Research Article

Fragment of the Ancient *RbcL* Gene from the Miocene

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The development of molecular biology techniques has allowed a new approach to palaeontology and studies on ancient DNA. As a plant fossil resin, amber provided a good matrix for preserving ancient biological material. Some difficulties arise when experimental work is done to extract information concerning these preserved specimens. The major risks in this type of works are the contamination with modern DNA and the degradation of the ancient DNA. A safe method to sterilize amber stones has been designed allowing the amplification of a fragment of the ancient *RbcL* gene from the Miocene (*c.a.* 25 million years). Presumably, the gene was from *Hymenaea protera*, an extinct member of the *Leguminosae* family. The phylogenetic tree and divergence rates indicate that since although it is a well-conserved gene, and then should be a good candidate for studying the evolution of plant macrogroups, probably it is not good enough for analyzing divergence among closely related species.

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1. Introduction

The development of PCR-based techniques has prompted studies on fossil DNA as well as in molecular palaeontology. In the pursuit of such goals, a crucial issue is the availability of a good supply of well-preserved ancient DNA, amber being one of the best such sources because of its high capacity of specimen preservation.

This material is formed through the fossilization of resins secreted by conifers and angiosperms [1]. The amber from Baltic was formed during the Oligocene from the members of conifers mainly (*c.a.* 40–50 million years) [2]. The amber from the Dominican Republic was mainly formed with resin from the *Leguminosae* during the Miocene (*c.a.* 25 million years), mainly from members of the genus *Hymenaea* (i.e., *Hymenaea protera*), an extinct member of the *Leguminosae* family.

There are two areas with major amber deposits in the Dominican Republic: the Northern area (Santiago de los Caballeros) and the Eastern area (Cordillera Oriental) (Figure 1(a)). Paleogeographically, both areas form part of the same sedimentary basin, which was disrupted by movements along major faults (Figure 1(b)).

During the amber-forming process, a variety of samples, including insects, pollen, and microorganisms, became

embedded in the resin and remained unaltered for millions of years despite deleterious external agents as water or ultraviolet radiations [3–5].

The current problems involved in working with this type of ancient DNA are contamination by contemporaneous DNA, the occurrence—in the sample—of PCR-inhibiting agents [6], and the degradation of the DNA itself in the amber [7, 8]. The prevention of such contamination is crucial in this type of studies and must be carried out via an appropriate sterilization procedure that is sufficiently aggressive to sterilize the surface of the amber and yet mild enough to preserve the ancient DNA.

Choice of the ancestral genes to be studied is a critical point in the outcome of these studies, and, in the present work, we decided to use oligonucleotides able to amplify *RbcL* gene (RuBisCo large subunit), an essential and highly conserved gene from plants.

2. Material and Methods

2.1. Amber Samples. The present work was carried out using samples from amber nuggets from Santiago de Los Caballeros Mountain (Dominican Republic), dated stratigraphically as 15–30 million years old [9].

FIGURE 1: (a) Map of the amber mining in Dominican Republic. (b) Stratigraphic layers of amber deposits.

2.2. Sterilization Procedure. This was basically according to Lambert et al. [10], with some modifications described by Veiga-Crespo et al. [9]. The incubation time in 2% glutaraldehyde (Merck, USA) at 40°C was divided into two 24 hours periods, and after the first period the solution was changed. The ultrasound treatments (Ultrasons P, Selecta, Spain) were for 30 minutes and this ultrasonic procedure was implemented throughout the sterilization procedure (24 hours period in 10% calcium chloride at 25°C and 24 hours period in 70% ethanol at room temperature) being interrupted before changing the amber nuggets to brain heart infusion broth medium (B.H.I.B.; Biolife).

2.3. Fracture of Nuggets. Before fracturing, the samples were kept for fifteen days at 21°C, fifteen days at 30°C and fifteen additional days at 37°C in B.H.I.B. in order to ensure a lack of microbial growth at the three temperatures. The amber was then frozen in liquid nitrogen and ground in a mortar. The powder was resuspended in B.H.I.B. and the aliquots kept at -70°C until use.

2.4. Extraction of Fossil DNA. Extraction of fossil DNA was accomplished using the Ancient DNA kit (GeneClean, Bio 101).

2.5. PCR Reactions and PCR-Products Purification. Oligonucleotide design was done according to the current sequence of the *RbcL* gene from *Pinus edulis* (Genbank accession code: X58137). The oligonucleotides used were *RbcL*-forward: 5'-ATGTCACCAAAAACAGAGAC-3' and *RbcL*-reverse: 5'-ATGTCACCAAAAACAGAGAC-3'.

