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Ultrastructure of 30–40 million year old leaflets from Dominican amber (*Hymenaea protera*, Fabaceae: Angiospermae)

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Abstract. *Hymenaea protera* leaflet fossils entombed in amber, dated at 30 to 40 million years (mine strata and exomethylene dating) were observed by both light and transmission electron microscopy. Ultrastructure preservation in these leaflets shows the presence of chloroplasts with thylakoid membranes, cell walls, mitochondria with associated endoplasmic reticulum, nuclei, and xylem tissue. Tissues show varying degrees of degradation; however, natural resin, which has perfused the cells, seems to maintain the structural integrity of the membranes and walls. We conclude that preservation of amber entombed organisms results from dehydration and slow fixative properties leaving the ultrastructure in excellent condition. These findings parallel reports on the exceptional preservation of amino acids and of DNA in amber-entombed organisms.
Key words. Fossil plants; amber; *Hymenaea* leaflets; ultrastructure of fossil material; amber preserved tissues; molecular preservation.

Amber is a fossilized resin formed over millions of years. It is known to preserve a wide variety of organisms in an excellent state[1,2]. In addition to the preservation of external features of microorganisms, animals and plants, there are recent reports of non-racemized amino acids[3] as well as of DNA recovery[4,5]. These reports contradict theoretical expectations on DNA longevity[6,7] and have yet to be shown to be reproducible, as has been shown for other ancient material[8].

The factors involved in long term preservation of ultrastructure and molecular components in amber are not completely known, nor have the chemical components of resins which become amber and their roles in preservation been completely elucidated. *Agathis* resin[9,10], which is responsible for the formation of Baltic amber, contains components that might explain long-term preservation. For example the presence of sugars like glucose, and alcohols like fenchyl, may be involved in rapid desiccation of the tissues. Terpenes may be involved in bacterial, fungal and enzyme inhibition[11]. *Hymenaea* resin, which becomes Dominican amber, is similar in terpene composition to *Agathis* resin[12]; however its complete makeup has still to be elucidated. Despite the components that may be important in molecular preservation, amber is not impervious to slow oxygen diffusion[13], and therefore slow and retarded oxidative processes may be taking place over long geological time periods.

Ultrastructure in fossils from the Eocene has been reported for a 40 million year old Baltic amber-preserved fungus gnat[14] and some other mummified insect tissues in amber[15], as well as plant tissues from the proclastic deposits of the St. Maries river (Clarkia) area (17 million years old)[16–18]. Here we report on the oldest ultrastructural preservation of plant fossils to date, portions of two leaflets from the extinct species, *Hymenaea protera*[19] from Dominican amber dated between 30 and 40 million years old[20].

Materials and methods

Pieces of amber containing leaflets of *Hymenaea protera* originated from La Toca mine, located between Santiago and Puerto Plata in the Cordillera Septentrionalle of the Dominican Republic. The La Toca mine contains amber that has been estimated at 30 to 40 million years old by mine strata as well as exomethylene dating[2,20].

The pieces of amber containing *Hymenaea protera* were independently opened and examined at both the University of California at Berkeley (UCB) and the Institute for Zoology at the University of Munich (LMU). The pieces were surface sterilized in sodium hyperchlorite and rinsed in doubly distilled water, followed by a brief rinsing in 70% ethanol. The pieces were opened by the addition of liquid nitrogen, which was allowed to boil off followed by hot sterile physiological saline. Once open small portions of the tissue were removed with sterile forceps and directly embedded into Araldite 6005 (Ted Pella Inc., Reading, California, USA) (UCB) or Glycidether 100 (Roth, Karlsruhe, Germany) (LMU). Mixtures of acetone and ethanol were not used with the embedding media. Both synthetic resins, Araldite and Glycidether, have approximately the same

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hardness as the natural resin, amber, and for this reason they were used for embedding. There was no need to remove the resin associated with the leaflets. Both synthetic resins were left to polymerize for 8 h at 60 °C. The Araldite block was trimmed and sectioned with a diamond knife on a Reichert ULTRA CUT E Microtome (UCB) and the Glycidether block with glass knives on an LKB Ultrotome III (LMU). The diamond sections (60 nm thick) were treated with saturated uranyl acetate in water for 20 min, and then with lead citrate for 5 min. The glass sections (70–100 nm) were stained on an LKB 2168 Ultrostainer with uranyl acetate (Leica uranyl acetate mix) for 60 min at 40 °C, and lead citrate (Leica lead citrate mix) 30 min at 26 °C. The sections were then mounted on slot grids and observed in either a JEOL.100CX EM (UCB) or a Philips CM10 (LMU) electron microscope at an accelerating voltage of 80 kV.

