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Study on Rubber Elongation Factor Gene (*ref*) Related Sequences from *Hevea nitida*

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ABSTRACT

Rubber elongation factor (*ref*) gene encodes for a protein in latex which has a key role in the biosynthesis of natural rubber (*Cis*-1, 4-polyisoprene) of *Hevea brasiliensis*. Genomic DNA was extracted from immature leaves of *Hevea nitida*, a wild *Hevea* species using CTAB method. Polymerase Chain Reaction (PCR) amplification of DNA from *Hevea nitida*, with *ref* gene specific primers amplified five fragments, out of which 670 bp fragment was prominent. The amplified 670 bp product was sequenced. Homology of nucleotide sequence with the existing sequences were analyzed using NCBI Blast tool. The nucleotide sequence was converted to amino acid sequence and that was compared with the amino acid sequences of *ref* gene sequence of RRIC 121 and *Hevea spruceana* integrase-like gene. The quality of the extracted DNA was poor with the presence of proteins and RNA suggesting the need for improvement of the CTAB extraction protocol used in DNA extraction. The amplified 670 bp nucleotide sequence shared 80% homology with the *Hevea spruceana* integrase-like gene, 64% homology with *Pisum sativum* clone cID58-6 centromeric retrotransposon and 57% with *Pisum sativum* clone cID58-2 centromeric retrotransposon. Rubber elongation factor gene (*ref*) of *Hevea brasiliensis* clones had no homology with nucleotide sequence obtained from *Hevea nitida*. The derived amino acid sequence showed no homology with the amino acid sequence of *ref* gene of RRIC 121, while it was homologous with *Hevea spruceana* integrase-like gene at amino acid level. By this study it is postulated that the *ref* related gene sequence obtained from *Hevea nitida* has no homology to *ref* gene from *Hevea brasiliensis*, however has homology to integrase like sequence found in wild *Hevea* species, *Hevea spruceana* and retrotransposon sequences of *Pisum sativum*.

KEYWORDS: *Hevea nitida, Hevea spruceana,* Integrase-like gene, Rubber elongation factor

INTRODUCTION

Hevea brasiliensis belongs to family *Euphorbiaceae*, and genus *Hevea*. Genus *Hevea* consists of ten species, *Hevea benthamiana, Hevea brasiliensis, Hevea camargoana, Hevea camporum, Hevea guianensis, Hevea microphyla, Hevea nitida, Hevea pauciflora, Hevea rigidiflora* and *Hevea spruceana*. *Hevea brasiliensis* is the extensively used commercial Natural Rubber (NR) producing plant amongst over 2,000 species which produce NR (Webster *et al.*, 1989). NR is chemically known as *cis*-1, 4 polyisoprene and it satisfies 40% for global demand of elastomers (Priya *et al.*, 2006).

Based on the export earnings, rubber is the 3rd largest plantation crop in Sri Lanka contributing 0.1% to Gross Domestic Production. Rubber industry has contributed 8% for the total export earnings of Sri Lanka amounting 935 million US dollars (Central Bank Annual Report, 2014).

Natural Rubber is produced and stored in laticifer cells or latex vessels. Laticifer cells are located in the outer bark of the tree. Latex is present in all parts of the tree, but laticifers in the bark are utilized commercially. Rubber particles found in latex are tightly bound to a protein, rubber elongation factor protein. The cDNA sequence of rubber elongation factor gene was reported by Attanayaka *et al.*, (1991). The genomic sequence of *ref* gene is 1367 bp long and it has three exons interrupted by two introns (Attanayaka *et al.*, 1996).

Rubber elongation factor protein is 137 amino acids long and has a molecular mass of 14.5 KD (Dennis and Light, 1989). *Hevea brasiliensis* clones show differential expression pattern of *ref* gene (Attanayaka *et al.*, 1996).

Hevea nitida, a member of the genus *Hevea* considered as non-rubber producing species because its poor yield. It is grown in periodically heavily flooded swamps but also on rocky hillsides near falls and rapids and in highland forest sands above the annual flood level (Sethuraj *et al.*, 1992). *Hevea nitida* is usually a medium-sized, evergreen tree with a sparse crown. It can be found in Northern and Southern America - northern Brazil, Colombia. The seeds are an important food source for native people in some parts of the Amazon, though in other areas they are used as a food in times of famine, when better foods are not available. The seeds contain cyanic compounds are poisonous to humans unless treated (Anon, 2016a).

Previous studies have proved the presence of *ref* gene variation among the *Hevea brasiliensis* clones. Presence of *ref* related sequences in a non-rubber producing wild *Hevea* species i.e. *Hevea spruceana* has also been reported (Accession no, KC864789.1)

(Anon, 2016c). In this investigation, it is intended to study the *Hevea nitida* genome for the presence of the *ref* gene related sequence which has direct implification for the rubber biosynthesis ability of the *Hevea* species.

