.6 kb corresponding to elongation factor-1alpha gene fragment was amplified from isolated genomic DNA using degenerate primers. The sequencing results from both the directions were aligned using Bio Edit software. This gave a 636 bp nucleotide sequence On BLAST analysis it shows homology with that of *B. napus* and *A. thaliana* sequences. A boot-strap phylogenetic tree shows its evolutionary relationship with other sequences in the database.

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