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LEBANESE AMBER : EXTINCT ZOOLOGICAL & BOTANICAL LIFE

By RAIF K. MILKI

Management Educational and Health Services Master Plan

November 1998
Beirut, Lebanon

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دراسة حول الحياة البيولوجية و النباتية في العنبر اللبناني

ملخص

العنبر اللبناني ، و هو أقدم و أغنى عنبر في العالم لما يحتويه من حيوانات و نباتات منقرضة ، يرجع عمره إلى 120 – 135 مليون سنة ، و ينحصر وجوده في لبنان بطبقة جيولوجية معروفة بالطبقة الطباشيرية أو الطبقة الكريتاسية . و يوجد 27 نوع من العنبر اللبناني يتراوح بين الأصفر الشفاف و الأحمر المعتم .

العنبر هو مادة صمغية تفرزها أشجار صنوبرية كانت تعيش منذ 120 – 136 مليون سنة و قد إنقرضت كليا منذ ذاك الوقت . هذه المادة الصمغية تمر بمراحل كيميائية و بيولوجية و فيزيائية، و مع الزمن تتأكسد و تتبخر منها مواد قابلة للتبخر مثل التربنتين و تتحجر لتصبح عنبرا . هذه المادة في مراحلها الأولى تكون لزجة تلتقط كل ما تلامسه من نباتات و حشرات ، فتغلفها و تحفظها دون أي تغيير .

العنبر اللبناني وجد في أربع مناطق من لبنان و هي جزين ، ضهر البيدر ، فالوغا ، و ميروبا . و قد أرسلت عينات منه إلى إنكلترا و الولايات المتحدة الأميركية . و من الدراسات الأولية ، تم كشف أهمية العنبر اللبناني من ناحية عمره و المنقرضات المحفوظة بداخله . و قد دعي الأستاذ رئيف ملكي إلى جامعة كاليفورنيا في بيركلي و جامعة أوريغون لدراسة عينات من العنبر اللبناني التي إصطحبها معه . و هناك ، تم تصنيف مئات من الحشرات المنقرضة . و قد كرم الأستاذ ملكي في الولايات المتحدة الأميركية حيث سميت إحدى المنقرضات على إسم لبنان و إسمه – ليبانوبايثوس ملكي . بعد ذلك نشرت الدراسة في أشهر المجلات العلمية كما تم جلب شجرة العنبر اللبناني لزرعها في لبنان . بالإضافة إلى ذلك ، تم دراسة إحدى المنقرضات في ألمانيا و سميت أيضا على إسم لبنان – ليبانانسيس – و نشرت الدراسة أيضا في مجلة علمية متخصصة بالمنقرضات .

و يواصل الأستاذ ملكي زياراته إلى الولايات المتحدة الأميركية لتوسيع الدراسة التي تشمل P.C.R. و هي آلة متخصصة لتكثيف و تنظيم الجينات الوراثية الموجودة في الدم المتجمد داخل الحشرات الماصة للدماء التي قد تكون حصلت على آخر وجبة طعام لها من دم الديناصور .

و سوف تشمل الدراسات اللاحقة النباتات و الهواء الموجودين داخل العنبر اللبناني و الذي سيعطي صورة واضحة عن البيئة في لبنان منذ ملايين السنين .

OUTLINE

A. Introduction - Lebanese Amber
(Appendix I & II)

B. Completion of the Classification of Amber Specimens, Preliminary Prepared in Lebanon, and in Cooperation with Oregon University
(Appendix III & V)

C. Preservation and Presentation of Samples in Bio-Plastic Media

D. Amber Specimens : Methods of Genetic Sequencing

E. Extinct Amber Tree in Lebanon for Re-Propagation

F. Samples of Amber Stamps
(Polish Amber, 30 Million Years)

G. Outline of Proposed Book on Lebanese Amber
(Appendix IV)

H. Future Plans

A. INTRODUCTION - LEBANESE AMBER (Appendix I & II)

WHAT IS AMBER ?

Amber, a gemstone sought after by ancient Stone Age sun worshippers because its beautiful radiance resembled the sun's rays, well deserves the title "golden gem of the ages". In the civilization of the early Greeks and Romans, amber was so reserved so it was available only to nobility. Ladies of the Roman court desired it for the protection from evil. Throughout Europe, amber was worn as protection against illness. Ancient man and later civilizations treasured amber as highly as gold. During the 1920's, amber was second to diamonds in the United States. Throughout these early periods and civilizations, little was known about amber and its origin until the age of science brought proof that it originated from sticky resin which flowed from prehistoric trees. Few gems match amber in respect to its mode of creation, the depth of its history, and scientific information science can withdraw from it. Over the centuries, amber primarily came into the hands of man from the seashores near the Baltic Sea.

During the 1800's, scientists began studying the insects and other faunae and flora entrapped in amber resin. Since then, research on amber has focused on the study of fossil inclusions, and the genetic relationships between the ancient fossilized resins and those being produced today by living trees.

SCIENTIFIC STUDIES

Sophisticated methods and techniques, such as Infrared Spectroscopy, X-ray diffraction, mass spectroscopy, and gas-liquid chromatography are being used today. The value of amber was also the concern and interest of paleobotanists, paleozoologists, geologists, archeologists, and anthropologists.

COLLECTION (See Photos I, II, III)

Amber is often collected directly from the waters of the Baltic Sea since it is just buoyaret enough to float in salt water. Such floating amber is already semi-polished because of the action of waves and beach sands. In contrast, mined amber or the amber which is removed from soil in its raw stage has to be carefully polished to

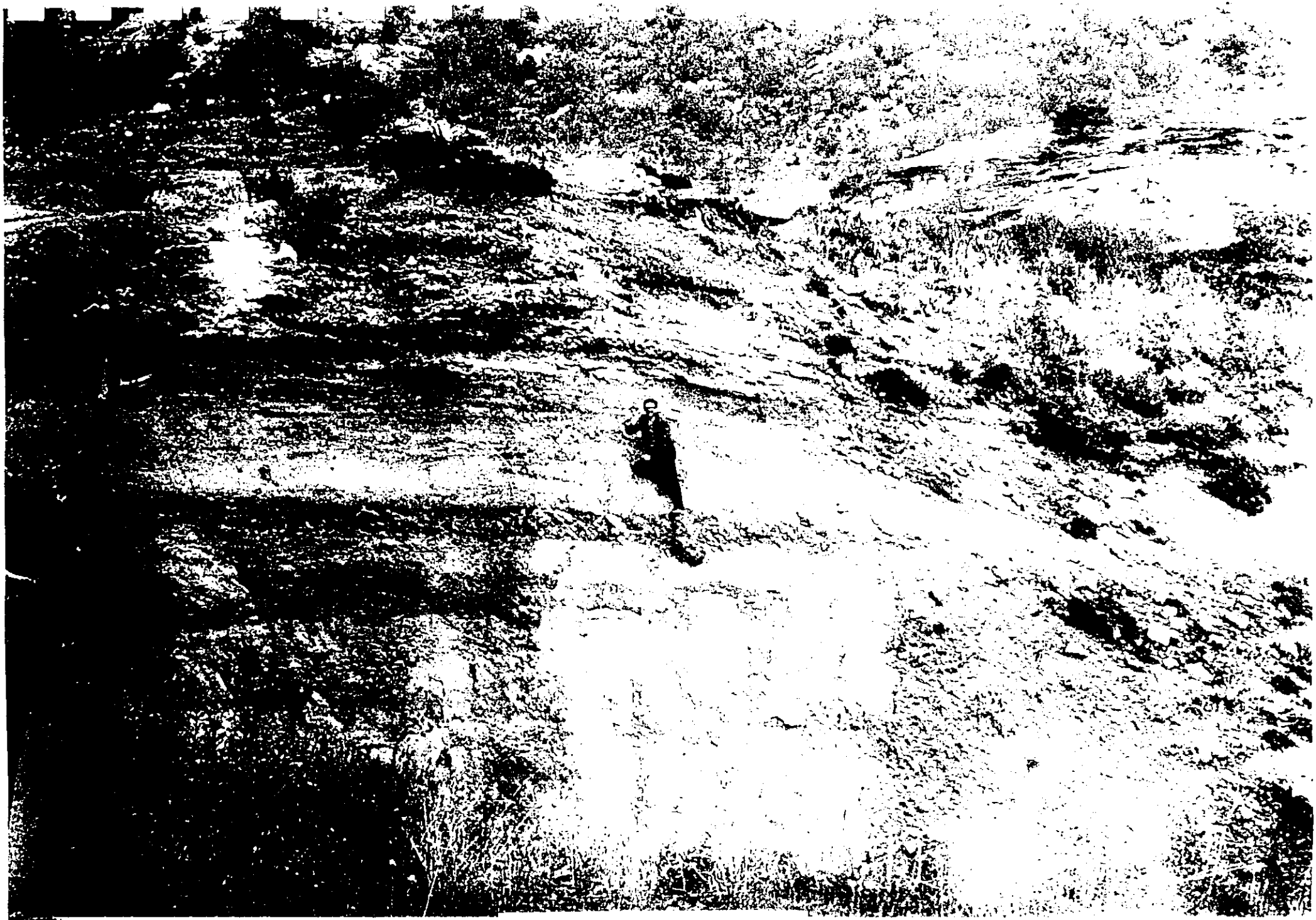


Photo 1 Amber Location

Photo II Amber Collection

2c Photo III Amber Location

remove the brown layers and make it available for microscopic study to discover the quality of material within; and this is what is being done on the Lebanese amber.