MATERIALS AND METHODS

Experimental Location

The study was conducted at the Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka from December 2015 to May 2016.

Plant Materials

Apple green stage immature leaf material from *Hevea nitida* was collected from the Genetics and Plant Breeding Department of the Rubber Research Institute of Sri Lanka.

Genomic DNA Extraction

Genomic DNA was extracted from *Hevea nitida* leaves according to the CTAB DNA extraction method as described by Edirisinghe *et al.* (2014). Twenty milligrams of leaf samples were ground with 400 µL of extraction buffer (2X CTAB buffer - 2% CTAB, 1.4 M NaCl, 20 mM EDTA (pH 8.0), 0.1 M Tris-Cl (pH 8.0), 1% 2-mercaptoethanol) using mortar and pestle. Another 400 µL of extraction buffer was added and materials were ground further. Then 750 µL of homogenate was spun at 8,000×g for 20 min at room temperature. The supernatant was gently mixed with 750 µL of chloroform for 5min and was spun at 8,000×g for 20 min at room temperature. Then the upper aqueous layer was collected and was gently mixed with 700 µL of absolute alcohol for 2 min and centrifuged at 7,500 rpm for 4 min. Finally the DNA pellet was washed twice with 70% ethanol and was re-suspended in 50 µL of TE buffer, and stored at -20 °C. Quality and concentration of the isolated DNA was checked on 0.8% agarose gel.

Column Purification of Genomic DNA

Extracted DNA was purified using a DNA purification kit (PureLink™ Quick Gel Extraction and PCR Purification Combo Kit). Two elutions were performed following manufacturer's instructions.

Optimization of PCR Conditions

The PCR conditions were optimized by doing a gradient PCR to find out the optimum annealing temperature for PCR using *ref* gene specific primers on *Hevea nitida* genomic DNA. In order to determine the optimum annealing temperatures for the primers, four annealing temperatures (48, 50, 52 and 55 °C) were applied. Different Taq polymerase concentrations (2.5, 5 and 6.5 unit) were applied to identify the best Taq polymerase amount.

PCR Amplification of ref Gene

Amplification of PCR was done in a 25 µL volume which contained 50 ng of DNA, 200 µM dNTP, 2.5 mM $MgCl_2$, 1X PCR buffer, 5 unit of Taq polymerase (SIGMA), and 1 µL each of the designed 10 µM forward and reverse primers of the *ref* gene. The primer sequences were; forward 5' ACG CGA ATT CGG AGG TTC GAT TAT GGC TGA AGA CG 3' reverse 5'ACG CGT CGA CTT GGG GCT CAA TTC TCT CCA 3' (Herath *et al.*, 2013).

PCR amplification was done with a BioRad thermocycler with an initial denaturation step at 96 °C for 5 min followed by 30 cycles of 96 °C for 15 sec, 48 °C for 30 sec and 72 °C for 1 min and 30 sec. final extension step at 72 °C for 1 min was also included.

Analysis of PCR Products

The PCR products were analyzed on a 0.8% agarose gel stained with ethidium bromide. Promega DNA marker 100 bp ladder (100-1500 bp) was used as DNA size marker. Electrophoresed gel was visualized and photographed using gel documentation apparatus (Quantum ST 4).

Gel Purification of the Amplified DNA Fragments

The amplified *ref* related DNA fragments were excised from the agarose gel and purified using an agarose gel purification kit (PureLink™ Quick Gel Extraction and PCR Purification Combo Kit).

Analysis of Purified PCR Products

Purified DNA was analyzed on 0.8% agarose gel stained with ethidium bromide. Dilution series of λ-DNA marker was used as a quantitative marker and they were visualized using gel documentation apparatus.

Sequencing of ref Related Sequence

Amplified *ref* gene related sequence of *Hevea nitida* was sequenced at Macrogen DNA sequencing, Korea.

Characterization and Comparison of ref Related Sequences

Sequenced gene fragment was analyzed using the mega 4 sequence alignment software. Similarities and dissimilarities of the sequence with existing gene sequences were performed using the NCBI data base (Anon, 2016c). Characterized nucleotide sequence was converted in to amino acid sequence with the

help of translate tool in ExPASy database (Anon, 2016b) and that was compared with the amino acid sequences of rubber elongation factor protein available in the database.

RESULTS AND DISCUSSION

Genomic DNA Extraction

Quantity and the quality of the extracted DNA using the CTAB extraction protocol were not adequate. Impurities, proteins and RNA content were much higher in the *Hevea nitida* DNA extraction. The yield of DNA extracted from *Hevea nitida* was very low (125 ng/mg) compared with RRIC 121 clone (500 ng/mg) (Erandika *et al.*, 2014; Figure 1).