Results

In this project, we are dealing with several types of resin. The leaf was embedded in trunk resin that was extruded from the *Hymenaea protera* tree. Within the leaf is the resin originating from leaf pocket cells, and finally the leaf was placed into synthetic resin for electron microscopic studies. We were not able to distinguish accurately between the natural trunk resin that may have perfused the leaflets and leaf pocket resin within the leaflets. We could, however, distinguish between the natural resins and the synthetic resins used for embedding.

The natural leaf pocket resin created no problems during the embedding or polymerization process. The trunk resin was adjacent to the leaf surface but did not adhere tightly to the cuticle (perhaps due to the waxy surface), since the leaflet was easily removed from the matrix once the amber piece was opened. Diamond-cut sections of the leaf tissue embedded in araldite were made without difficulty. Some shattering occurred with the glass knife-cut sections of the leaf tissue embedded in glycid ether. This difference could be explained by the presence of polymerized resin from the leaf pocket cells within the leaflet, or some perfused natural resin.

Observations with the light microscope indicated that no replacement or permineralization had occurred in any of the leaflets, or in any plant material found in Dominican amber. The leaflets were preserved in their natural three dimensional state (fig. 1A). The dark color of both leaflets could have been due to 1) a darkening of the chlorophyll, 2) the presence of tannins, or 3) slow oxidative effects that the leaf underwent over time. Occasionally green leaves do occur in amber but *Hymenaea protera* fossils with this color have never been observed. Other than darkening reactions, no damage from microbial decomposition or physical distortion was noted. The leaflets most likely fell from the tree directly into the natural trunk resin and were subsequently covered relatively quickly.

Electron micrographs from both leaflets demonstrated that the leaf had an internal structure typical for an angiosperm, i.e. an outer cuticle, underlying epidermis and a central portion of palisade and mesophyll tissues (fig. 1B). Although the parenchyma cells appeared to be collapsed, the outline of the cell walls was often present.