VARIETIES AND CHARACTERISTICS

Although there are about 250 colored varieties of amber which are known, the most common colors are yellow, orange, and dark brown. The Lebanese amber which covers the above mentioned three varieties, includes also the red amber and many other color varieties ranging from the yellow transparent to the dark red. When amber is burned, a smoke with pleasant resinous odor is being produced. If amber is rubbed vigorously, the friction produces electrical charge to attract light objects. The word electricity was actually derived from "elektron", the Greek name for amber. Modern studies of the botanical origin of the resins also use succinic acid content for identifying amber varieties. Based on its presence, amber resins have been placed in two classes : the succintes; those containing succinic acid, and the retinites; those which do not contain succinic acid.

LOCATION

Recent studies have also shown that the natural formation of amber (production by resinous prehistoric trees) was similar in all regions which applies to all Baltic amber - amber from the Baltic area which comprises today Denmark, Sweden, Northern Germany, Poland, and Russia.

Small quantities of amber have been found elsewhere like Sicily, Romania, China, Burma, Thailand, Japan, Canada, Mexico, and USA. In addition to color varieties, Lebanese amber can be classified as clear or transparent, and cloudy. The transparent varieties are the ones rich in insect inclusions in which you can see details of insect morphology like the compound eyes, wing venation, appendages, hairs, reproductive organs.

ORIGIN

Although paleobotanists are not in agreement as to the exact species of trees from which the resin flowed which formed the amber, recent scientific studies indicate

the probable existence of 20 to 40 varieties of coniferous trees all of which produce resins from which amber is the fossilized product. It has been found, however, that one tree similar to our Arborvitae or White Cedar (Thuja occidentales) flourished in greater abundance than other conifers; this tree was the major producer of resins which later formed Baltic amber.

FOSSILIZATION

The fossilization process of amber is greatly different from that which converts wood into pertified wood whereby the woody cells have been replaced wholly or in part by mineral substances. While amber retains basically the same organic substances present in the original resin. The volatile compounds which imparted stickiness to the resin escaped and evaporated so slowly. This slow process prevented the resin from cracking. During the lengthy period which lead to fossilization, molecules were forced to polymerize - rearrange themselves which helped the resin to solidify forming a compound with greater stability and hardness.

AMBER TRADE

The ancient Phoenicians a hardy commercial race, were probably the first sailors to rade amber among the Mediterranean countries as well as to pioneer routes to the Atlantic shores of Northern Europe to obtain amber and exchange it for bronze between the thirteenth and sixth centuries BC. The Phoenicians were clever traders and fabricated tales of sea monsters and other dangers encountered on their sea voyages. In modern times, there has been many tricks being applied whereby false substances are being sold on the basis they are amber. The semi-fossilized resin known as Copal is being sold on the basis they are amber. Amberoid which is the synthetic amber made by heating small pieces of amber under pressure is sold to be amber.

B. COMPLETION OF THE CLASSIFICATION OF AMBER SPECIMENS, PRELIMINARY PREPARED IN LEBANON, AND IN COOPERATION WITH OREGON UNIVERSITY

(Appendix III & V)

NAMES OF AMBER

1. Succinum by Romans
2. Elektron by Greeks
3. Bernstein (burning stone) by Germans
4. Amber, an English word derived from Anber
5. Anber, the Arabic Name; also Kuhruman, Kuhruba, Kawrab, are Arabic names; sandarus is the name in certain villages in South Lebanon - in connection with Sandarac tree that exudes resin.
6. Ambre Jaune (fossilized resin), Ambre gris (product of spermwhale), French names.
7. Yantar, Russian name.

LEBANESE AMBER

The Lebanese amber beds are located in four areas in Lebanon namely Jezzine, Dahr Al-Baydar, Ain Al-Suhha (Falougha), and Meyrouba - Hrajel. Geologically, all the areas belong to the lower or early cretaceous period, and Lebanese amber has the distinction of being the oldest extensive fossiliferous amber known and was deposited some 136-120 million years ago. This period overlaps with the period of the dinosaurs. This implies that any blood sucking insect being preserved in Lebanese amber could have had their last meal on dinosaurs' blood.

Only recently early 1990's, Lebanese amber started withdrawing the attention of scientists in USA, Canada, England, and Germany - being the oldest amber and the richest in animal and plant inclusions.

AMBER COLLECTION (See Photo IV)

Collection of amber from specified areas takes place during winter season while the soil is wet. One of the characteristics of lower cretaceous soil is that when it is dry, it is so solid, that you can not dig in it, and if you dig you may expose the buried pieces of amber for breakage. When the soil is wet, you can scrape or dig with the least damage to amber pieces. If the area has slopes, the biggest amount of amber can be gathered during the first visit after heavy rain as a result of soil erosion which exposes the buried pieces of amber to the top soil surface. While digging through the soil, a lot

5a Photo IV Amber Embedded in Rocks

of attention is given to the technique of digging using soft tools like long knife or screw driver. If a piece of amber appears, digging should occur around it to permit the removal of the whole piece and to avoid breakage. In one location only, petrified wood was found with the amber. Also, you can spot a variety of colors in one location ranging from transparent yellow to dark red.

CLEANING

When the pieces are collected, they are covered with soil and require careful cleaning. These muddy amber pieces are soaked in water for 30 minutes and then the soil can be removed partly with fingers through soft rubbing. Since Lebanese amber is fragile, pressure on the pieces should be avoided. If a big piece is broken, all the fragments should be kept as one unit; this is important in later stages during microscopic studies while looking for symbiotic life. If there are cracks in the amber, and usually there are many wholes and cracks, these are filled with soil which can not be removed by soaking and finger rubbing; a soft toothbrush can be used applying a very light and soft technique moving the brush in the direction of the groove, the yellow transparent pieces are put aside for the first stage of microscopic study.

MICROSCOPIC STUDY

Before applying any polishing technique, the amber piece is studied under the microscope using dissecting microscope - low power magnification. During this phase, it is not difficult to detect any plant or animal inclusion entombed in amber pieces. When the object is detected, whether insect or plant, orientation of the piece is important so that when you want to polish you will be aiming to have the polished surface area exposes most of the insect or plant detected earlier.

POLISHING

After orientation of the piece of amber, polishing technique becomes the most critical and dangerous phase during which the amber piece may break or be damaged. A variety of emery papers of different grades are being used, the finest is 400 softness.

Starting with the coarse emery papers, it is recommended to conduct the polishing with running water, because this is the phase when the hidden soil, sand gets exposed and causes damage during rubbing; this danger is reduced to a minimum if it is done with running water. It is also important to do the rubbing in one orientation from left to right to left and not in a circular manner. Then, you shift to the second finer emery paper but at different intervals you should check how deep you went through to avoid touching the inclusions detected earlier. When you reach the last stage of polishing, the fine powder of amber appearing as a result of rubbing, helps in creating a very smooth surface of the amber. Even after this stage, there are certain linings appearing under the microscope. These can be removed by using polishing cream (liquid polish Al_2O_3) which is applied on a piece of cloth and then, rubbing the piece against the cream several times creates a very smooth surface that allows easy microscopic study as well as good photo-microscopy.

MICROSCOPIC STUDY AND IDENTIFICATION

During microscopic study, a drop of glycerine or oil on the surface will create better vision. Playing with the side lamps to allow proper angle penetration of light will improve the vision also. The study of the inclusion can be done on low, medium, or high power magnification using different color backgrounds; white, yellow, blue, or green which will also improve seeing the parts better.

All these stages, in addition to the preliminary identification of insects, are done in Lebanon where the orders of insects are identified then photo-microscopy is conducted after which the piece of amber is ready to be taken to USA using a very safe packaging procedure. The piece is given an identification number.

C. PRESERVATION AND PRESENTATIOM OF SAMPLES IN BIO - PLASTIC MEDIA

BIO-PLASTIC EMBEDDING

Some of the amber pieces appear to be very fragile with so many fissures, cracks, or the insect or plant specimen is very near the amber surface due to polishing. In these conditions, the specimens are embedded in Bio-plastic medium for protection. This embedding facilitates handling the amber pieces in different directions during detailed classifications without exposing the piece to breakage. Embedding in Bio-plastic not only allows easy handling of the amber piece safely, but also gives better photographs. The procedure to be summarized here is the result of so many experiments and trials conducted at University of California, Berkley and at the American University of Beirut, and after so many errors and bad results. The best results of embedding can be summarized as follows.