Column Purification of Genomic DNA

Purified DNA was free of RNA and protein. First elution resulted 100 ng/µL of DNA while second elution yielded approximately 75 ng/µL (Figure 2).

Optimization of PCR Conditions

Out of the four annealing temperatures (48 to 55 °C) 48 °C was the best annealing temperature for the PCR amplification. When considering the three Taq polymerase concentrations, 5 units of Taq was the best for the amplification (Figure 3).

PCR Amplification of ref Gene

A total of five bands were observed in PCR amplification. The most persistent band had the length of approximately 600 bp. The size of the band corresponding with the *Hevea spuruceana* integrase-like gene size deposited in NCBI data base (Figure 4).

Gel Purification of the Amplified DNA Fragments

The yield of the purified DNA band was about 25 ng/µL.

Sequencing of ref Related Sequence

The gel purified fragment was sequenced and 670 bp long sequence was obtained.

Characterization and Comparison of ref Related Sequences

The *ref* related sequence obtained from *Hevea nitida* showed no homology with the *ref* gene sequences deposited in the GenBank. This indicates the *ref* related sequence amplified from *Hevea nitida* to be different from the *ref* gene sequence present in the commercial rubber tree *Hevea brasiliensis*. The *ref* gene has been identified as an important gene involved in the biosynthesis of natural rubber. The absence of *ref* gene homology in *Hevea nitida* genome may suggest as a reason for the inability of this species to biosynthesis of natural rubber. The sequence obtained from *Hevea nitida* to have 80% homology with an integrase like sequence obtained from another *Hevea* species, *Hevea spruceana* (Figure 5). Then two sequences showed significant homology to a retrotransposon found in *Pisum sataivum* (*Pisum sativum* clone cID58-6 centromeric retrotransposon - 64% and the *Pisum sativum* clone cID58-2 centromeric retrotransposon - 57%).

The protein blast of the two retrotransposon like sequences from *Hevea nitida* and *H. spruceana* too showed 70% homology at the amino acid level.

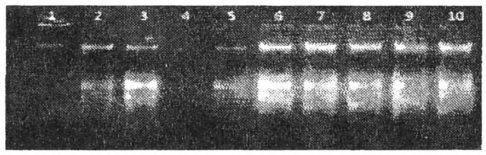

Figure 1. Agarose gel electrophoresis of *Hevea nitida* genomic DNA; *Lane 1-λ DNA (50 ng/µL), Lane 2, 3, 4, 5, 6, 7, 8, 9, 10- Hevea nitida genomic DNA*

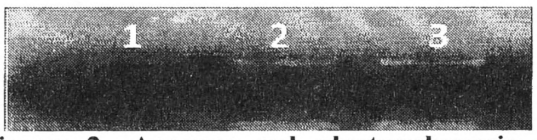

Figure 2. Agarose gel electrophoresis of column purified genomic DNA; *Lane 1 –λ DNA (50 ng/µL), 2,3 – purified Hevea nitida genomic DNA*

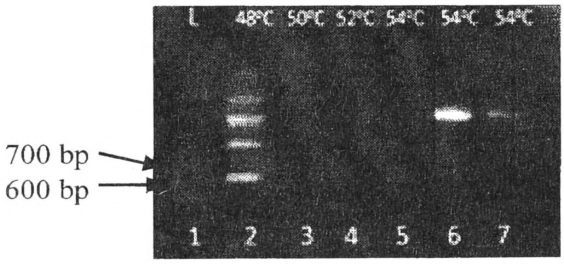

Figure 3. Agarose gel electrophoresis of PCR products amplified with *ref* gene primers at different annealing temperatures; *Lane 1 – ladder (100 bp), Lane 2, 3, 4, 5 – amplified bands of Hevea nitida, 6,7 – RRIC121*

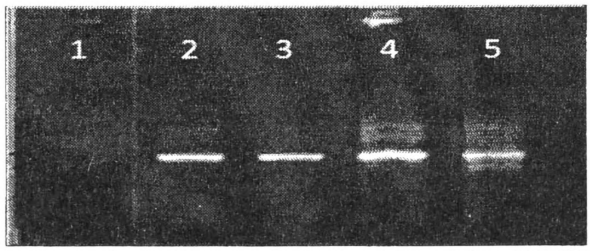

Figure 4. Agarose gel electrophoresis of PCR products of *Hevea nitida* at 48 °C; *Lane 1 – 100 bp ladder, 2,3,4,5 – amplified bands of Hevea nitida*