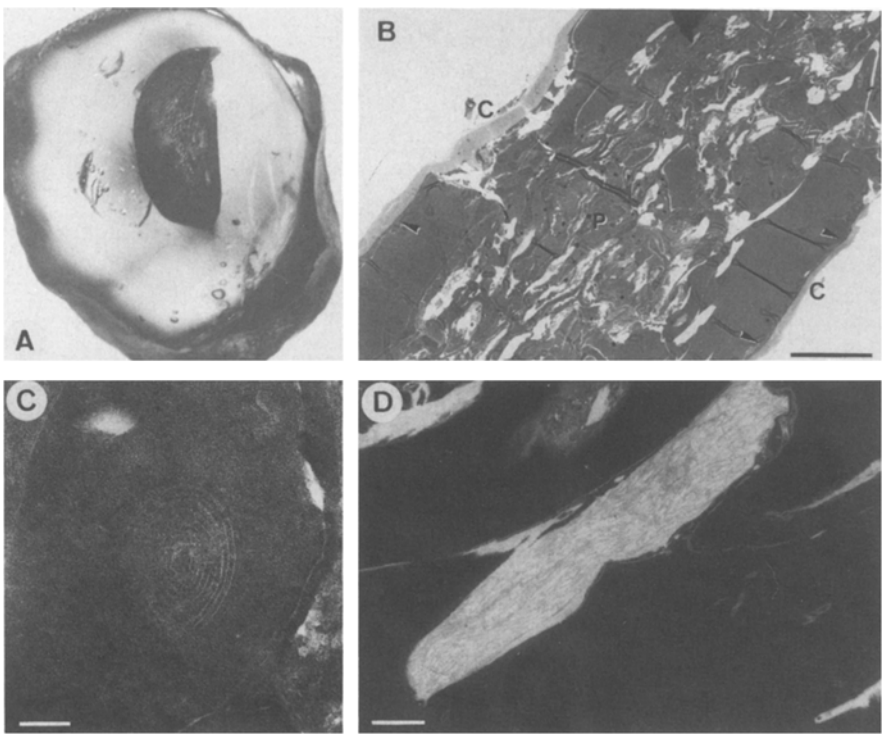

Figure 1. *A)* A leaflet of *Hymenaea protera* entombed in Dominican amber (3.81 cm). The pieces were collected from La Toca mine in the Dominican Republic, which has been dated at 30 to 40 million years old. The leaflets were darkened yet retained their three dimensional shape.
B) Transmission electron micrograph of (c) outer cuticle, with an underlying epidermis (arrowheads) and partially collapsed parenchyma (p) cells (× 1600). Bar = 10 μm.
C) Circularized membranes, such as this one, were commonly observed throughout the sectioned tissues. They show evidence of autolysis (× 40,000). Bar = 0.25 μm.
D) Resin pockets or ducts common to *Hymenaea protera* and often seen throughout tissues (× 50,000). Bar = 0.20 μm.

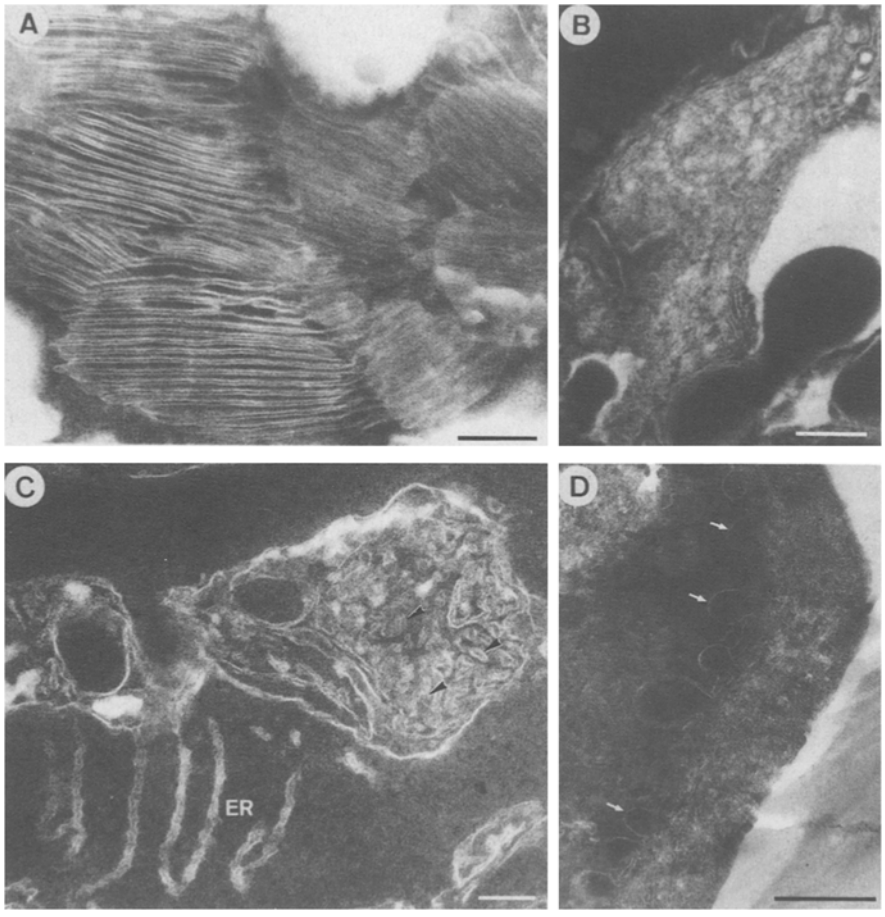

Figure 2. *A)* Chloroplast with internal detail showing membrane intactness, as well as uncollapsed thylakoids. The chloroplasts showed negative staining due to fixation or perhaps oxidation of membranes over long time periods (× 73,000). Bar = 0.2 µm.
B) Nucleus-like bodies (× 66,000). Bar = 0.2 µm.
C) Mitochondrion with visible cristae (arrowheads), as well as remnants of endoplasmic reticulum (ER) (× 55,000). Bar = 0.2 µm.
D) Intact membranes of xylem tissue (arrowheads); notice the natural resin lying on top of the membranes and causing little distortion (× 39,000). Bar = 0.5 µm.