The amber piece after final polishing with liquid polish (cream), is washed carefully and thoroughly with water to remove all liquid polish which is on the surface or may have penetrated some cracks; a soft tooth brush may be used observing the cleaning procedure under the dissecting microscope. After cleaning, the specimens have to be dried completely from water. Then, the specimen is dipped in the Bio-plastic without the catalyst so as to keep the Bio-plastic in the liquid form. This will allow the Bio-plastic to penetrate and fill all the empty gaps, cuts, fissures, and cracks which usually hinder the proper light penetration thus forming an obstacle during detailed classification and photo-microscopy. There are ready-made magnifying boxes of two sizes and two magnifications. These boxes are used as a container and magnifier for embedding. Depending upon the size of the amber piece, the box size is selected. A needed volume of Bio-plastic liquid is taken into a beaker, and according to the volume, a specified volume of a catalyst is added. The function of the catalyst is to undergo a chemical reaction with the liquid Bio-plastic, changing it slowly from the liquid form to the solid form. The Bio-plastic and the catalyst are mixed slowly with a glass rod to avoid air bubble formation as much as possible. The mixed medium is poured into the magnifying boxes to cover 2 cm of the base. This layer will be the basic layer on which the amber piece will be anchored in a selected orientation. This layer is covered in a way to allow ventilation and to prevent dust from falling on the surface.

When the layer starts solidifying, usually 2-3 hours, the piece of amber is taken from the Bio-plastic medium and laid on the surface of the solid Bio-plastic layer.

Orientation of the specimen, to allow the maximum desired part of the insect appearing, is best done under the microscope. The piece is then left for another 1-2 hours to be stuck well to the base and another newly prepared volume of catalyzed Bio-plastic is poured over the amber piece to cover it completely. All this procedure should be done slowly to avoid the formation of air bubbles. This is left covered overnight for complete solidification. Then, the magnifying cover is fixed and the specimen is ready for final classification and photo-microscopy.

D. AMBER SPECIMENS : METHODS OF GENETIC SEQUENCING

(Appendix VI)

JOINT RESEARCH PROGRAM : POINAR - MILKI

The joint research program with Dr. George Poinar, University of California (U.C.), Berkley, started in the early 1990's. Milki has paid two visits to U.C. Berkley in September 1993 and September 1994. During these visits, an outline of future work was finalized which included :

1. Starting joint study with Oregon State University, Corvallis which Dr. Poinar already made contacts with.
2. New publication on an extinct insect which was called after Lebanon and Milki (*Lebanobythus milkii*).
3. Send samples to Leipzik University, Germany for classification and genetic study.
4. During the two visits of Milki, he was exposed to the P.C.R. machine (Polymerase Chain Reactor) whose function is to amplify and sequence the genes of inclusions present in amber (plant or animal).
5. Send samples to Canada - contacts have already been made.
6. During these two years of visits, several hundreds of insects entombed in amber were classified, some finally and others need specialists in the specific order to reach the genus and species.
7. In 1994, Milki was honored in California (See Photo V) and an insect was named *Lebanobythus milkii* and the publication was sent to a publishing company (See Appendix I & Photo VI).
8. Another extinct insect which was sent to Germany was identified and called *Cretaceomachilis libanenthis*, then an article about the issue was published with acknowledgments to Milki (Appendix II).

In September 1995, Milki visited Oregon State University for similar purposes, and in 1996 a new outline of joint work was set up and includes (Appendix III) :

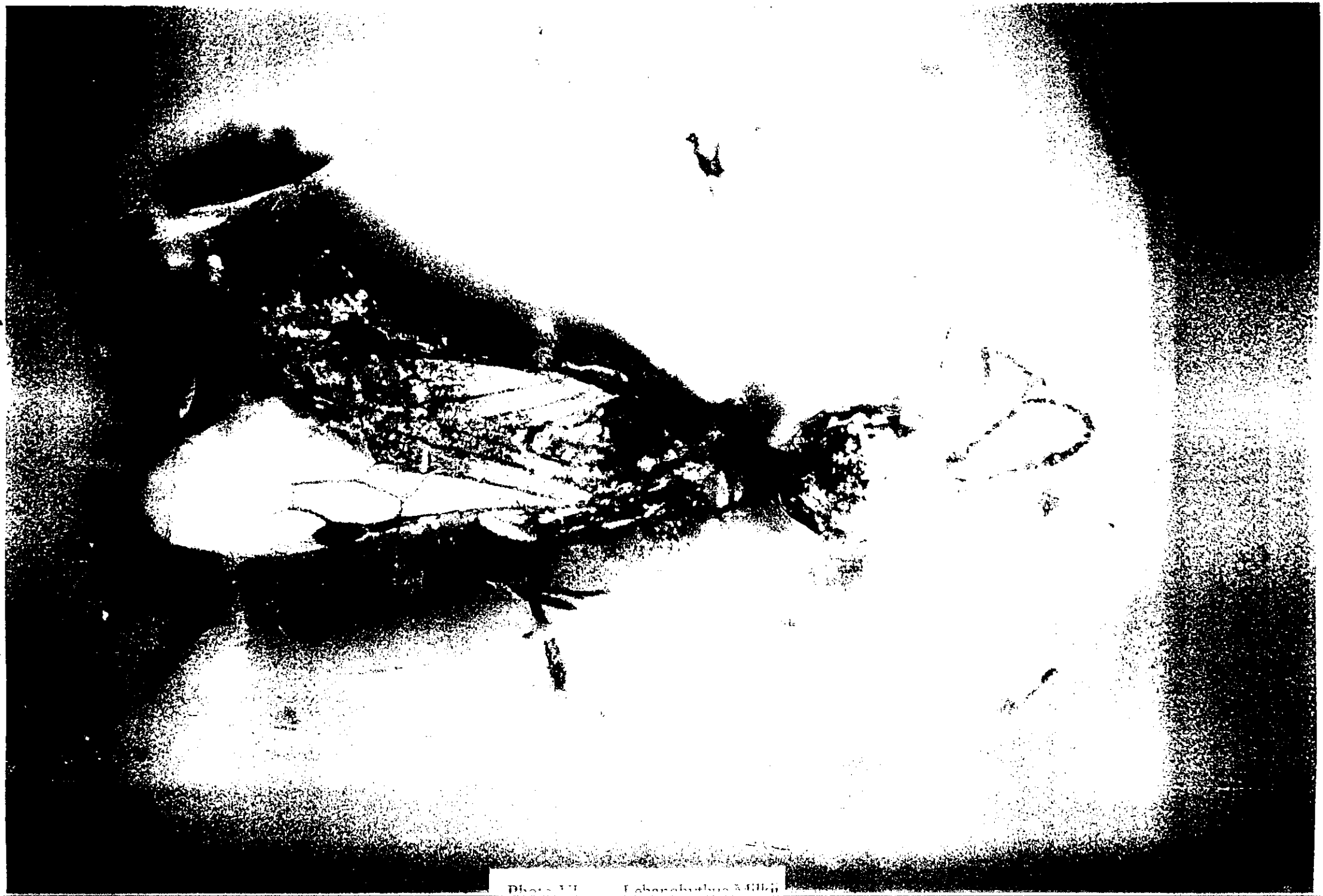
1. Identification of amber fossils in the Milki Collection (Appendix IV).
2. Description of amber sites in Lebanon.
3. Preparation of a book on this subject.
4. Preparation of drawings and photographs to illustrate the book.
5. Discussion of DNA studies with material from the Milki Collection.

In October 1998, the visit of Milki to Corvallis, USA was very fruitful since he brought with him the following :