The PCR mixture was 1 U Taq polymerase (Takara Shuto Co.), 2 ng/ μ L BSA (Promega), 0.5 μ M of each oligonucleotide

(Invitrogen), 2 mM MgCl₂ (Takara Shuto Co., Japan), 0.2 mM dNTPs mix (Takara Shuto Co.), and deionized sterile water to a final volume of 50 μ L. The reaction was accomplished in a Robocycler Gradient 96 device (Stratagene) with the following program: 1 cycle of 5 minutes at 94°C; 35 cycles of 1 minute at 94°C, 30 seconds at 56°C and 1 minute at 72°C. The process was ended with a cycle of 10 minutes at 72°C.

The PCR-products were purified using Wizard PCR preps kit (Promega, USA). DNA sequencing was performed according to Sanger's method [11], employing duplex DNA and using the T7 Sequenase v2.0 PCR Product sequencing kit (Amersham Pharmacia Biotech, Spain), following the manufacturer's instructions. Labelling was done with Redivue [35S]-dATP.

2.6. Sequence Analyses. Sequence analyses and determination of phylogenetic relationships were performed using the ClustalW application, included in the software VectorNTI Advance Suite v9.0 (Informax). The distance matrixes were performed using the Biology WorkBench 3.2 from San Diego Supercomputer Center, employed the ClustalW algorithm [12] and the PHYLIP algorithm [13].

2.7. Controls of Contamination. Working surfaces were periodically treated with ethanol (70%) and before and after each work session the nuggets were treated with 10% sodium hypochlorite (Merck). All culture media were maintained for 15 days at 21, 30, and 37°C before use. All solutions used to sterilize the amber stone surfaces were previously filtered through 0.22 μ m membranes (three times) that had been previously sterilized. Before stone grinding, the samples were incubated in B.H.I.B. medium and subjected to the

H._protera_sp.n	- - - ATGTCACCACAAACAGAAACAAAGCAAAG- GTTGGGTTCAAAGCTGGTGTAAAG
This_work	ATGTTTCACCCCAAAAAACAGAGACTAAAGCANAGTGTGGGTTCAAAGCTGGTGTAAAG
P.edulis_RbcL	- - - ATGTCACCAAAAAACAGAGACTAAAGC- TAGTGTGGGTTCAAAGCTGGTGTAAAG
H._protera_sp.n	ATTATAAATTGACTTATTATACTCCTGACTATCAAACCAAGGATACTGTTGGCATATCAG
This_work	AGTATAAATTGACTTACTATACTCCTGAATATCAAACCAAGGATACTGATATCTTGGCAG
P.edulis_RbcL	ATTACAGATTAACCTTATTATACTCCTGAATATCAGACCAAAGATAACGATATCTTGGCAG
H._protera_sp.n	CATTCCGAGTAACCTCCTCAACCCGGAGTTCGCCCGAAGAAACAGGTGCCCGCAGAGCTG
This_work	CATTCCGGTTAACTCCTCAACCTGGAGTTCACCTGAAGAAGCAGGCGCTGCCGTAGCTG
P.edulis_RbcL	CATTCCGAGTAACCTCCTCAACCTGGGTCACAGCCGAGGAAGCGGTGCAGCAGTAGCTG
H._protera_sp.n	CTGAATCTTCTACTGGTACATGGACAACTGTGTGGACCGATCGGTTACCAGCCTTGATC
This_work	CTGAATCTTCTACTGGTACATGGACAACTGTGTGGACCGATCGCTTACCAAGCC- - ATC
P.edulis_RbcL	CTGAATCTTCCACCGGTACATGGACACTGTTTGGACCGATGGACTTACTAGTCTCGATC
H._protera_sp.n	GTTACAAAGGACGCTGCTACCACATCGAGCC- CGTTCTGGAGAAGAAATCAATATATT
This_work	GTTACAAAGGACGATGCTACC- CATCGAGCCCGGTTCCAGGAGAAGAACTCAATTTATT
P.edulis_RbcL	GTTACAAAGGGCGATGCTATGACATTGAGCC- CGTTCTGGAGAAGAAATCAATTTATT
H._protera_sp.n	GCTTATGTAGCTTATCCCTTAGA- - CCTTTTTGAAGAAGAGGGTTCTGTAACTAT- - -
This_work	GCGTATGTAGCTTATCCCTTAGAAGCCTTTTTGAAGAAGGTTCCGTTACTAACATGTTTA
P.edulis_RbcL	GCTTATGTAGCTTATCCCTTAGA- - CCTTTTTGAAGAAGGTTCTGTACTAACCCTGTTCA
H._protera_sp.n	-----
This_work	CCTCGATTGTGGTAATGTATTTGGGTTCAAAGGCCCTGGCTGCTCTACGTCTAGAGGATC
P.edulis_RbcL	CTTCCATTGTAGGTAATGTATTTGGATTCAAGGCCCTACGGGCTCTACGTTTGAAGATT
H._protera_sp.n	-----
This_work	TGCGAATCCCTCCTGCTTATACTAAAGCTTTCCAAGGACCACCTCATGGTATC
P.edulis_RbcL	TGCGG-----

FIGURE 2: Alignment between the three sequences.

same temperature cycle in order to discard any possible contamination. After grinding, microbial contamination was investigated again. The solutions used for DNA extraction and PCR were periodically controlled for fortuitous contamination. Throughout the process, particular care was taken in order not to use glassware or equipment that had been previously in contact with current DNA.