We noted variously sized ducts filled with natural resin evenly dispersed throughout the leaflets, which we believe are resin ducts or pockets (fig. 1D), common to this tree[21]. Within the resin in these ducts were unknown electron dense fibrillary structures. Introgressed, multilamellar bodies, or circularized membranes (fig. 1C) were frequently observed, suggesting that slow yet significant autolysis had taken place, as is typically seen in tissues that undergo degradation post mortem.

Especially noteworthy were the remains of chloroplasts (fig. 2A), frequently seen in the parenchyma cells along with quite distinct thylakoid membranes. Also of interest were nucleus-like bodies noted in some cells (fig. 2B), as well as membranous arrays characteristic of endoplasmic reticulum (ER) cisternae and bodies resembling mitochondria (fig. 2C) with cristae (arrowheads). The presence of xylem tissue (fig. 2D) also showed intact membranes, along with natural resin which bordered and yet did not seem to distort the surrounding plant tissues.

Discussion

The discovery of chloroplasts, nuclei, mitochondria and other intracellular components in *Hymenaea protera* leaflet tissues from 30 to 40 million year old Dominican amber demonstrates the remarkable preservative qualities of the natural resin on plant material. These findings would seem to parallel reports of well-preserved amino acids and DNA.

The 17 million year old Clarkia leaves[16–18] indicated preferential preservation in the order of chloroplasts, cell walls, and in a few cases mitochondria and nuclei. We have seen a similar order of preservation, with chloroplasts clearly remaining most intact, along with cell walls. Chloroplast preservation may be attributed to its double membrane and its internal thylakoid membrane stability. To a lesser extent we have also noted nuclei and mitochondria with associated endoplasmic reticulum, which may also be afforded better protection due to their multiple membranes.

The tissues of the amber-preserved leaflets demonstrate negative staining, characteristic of the Clarkia fossils, which may be attributed to fixation by terpenes in the natural resin or by tannins in the leaflet tissues. Tannins are known to occur naturally in some plant tissues and bind to proteinacious material, thereby creating a type of autofixation[16].

It is obvious that the slow embalming and polymeriza-

tion method of the natural resin has an immense effect on long term preservation. No other fossils of this age, as far as we know, show this type of ultrastructural and amino acid integrity.

Degradation of tissues post mortem is not well understood, but the process is considered to consist of the following stages[22]. The first stage (apart from autolysis of various components within the organism) is degradation of tissues by microbial attack. Bacteria in soil contain enzymes that can actively degrade the molecular components of a cell very effectively. In cases where bacterial degradation is slowed or halted (e.g. frozen, acidic bogs) the next degradative process is presumably hydrolysis, so tissues that are perfused by water are most susceptible to degradation and permineralization after a few thousand years. Tissues that can dehydrate quickly and remain that way are the most likely to 'survive' extended periods of time. Theoretically, amber would seem an ideal environment for fossil storage.

The degree of preservation seen in these leaflets, and in amber tissues in general, may therefore be attributed to the following factors: 1) rapid burial of the organism in the natural resin, thereby stopping microbial and enzymatic degradation[23]; 2) inert dehydration as a result of the high sugar and alcohol content of the natural resin; 3) protein integrity through fixation by aldehydes[10] and possibly sugars in the resin. Sugars in natural resin, such as glucose, could interact directly with proteins and other molecular components through non-enzymatic glycosylation, which has been shown in vitro[24,25].

The exact components of the *Hymenaea* resin and the chemistry involved in long term preservation are not completely known at the moment, but it has been shown that in plant tissues 30 to 40 million years old preservation follows a characteristic pattern, with chloroplasts and cell walls being the most frequently preserved structures, followed by nuclei and mitochondria. The tissues shown here, although twice as old as the Clarkia fossils, show better ultrastructural integrity. This would indicate that mode of preservation rather than age is important in long term conservation. The present study therefore shows that plant material preserved in amber can retain intracellular details that are normally lost in other types of fossilization.

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