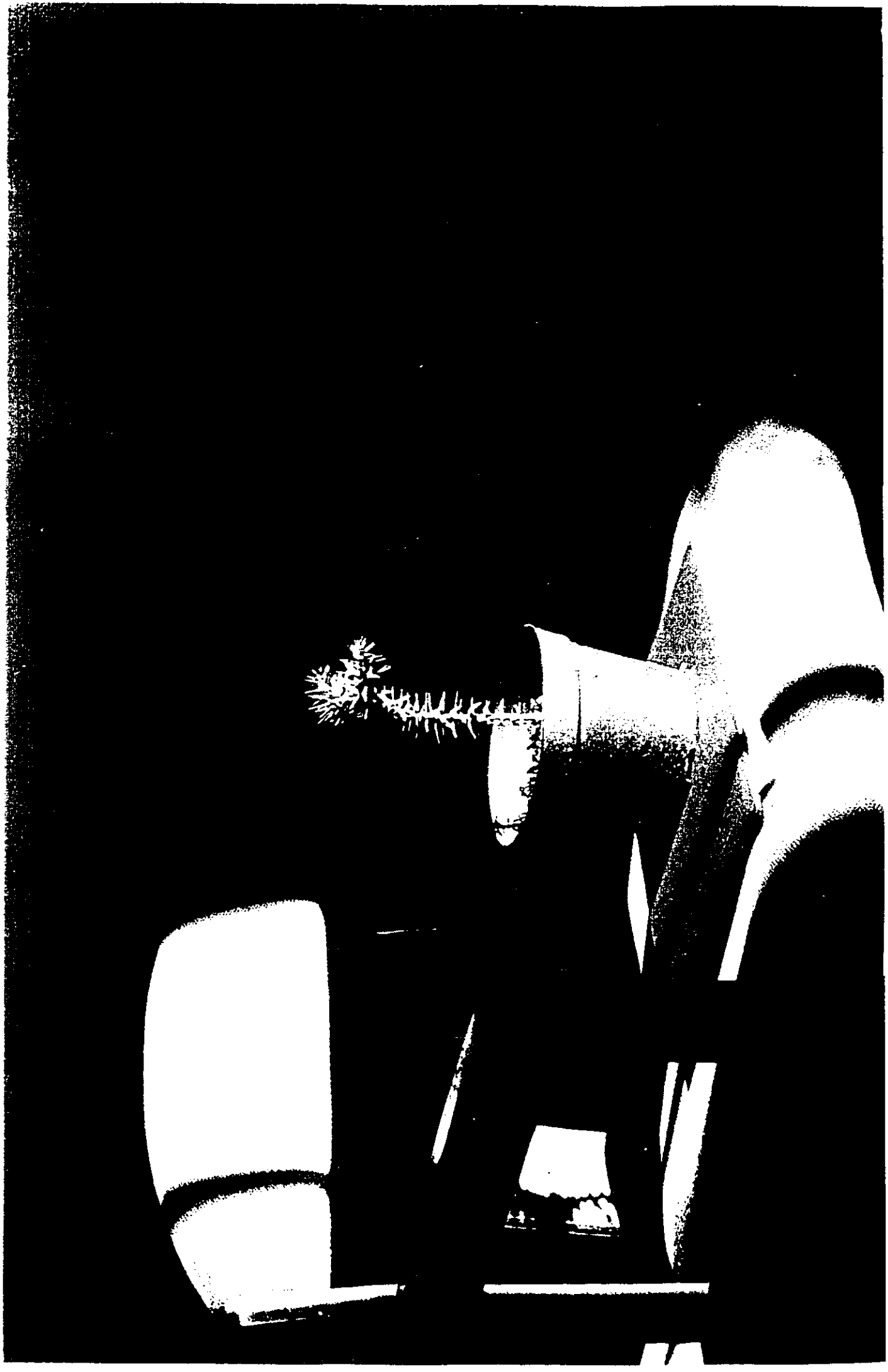
1. An amber tree, Areaucariaceae, Agathis (Kauri pine tree) to be propagated in Lebanon (See Photos VII & VIII).
2. Amber seeds.
3. Polish stamps (See Photos IX & X).
4. Copies of the publications.
5. Outline of the book (Appendix V).

Note : Photos XI to XVI are some of the identified insects embedded in Lebanese Amber.

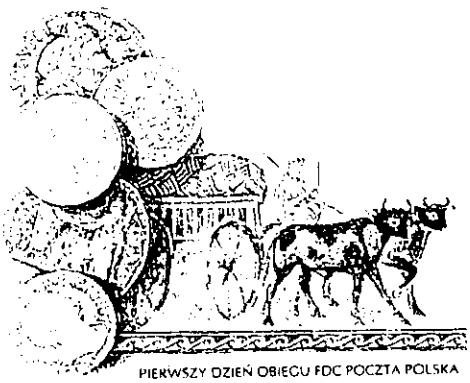
Photo V Honoring Milki by Poiner & Prentise In U.C. Berkley



E. EXTINCT AMBER TREE IN LEBANON FOR RE - PROPAGATION



F. SAMPLES OF AMBER STAMPS (Polish Amber, 30 Million Years)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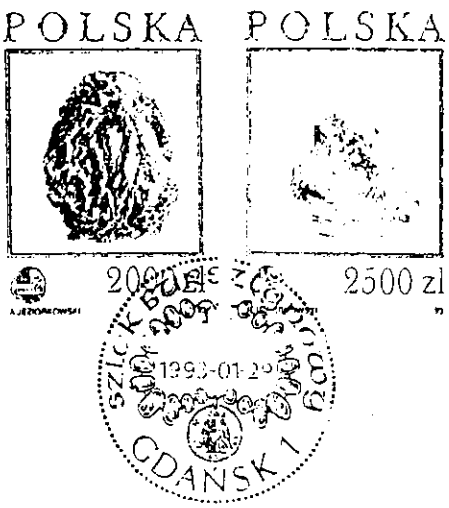
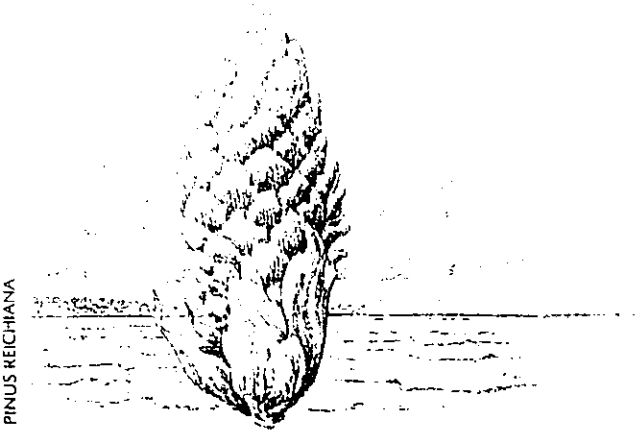
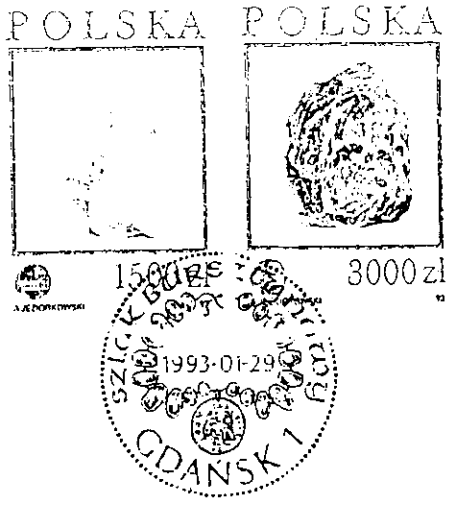




PINUS KEICHIANA

PIERWSZY DZIEŃ OBIEGU FDC POCZTA POLSKA



G. OUTLINE OF PROPOSED BOOK ON LEBANESE AMBER
(Appendix IV)

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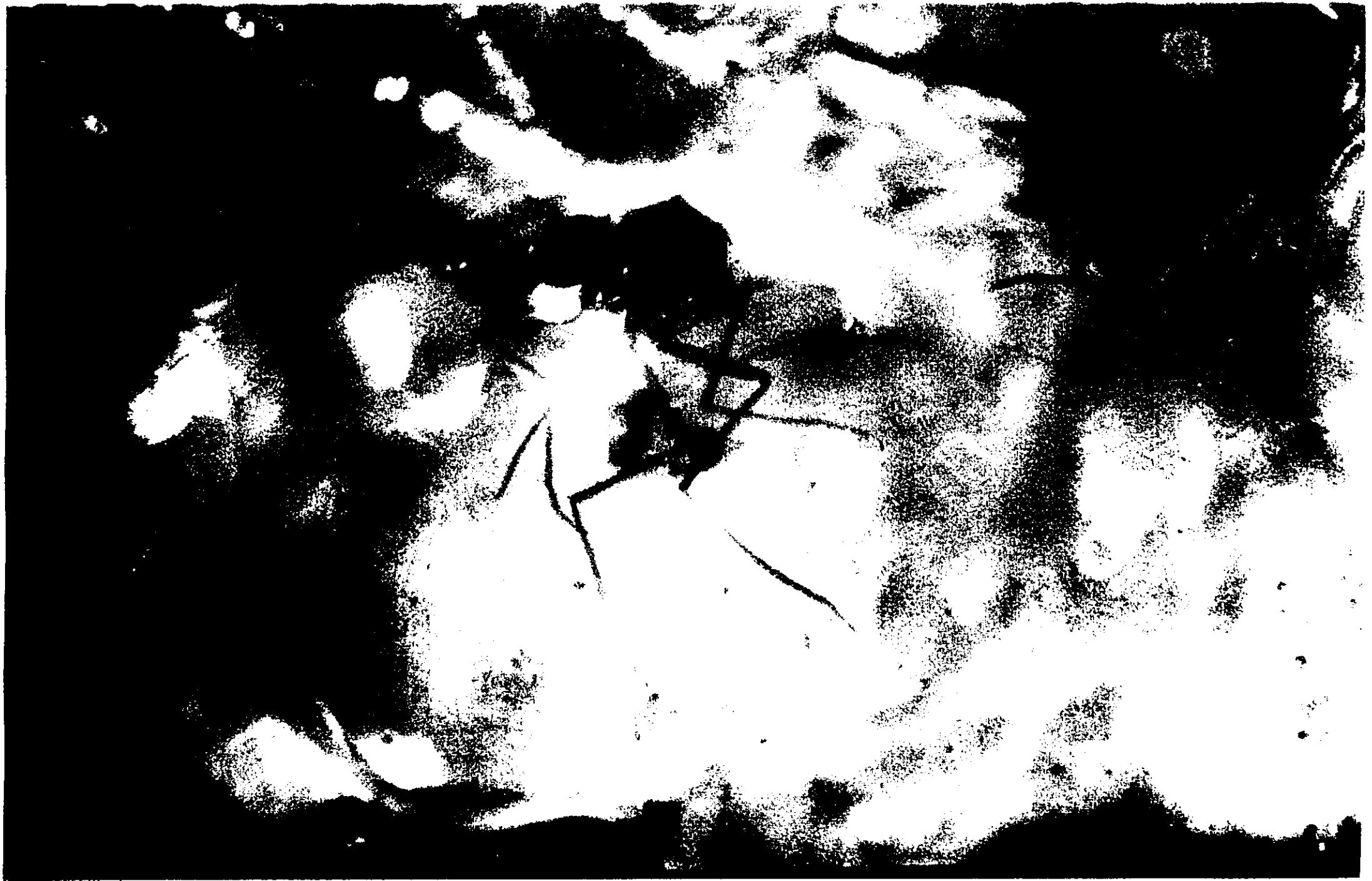