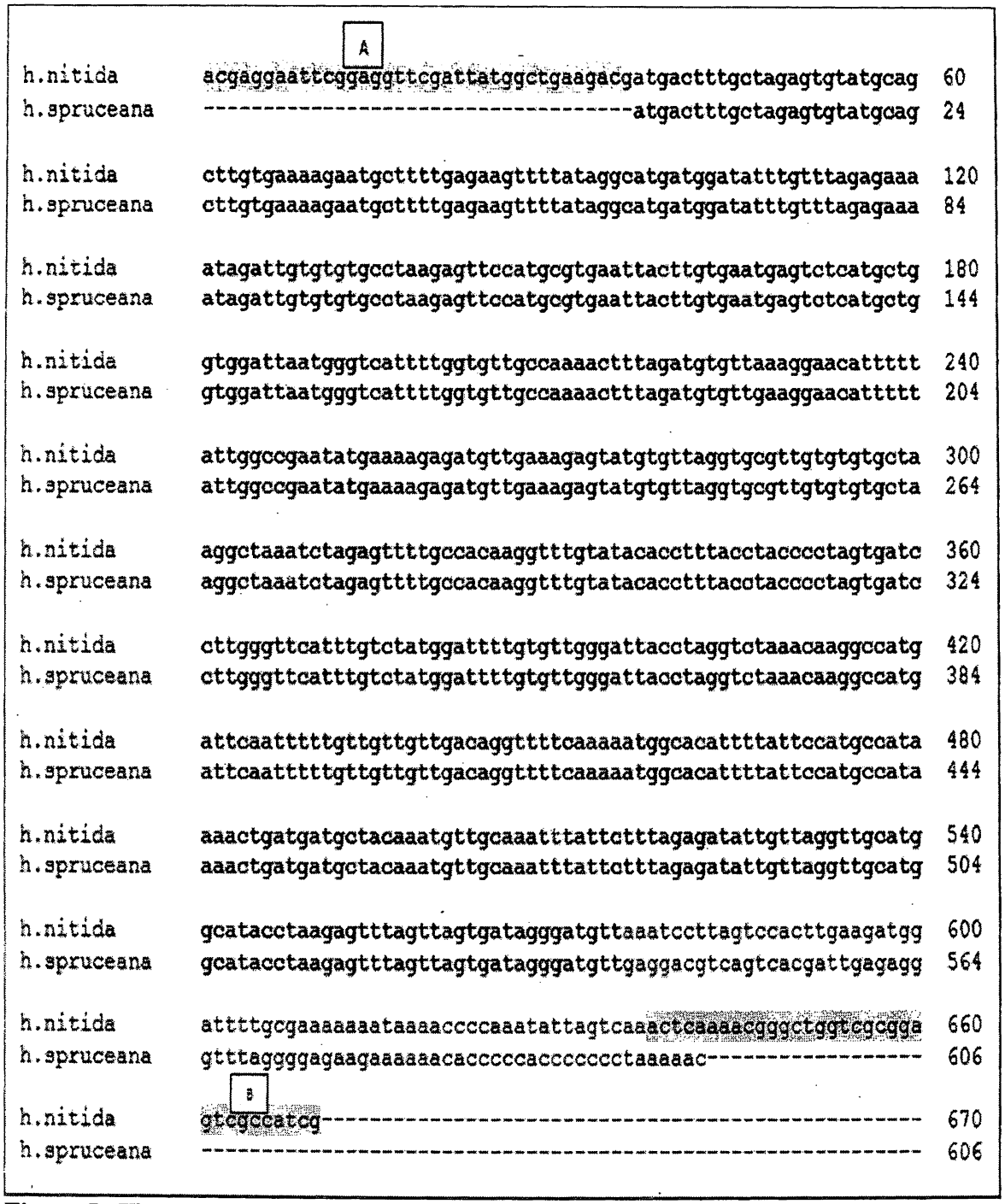

Figure 5. Homologous sequence to *Hevea nitida* and *Hevea spruceana* integrase-like gene; *Bold region A – forward primer binding site, Bold region B – reverse primer binding site, base number 1 to base number 539 of Hevea spruceana integrase like gene – homologus region for both H. nitida and H. spruceana*

CONCLUSIONS

The DNA extraction method used in this study to extract DNA from *Hevea nitida* was not adequately successful and need to be improved. The optimum annealing temperature to amplify *ref* related sequence from *H. nitida* was 48 °C. The *ref* related 670 bp sequences obtained from *Hevea nitida* was not related to the *ref* gene reported from the commercial rubber tree *Hevea brasiliensis*, but it was related to a retrotransposon sequence present in *H. spruceana* and *Pisum sataivum*. Further studies are necessary to investigate the hypothesis that non-existence of *ref* gene in *Hevea nitida* may have caused inability of this species to synthesis natural rubber.

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