2.8. Criteria of Sequence Validity. The following criteria were adopted (i) DNA was extracted only from stones that had passed all the contamination checks; (ii) samples from the same stones had to show similar results; (iii) ancestral sequences had to show homology with current ones and had to display phylogenetic coherence, and (iv) large DNA fragments (longer than 1 Kb) had to be discarded to avoid either sample contamination with current DNA or jumping-PCR phenomena.

3. Results and Discussion

Because of the size of the gene and the state of degradation of the ancient DNA, amplification of the complete gene from the samples was unsuccessful. It was, therefore, necessary to design internal oligonucleotides from the initial region of the gene.

Amplification of a segment of the ancient *RbcL* gene was positive in Miocene stones from the Dominican Republic but negative in Oligocene amber samples (*c.a.* 40 million years old), which were positive for other genes [9, 14]. A consensus sequence was elaborated (Genebank accession code: AY484432) from all the ancient DNA sequences. This Miocene sequence showed 82% similarity to the current

Pinus edulis RbcL gene and it was by far the most highly conserved sequence in all the genes found in both Miocene and Oligocene stones [9, 14].

When the obtained consensus sequences were compared with the actual ones of *P. edulis*, it was seen that 1% of the total changes were insertions or deletions (22% and 78%, resp.) and the rest corresponded to transversions or transitions (42% and 58%, resp.). As a result of all these changes, the actual gene fragment is thirteen nucleotides shorter than the miocenian one; it would be worth trying to corroborate this tendency to shorten gene size through cloning of new *RbcL* fragments.

The new amplified fragment was 122 pb longer than the previous one reported by Poinar Jr. [15] for *Hymenaea protera* sp. n. (GenBank DataAccess: L08477). When both fragments were compared with each other, it was seen that 15% of the total changes were insertions or deletions whereas the rest corresponded to transversions or transitions.

When the three sequences were aligned (Figure 2) and the distance matrix elaborated, it was observed that the both miocenian sequences were closer than actual *P. edulis* one. These distance values were observed independently of the algorithm used for calculation the distance (Table 1).

Next, a phylogenetic tree was drawn according to the data in GenBank (Figure 3). Phylogenetic analysis was done using the neighbor-joining method with the lowest possible evolutionary events. Ambiguous residues were resolved as "gaps" and transversion/transition substitutions were given more importance than insertion/deletion events.

The *RbcL* gene is highly conserved among plants and, as it is subjected to high selective pressure, it shows a low degree of evolutionary divergence; it is thus a good candidate for

FIGURE 3: Phylogenetic tree of *RbcL* gene.

TABLE 1: Distance matrixes of *P. edulis*, *H. protera* sp. n. and miocenic sequences. (a): by ClustalW algorithm; (b): by Phylip algorithm.

(a)			
	<i>H. protera</i>	<i>This_work</i>	<i>P.edulis</i>
<i>H. protera</i>	0.000	0.154	0.182
<i>This_work</i>	0.154	0.0000	0.154
<i>P.edulis</i>	0.182	0.162	0.0000
(b)			
	<i>H. protera</i>	<i>This_work</i>	<i>P.edulis</i>
<i>H. protera</i>	0.0000	0.1806	0.2127
<i>This_work</i>	0.1806	0.0000	0.1864
<i>P.edulis</i>	0.2127	0.1864	0.0000

fossil DNA work. In the phylogenetic tree, it may be seen that in general the different groups do appear correctly located except for the sequence of *Pinus halepensis*, which is located at an algal junction. This analysis again confirms the position of the amplicon in the group formed by the actual and

extinct members of genus *Hymeneaea* and it keeps it away from the actual sequences belonging to the conifer members despite the fact that these latter sequences were employed for oligonucleotide design and the high conservation of *RbcL* gene.

The fact that our Miocene sequence was found among dicot plants instead of among gymnosperms may be attributed to the abovementioned highly conserved sequence, although it should be borne in mind that dicots were the precursors of monocots, either through syncotyl events or through heterocotyl events after a chain of progressive anisocotyl events [16].

Since the Miocene sequence and that of *Pinus edulis* (used for oligonucleotide synthesis) exhibited 82% of homology and the highest conserved grade between all the species studied in this work, we suggest that this gene may be useful for studying macroevolutionary lines in plants, although it may not be as suitable for studies of divergence and evolutionary rates among closely related species.

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