21 Photo XV Air Bubbles & Pollen in Lebanese Amber

H. FUTURE PLANS

FUTURE PLANS

It was agreed that more samples will be delivered to Dr. George Poinar for classification and P.C.R. studies. This will lead to more publications on Lebanese extinct species. More emphasis shall be given to botanical studies; samples of which have not yet been studied.

Studies shall be conducted on the recent technique of Amino Acid Racemization. The relationship of DNA and Amino Acid Racemization suggests that Amino Acid Racemization may be useful in assessing the extent of DNA chain breakage in ancient biological remains. The Zoological and Botanical studies, in addition to the studies sound and spore inclusions plus air bubbles will help to develop a clear picture about the Lebanese environment that dominated 136 million years ago.

Now that we have reached a stage at which we obtained strands of DNA from amber insects and have determined that amber is the best preserver of ancient protein, could we eventually bring back extinct life ? At present, we have to be content with bringing back segments of DNA which is just exciting in itself.

As science advances, it is possible to repair some of the DNA that exists in amber, and may be one day be able to tell a body cell to start acting like a fertilized egg and begin embryonic development all over again.

In conclusion, we can say with confidence that the Lebanese amber, which is the oldest and richest in the world, is an ecological museum for science and scientists all over the world.

APPENDICES

APPENDIX I

31 October 1996

PROC. ENTOMOL. SOC. WASH.
98(4), 1996, pp. 802–811

FOSSIL SCOLEBYTHIDS (HYMENOPTERA: SCOLEBYTHIDAE) FROM LEBANESE AND DOMINICAN AMBER

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Abstract.—The first two fossil species of Scolebythidae are described: **Libanobythus milkii,** n. gen., n. sp., from Lebanese amber of Early Cretaceous, and **Dominibythus inopinatus,** n. gen. n. sp., from Dominican amber of Late Eocene to Late Oligocene. The relationships of these tax to extant scolebythids are discussed.

The family Scolebythidae is a morphologically unusual and very rarely encountered group of chrysidoid wasps that has been only recently recognized (Evans 1963). The family may be immediately identified by its unique apomorphies that include a remarkably modified propectus. In the extant fauna, it is represented by three species, viz. *Clystopsenella longiventris* Kieffer, *Scolebythus madecassus* Evans, and *Ycaploca evansi* Nagy. The fossil *Cretabythus sibiricus* Evans was tentatively assigned to this family by Evans (1973), but is apparently not closely related to Scolebythidae (Rasnitsyn 1988). We describe below the first unquestionable fossil scolebythids, *Libanobythus milkii* gen. et sp. n. from Lebanese amber of Early Cretaceous age (135–120 Ma) and *Dominibythus inopinatus* gen. et sp. n. from Dominican amber of Late Eocene to Late Oligocene age (40–15 Ma). Considering the rarity of the family today, its occurrence in both ambers is noteworthy, particularly its presence in the Early Cretaceous.

MATERIALS AND METHODS

Morphological terms used here are those in general use in aculeate systematics (e.g. Michener 1944, Richards 1977). The terminology and modified drawing conventions of Mason (1986) for stages of venational reduction are used. For brevity, the seven metasomal tergites and eight sternites are referred to as TI–TVII and SI–SVIII, respectively. All illustrations, except those of the male genitalia, were made with a camera lucida from specimens submerged in safflower oil to minimise optical distortion. The male genitalia of *Dominibythus inopinatus* were illustrated with an ocular grid at 160× magnification. The illustrations were refined using the Adobe Illustrator computer program (Adobe Systems Inc.).

The piece of Lebanese amber containing the holotype of *Libanobythus milkii*, like most Lebanese amber, was very fragile and contained many internal fractures before preparation. To strengthen the amber for polishing and to improve the clarity of the piece, the specimen was embedded in Ward's bioplastic. After embedding, the piece was polished down by hand as nearly as possible to the level of the fossil to improve the visibility of the wasp. The piece was then re-embedded in plastic to cover exposed portions of amber. Because of the clarity of the Dominican amber containing the holotype of *D. inopinatus*, this speci-

men was not embedded in plastic for study. [For permanent preservation of amber fossils, however, it is recommended that they be mounted in some type of clear medium to protect them from eventual oxidation that would otherwise tend to cause discoloration and cracking (Poinar 1992).] After polishing, the pieces were studied in safflower oil.

Age of the Fossils

The Lebanese amber specimen of *L. milkii* came from beds located near the mountainous villages of Jezzine and Dar al-Baidha. These beds occur in primary deposits of the Neocomian Epoch of the Early Cretaceous as well as in secondary deposits of the Barremian and Aptian stages. These deposits are dated from 120 to 135 million years old (Schlee and Dietrich 1970, Schlee and Glockner 1978).

The Dominican amber specimen of *D. inopinatus* originated from mines located in the Cordillera Septentrional, between Santiago and Puerto Plata, in the Northern Portion of the Dominican Republic. These mines are in the Altamira facies of the El Mamey Formation (Upper Eocene), which is shale sandstone interspersed with a conglomerate of well rounded pebbles (Eberle et al. 1980). Amber from these mines have been estimated by Lambert et al. (1985) to range from 15 to 40 million years old.

Characters of the Family

The fossils may be recognized as Scolebythidae by their enlarged and exposed diamond-shaped prosternum and posteriorly produced fore coxae bearing the fore trochanters laterally (Figs. 3, 6). These propectal characters are unique apomorphies of Scolebythidae (Brothers 1975, Carpenter 1986) and provide the strongest evidence for the family-group identities of the fossils. Both species also have most other apomorphies of the family listed by Brothers (1975) including a small, transverse clypeus, reduced metanotum (only visible in *D. inopinatus*), enlarged femora, and a complete lack of middle tibial spines. They also possess short, broad mandibles and venation that may be readily derived from the typical scolebythid pattern (Figs. 5, 7). Like extant scolebythids, both fossil forms have a convex and smoothly rounded first metasomal sternite that does not form an articulation with the second metasomal sternite and have a noticeably concave anterior surface on the first metasomal tergite. Like extant female scolebythids, the female of *L. milkii* possesses enlarged genae. Loss of the pronotal collar, which is another apomorphy of the family listed both by Brothers (1975) and Carpenter (1986), is present in *D. inopinatus* but not *L. milkii*, which has a less declivous and more elongate pronotum. Given this list of features, there can be no other taxonomic placement for the fossils.

As members of Chrysidoidea, the fossils possess the standard features of the superfamily including reduced forewing venation, lack of any closed cells in the hind wing (true of all Chrysidoidea except Plumariidae), 13-segmented female antennae and no jugal lobe.

Key to Scolebythid Genera

1. R1 vein of forewing complete and fusing with Rs vein apically . . . . . extant genera, see Nagy (1975) for key
– R1 vein of forewing absent, not fusing with Rs vein apically (Figs. 5, 7) . . . . . extinct genera, 2
2. Discoidal cell defined by tubular veins, pronotum longer than scutum dorsally (Fig. 5) . . . . . *Libanobythus*, new genus
– Discoidal cell absent (Fig. 7), pronotum markedly shorter than scutum dorsally (Fig. 4) . . . . . *Dominibythus*, new genus

Dominibythus Prentice and Poinar, new genus

Type and only known species: *Dominibythus inopinatus*, new species.

Generic characters.—Frons elevated between antennal sockets, with carina arising at medial margin of antennal socket and extending outward dorsally creating two slightly depressed lateral areas that receive antennal scapes (Fig. 8). Orbits converging ventrally (Fig. 8). Ocelli positioned slightly

Figs. 1–2. 1, *Dominibythus inopinatus*, lateral view of left side. 2, *Libanobythus milkii*, dorsal view. Bars = 0.5 mm.

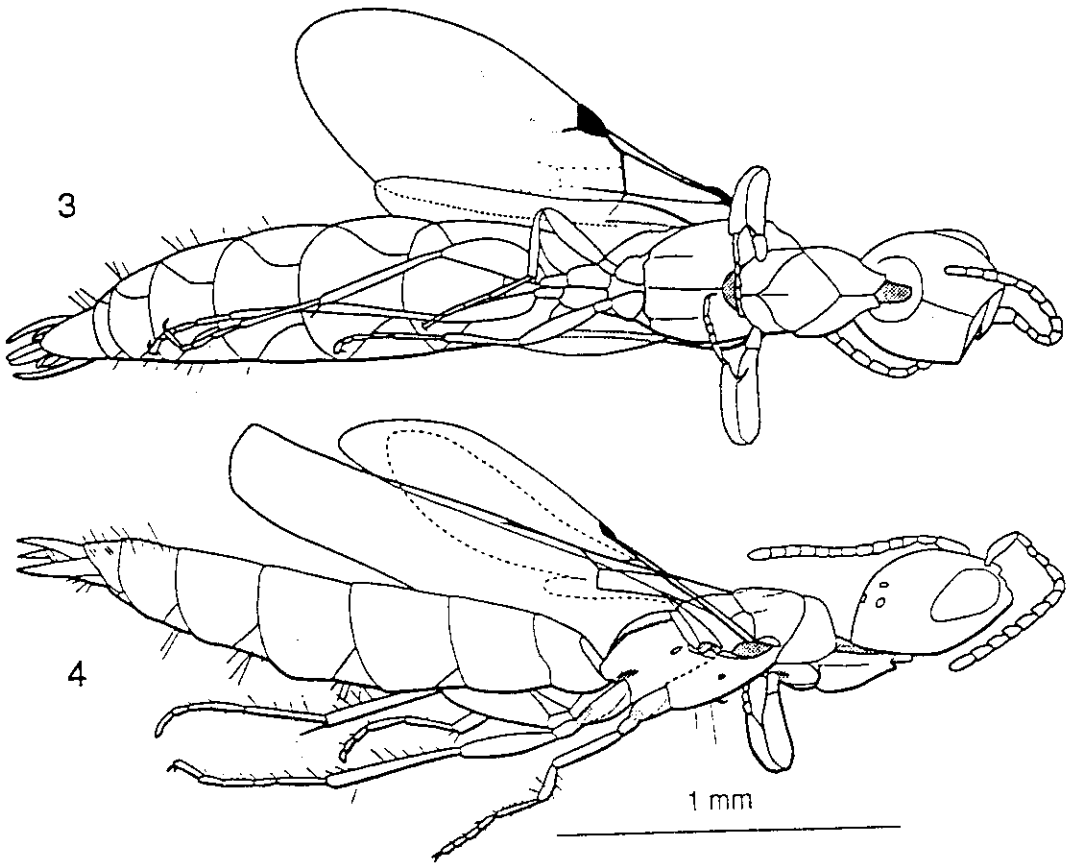
anterad of imaginary line connecting postero-dorsal margin of eyes. Clypeus conspicuously transverse, produced ventrally into a lobe that is about as wide as distance between outer margins of antennal sockets. Length of malar space equal to 0.5× basal mandibular width. Occipital carina present, at least ventrally, with gular sulcus extending along midline. Antenna arising very low on face, 13-segmented. Pronotum dorsally short, its length 0.2× length of mesoscutum, sharply declivous anteriorly; posterolateral lobes extending to tegulae. Propleuron well developed, anteriorly forming a neck separating head from pronotum, exposed dorsally. Prosternum large, diamond-shaped. Parapsidal lines long, positioned near lateral margins of mesoscutum. Notauli present only anteriorly. Mesoscutellum produced posteriorly as narrow rim overlying anterior margin of metanotum. Mesopleural signa comparatively long, extend-

ing from very near posterior margin of mesopleura half the distance to the antero-ventral margin of the mesopleura. Scrobe present and well-developed. Mesosternum simple, not produced as lamella over coxa. Metanotum short, its length 0.25× length of mesoscutellum. Propodeum with median longitudinal depression extending from anterior margin to near posterior rim, without evident metapostnotum dorsally. Tibial spur formula 1-1-1. Tarsal claws simple. Fore wing venation with R1 completely lacking; Rs+M as spectral vein; apical portion of Rs represented by an exceedingly light nebulous vein; first free abscissa of Cu nebulous, apically becoming spectral (Fig. 7); stigma conspicuous, slightly convex on costal margin. Hind wing venation reduced to costal vein extending 0.3× length of wing and to very base of anal vein. Jugal lobe absent. Metasoma with no constriction between first and second segments. Metasomal tergites dorsally comparatively flat in cross-section, sternites somewhat convex in cross-section; male with seven exposed tergites and eight exposed sternites. First metasomal tergite with a well formed depression on the anterior face fitting the posterior portion of the propodeum. First metasomal sternite evenly convex apically, not forming an articulation with SII.

Derivation of name.—The name *Dominibythus* is a combination of Dominican (referring to the Dominican Republic) and Scolebythus.

Dominibythus inopinatus Prentice and Poinar, new species (Figs. 1, 3, 4, 7–9)

Male.—Length 2.8 mm. Color: Head, mesosoma and metasoma brown; legs, antennae, mouthparts and genitalia tan; wings clear, veins tan. Sculpture and vestiture: Head smooth, with microscopic punctures that are about 2 ocellar diameters apart, with no visible vestiture; mesosomal cuticle microscopically areolate, with a few erect setae on venter; metasoma with some sparse, erect setae, 1–2 mid antennal flagellomeres in length; most metasomal setae on more apical segments. Fore, mid, and especially hindtibiae bearing setae whose length nearly averages that of tarsomere II. Fore and hind wing bearing minute, evenly spaced setae on apical half. Structure: Head 0.45 mm from vertex to ventral margin of clypeus and 0.45 mm in maximal width. Frontal line present, extending from near anterior ocellus to between antennal sockets. Ocellar triangle slightly elevated; ocello-ocular distance slightly greater than width of ocellar triangle; distance between lateral ocelli 1.5× diameter of a lateral ocellus. Width of gena 0.5× width of eye in lateral view. Proboscidial fossa short and wide, width 6× length. Occipital carina separated from proboscidial fossa by approximately the length of 4 mid-flagellomeres. Scape somewhat flattened in cross-section, length, including radicle, approximately 2× greatest width. Length of pedicel 2× length of first flagellomere, slightly less than length of scape without radicle; length of flagellomere II approximately 1.5× length of flagellomere I; flagellomeres III–X approximately equivalent in length and slightly longer than flagellomere II; length of flagellomere XI 1.3× length of flagellomere X. Mandibles relatively short, subapical teeth not visible although ventral one well produced apically. Mesosoma 0.9 mm in length. Mesoscutum about as long as mesoscutellum; median longitudinal impression evident. Epicnemial sulcus apparently present below pronotal lobe and extending ventrally to midline near very anterior margin of mesopleura. Propodeal spiracle with well-developed operculum and placed very near propodeal anterior margin, completely on lateral propodeal face. Fore femur robust, length slightly less than 3× width, widest distally; length of hindfemur slightly more than 3× width. Fore tibia about as long as fore femur; mid and hind tibiae slightly longer than respective femora. Length of hindtibial spur about 0.5× length of hindbasitarsus. Fore tarsus about as long as fore tibia; mid and hind tarsi longer than



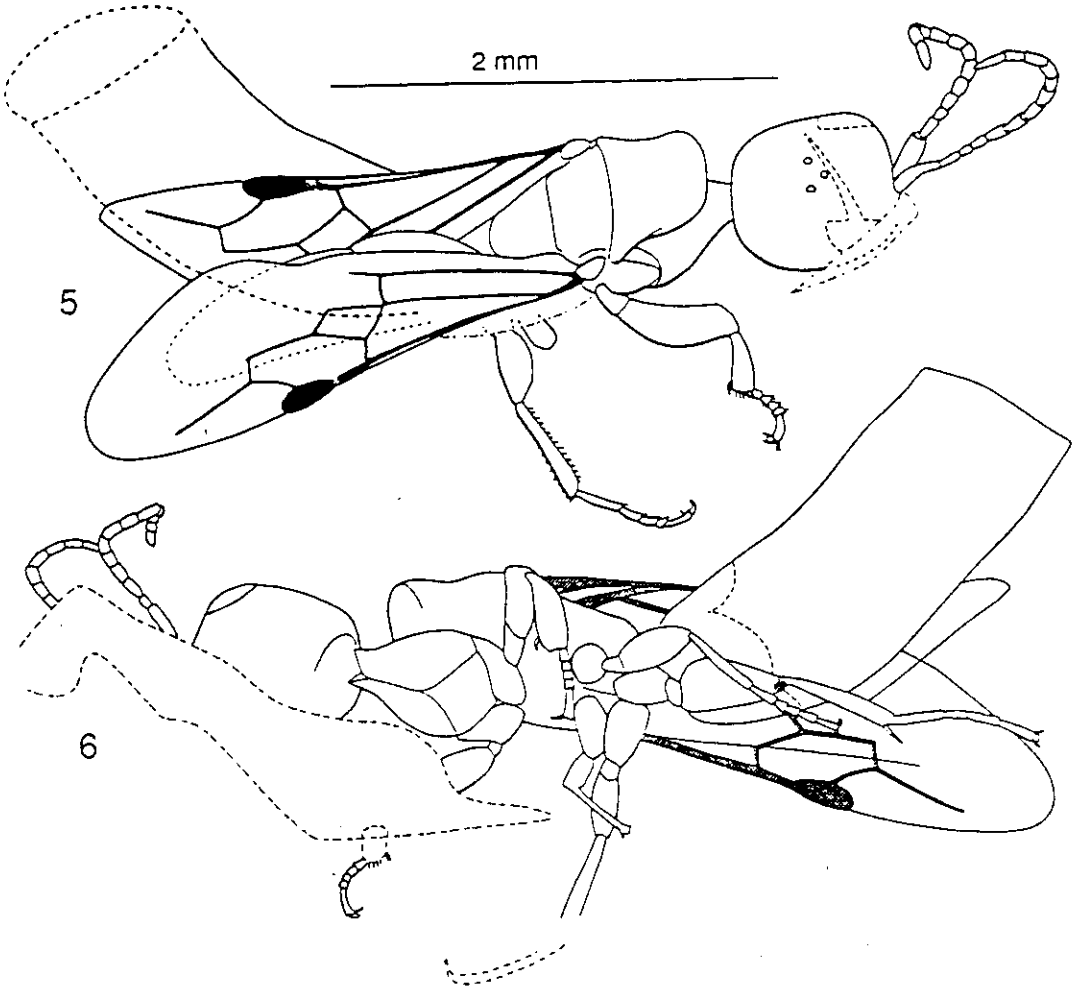
Figs. 3–4. *Dominibythus inopinatus*. 3, Ventral view. 4, Dorsolateral view.

their respective tibiae. Fore wing 1.4 mm in length; costal vein bearing a number of relatively well-developed setae that form a fringe approximately as wide as the prestigma. Hind wing with three hamuli; length of vannal lobe approximately 0.3× length of hind wing; posterior margin of hind wing bearing a fringe of well-developed setae that is as wide as the fringe on the costal margin of the fore wing. Metasoma elongate, 1.6 mm long (excluding genitalia). Length of second metasomal tergite approximately 1.3× length of TI. Length of first metasomal sernite about 1.5× length of TI. Subgenital plate clearly exposed and entirely convex on apical margin (Fig. 9). Aedeagus extending slightly beyond imaginary line drawn between apices of gonapophyses; gonapophyses bearing at least a few, very fine setae apically (Fig. 9); gonapophyseal setae about equivalent to width of gonapophyses near tip in length. The male genitalia of the holotype are exposed. In the normal position they would be concealed by TVII and SVIII.

Female.—Unknown.

Derivation of name.—The Latin *inopinatus*, meaning unexpected, refers to the seemingly improbable discovery of this wasp in Dominican amber given the rarity of the family in the extant fauna.

Material examined.—Holotype ♂: a superbly preserved wasp in Dominican amber with locality data as described under Age of the Fossils. It is in a clear piece of amber approximately 7 mm in length (specimen #7594, Smithsonian Dominican amber Brodzinsky/Lopez-Penha collection).

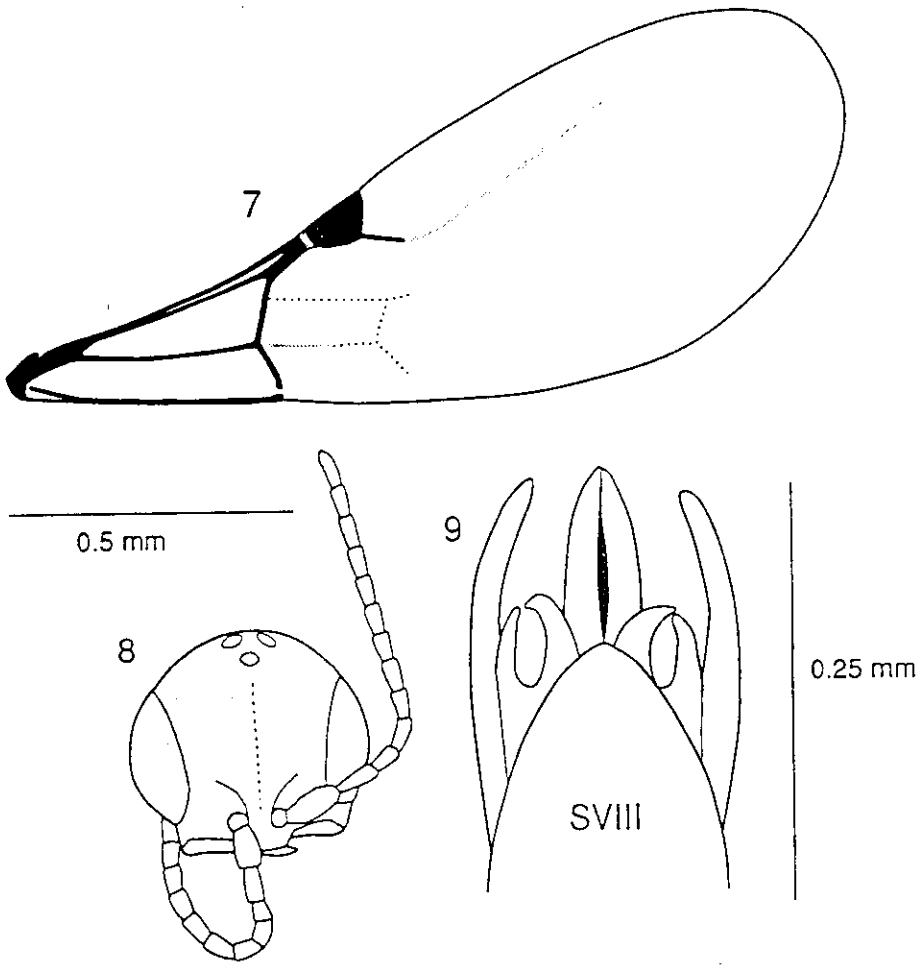


Figs. 5–6. *Libanobythus milkii*. 5, Dorsal view. 6, Ventral view.

Libanobythus Prentice and Poinar, new genus

Type and only known species: *Libanobythus milkii*, new species.

Generic characters.—Frons not elevated between antennal sockets with no evident carinae. Inner orbits evenly convex, closest near middle. Lateral ocelli positioned slightly posterad of imaginary line connecting postero-dorsal margin of compound eyes. Clypeus very short, transverse. Gena well developed, wider than eyes in lateral view. Occipital carina present, at least ventrally. Antennae placed very low on face, 13-segmented. Mandibles short, broad. Maxillary and labial palpi apparently 4-segmented. Pronotum nearly 1.5× as long as mesoscutum, not sharply declivous anteriorly; postero-lateral lobe of pronotum extending to tegula. Propleura well developed, anteriorly forming a neck that separates head from pronotum. Prosternum exposed and diamond-shaped. Parapsidal lines and notauli apparently absent. Mesosternum simple, not produced as lamellae over mid coxa. Tibial spur formula apparently 1-1-1. Tarsal claws simple. Forewing with vein R1 completely lacking; vein Cu absent beyond 1m-cu cross-vein; apical abscissa of anal vein slightly longer than cross-vein cu-a;



Figs. 7–9. *Dominibythus inopinatus*. 7, Right forewing, nebulous veins indicated by stiplings, spectral veins indicated by dotting. 8, Face. 9, Male genitalia, ventral aspect with outline of last sternum indicated.

apical portion of vein Rs extending close to costal margin, its length 3× length of crossvein 2r-rs, tubular throughout; stigma slightly convex on costal margin (Fig. 5). Hind wing vannal lobe extending 0.4× length of hindwing; jugal lobe absent. No constriction between first and second metasomal segments; segments wider than high. First metasomal tergite with depression fitting end of propodeum. First metasomal sternite evenly convex apically, not forming articulation with SII.

Derivation of name.—*Libanobythus* is a combination of the names Lebanon and Scolebythus and is in reference to the country of origin of the fossil.

Libanobythus milkii Prentice and Poinar, new species

(Figs. 2, 5, 6)

Female.—Body length approximately 4 mm not including apical metasomal segments which are missing from holotype. Color: Head, mesosoma and metasoma piceous; legs, antennae and mouthparts brown; wings clear, radial vein and stigma brown, other veins brownish-yellow. Sculpture and vestiture: Head smooth, with at least a few short, erect setae on genae. Pronotum and mesonotum smooth; pronotum bearing some erect setae approximately 0.05 mm in length. Metasoma bearing nu-

merous scattered setae, at least ventrally, approximately 0.1 mm in length. Legs with numerous scattered setae from 0.05 to 0.1 mm in length, best developed on mid and hind tibiae. Wings without evident setae. Hindwing bearing a fringe of setae approximately 0.02 mm in width. Fringe of same width extending from fore wing costal vein. Structure: Head 0.65 mm wide, 0.75 mm from vertex to ventral margin of clypeus. Width of ocellar triangle about equal to ocello-ocular distance; distance between lateral ocelli about 2.5× diameter of a lateral ocellus. Scape flattened, length including radicle, approximately 2.5× greatest width. Pedicel moderately long, its length 2× length of first flagellomere. Flagellomeres II–X nearly equal in length, approximately twice as long as flagellomere I; length of flagellomere XI 1.5× length of flagellomere X. Mesosoma, including propleura, approximately 1.4 mm in length. Pronotum subequal to mesoscutum in length. Fore femur robust, 2.4× as long as wide; mid femur robust, length slightly more than 2× width; hind femur very robust, length slightly less than twice greatest width. Fore tibia slightly shorter than fore femur; mid and hind tibiae approximately equal to respective femora in length. Length of hind tibial spur less than 0.5× length of hind basitarsus. Fore tarsus shorter than fore tibia; mid and hind tarsi longer than their respective tibiae. Fore wing 2.4 mm in length. Hind wing with four hamuli. Length of vannal lobe slightly less than 0.4× total hind wing length.

Male.—Unknown.

Derivation of name.—This species is named for Dr. Raif Milki.

Material examined.—Holotype ♀: a not well preserved specimen of an almost entire wasp from Lebanese amber with locality data as described under Age of the Fossils. The fossil is covered with numerous small air bubbles and cracks that obscure many details. The wasp is also lacking the last few metasomal segments. It is embedded in a piece of plastic measuring 1 cm × 1 cm × 0.5 cm. The specimen is part of Dr. Raif Milki's Lebanese amber collection maintained at the American University of Beirut.

Preservation.—The specimen lacks the end of the metasoma, but may be identified as a female due to its overall robust form, powerfully built legs (particularly the fore femora) and enlarged genae. Male scolebythids are more delicately built, with slenderer legs and less well developed genae. Because of the state of preservation of this wasp, its description is not entirely comparable with that of *D. inopinatus*.

Relationships

The elongate pronotum that retains a pronotal collar in *Libanobythus milkii* suggests a sister-group relationship between this species and other known Scolebythidae since the loss of the collar in other scolebythids is very likely derived within Chrysidoidea (Brothers 1975, Carpenter 1986). This sister-group relationship would not be unexpected given the age of the fossil, but, if true, means the complete absence of vein R1 in both *L. milkii* and *D. inopinatus* is convergent. The elongate pronotum and presence of a pronotal collar in *L. milkii* provides further support for the views of Brothers (1975) and Carpenter (1986) whose cladistic analyses indicated that the loss of the pronotal collar in Plumariidae (most probably the sister-group to other Chrysidoidea (Brothers 1975, Carpenter 1986)) and Scolebythidae is convergent. The alternative, that the absence of the collar in Plumariidae and some scolebythids is homologous and thus a groundplan feature of Chrysidoidea, as Koenigsmann (1978) believed, is now especially unlikely.

Dominibythus inopinatus appears to be most closely related to *Ycaploca evansi* as indicated by the presence in both taxa of a frontal prominence between the antennae that is marked laterally by two dorsally diverging carinae (Fig. 8). This feature is not evident in any of the other species of Scolebythidae. *Dominibythus inopinatus* also shares a well developed clypeal lobe, pro-

podeal groove and evenly convex subgenital plate with *Ycaploca*, but these features are also present in *Scolebythus*. An apparent tibial spur formula of 1-1-1 may be an additional character linking *D. inopinatus* with *Y. evansi* since *Scolebythus* and *Clystopsenella* have the ancestral tibial spur formula of 1-2-2. *Dominibythus inopinatus* may be distinguished from *Y. evansi*, and other extant scolebythids, by its much more reduced wing venation which does not include a complete marginal, submarginal or discoidal cell, by its more strongly diverging frontal carinae, by its lack of well developed notauli and by its lack of an evident metapostnotum on the propodeum.

Discussion

The addition of two new monotypic fossil scolebythid genera to the three extant monotypic genera might seem to unnecessarily clutter this small family with genera. However, on phenetic grounds, the recognition of five genera is justified. Neither fossil species may be placed in a currently defined genus and both are as morphologically distinct as any of their extant relatives. This taxonomic arrangement simply reflects the relictual status of this group and accords with the great range in age of the included species. Unfortunately, this scheme does not represent any cladistic or phenetic relationship between the species.

When Evans (1963) described the family, he speculated that the species attacked wood-boring beetle larvae based on their possession of some characteristics reminiscent of wasps, such as Aulacidae, having such a biology. Features mentioned by Evans (1963: 14) are "the broad mouth opening and powerful mandibles, the reduced clypeus, and the strong malar space; on the thorax the elongate proepisterna, perhaps the large prosternum, and the flattened coxae." Evans (1963: 8) also identified a well developed proepimeron in scolebythids that he stated as "... completely set off by sutures from the proepisterna." From studying his figures and specimens of *Clystopsenella longiventris* and *Ycaploca evansi*, as well as the fossil material, however, it appears that this "so-called" proepimeron is actually a part of the fore coxa that extends as a lobe basad of the insertion of the trochanter. Based on collection data from a number of specimens of *Y. evansi*, Nagy (1975) and Brothers (1981) have essentially confirmed Evans' (1963) original speculation. The information indicates that scolebythids develop on wood-boring beetle larvae (Cerambycidae) that are attacked in their host beetle's burrows. Of interest is the possibility that they are gregarious parasitoids (Brothers 1981), which, if true, is unusual for an aculeate. Given the nearly identical modifications of the fossil scolebythids to their extant relatives, it is very likely that they, too, attacked wood boring beetle larvae in the same manner as living scolebythids.

In addition to the features listed by Evans, other peculiar apomorphies of the family include the convexity of SI, the lack of an articulation between SI and SII, the deep concavity in TI that fits the end of the propodeum, the transverse flatness of the tergites as opposed to the convexity of the sternites, and the overall length of the metasoma. Possibly these features facilitate movement through their host's burrows, although it is curious that each modification would seem to allow the metasoma to be more freely articulated dorsad. Possibly this is an alternative means for bringing the sting to bear on the prey; if true, this would differ from the movement of other aculeate wasps which universally articulate the metasoma downward and below the body for stinging prey. If anything, the peculiar modifications of the propectus enhance this idea, for the form of the propectus allows the head to be strongly directed dorsad (as seen in the fossils and pinned scolebythids), and the fore legs to be brought forward; this could allow the mandibles and forelegs to grip the prey while stinging with the metasoma held up and over the body of the wasp.

By attacking wood boring beetle larvae, scolebythids are exposed to places (such as the bases of resin producing trees) where they may be trapped in resin having the possibility of subsequent fossilization. Thus, like many other aculeate wasp taxa that have been found in amber, the biology of scolebythids increases the probability that they may be found in amber. This may partially explain their occurrence in both Lebanese and Dominican ambers, although it is also possible that the family may have been more common in the geologic past.

Acknowledgments

We thank Dr. Karl V. Krombein of the National museum of Natural History, Smithsonian Institution, Washington, DC, for lending us specimens of *Scolebythus madecassus*, *Clystopsenella longiventris* and *Ycaploca evansi* for study. We also thank Dr. A. P. Rasnitsyn of the Paleontological Institute, Russian Academy of Sciences, Moscow, for the unique specimen of *Cretabythus sibiricus*, and Mr. Gary Hevel of the National Museum of Natural History for the Dominican amber specimen of *Dominibythus inopinatus*.

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APPENDIX II

Mitt. Mus. Nat.kd. Berl., Dtsch. entomol. Z. 45 (1998) 1: 43–48 25. 05. 1998

Cretaceomachilis libanensis, the Oldest Known Bristle-tail of the Family Meinertellidae (Machiloidea, Archaeognatha, Insecta) from the Lebanese Amber

Helmut Sturm[1] **& George O. Poinar Jr.**[2]

With 11 figures

Abstract

The oldest known member of the family Meinertellidae from Lebanese amber (Lower Cretaceous, 120–135 mill. years old) is described. The male specimen represents also the oldest bristletail which can be placed unquestionably in the Machiloidea. Taxonomic and biogeographic aspects are briefly discussed.

Key words: Fossil insects, Microcoryphia, Mesozoic amber

Introduction

Within the extant Archaeognatha, two families are distinguished, the Machilidae (45 genera and some 320 species) and the more derived Meinertellidae which occur predominantly in the southern hemisphere but also exist in the USA, Mexico and the Mediterranean area (16 genera and 150 species).

The fossil record of the Archaeognatha is scanty. In Dominican amber (20–25 mill. years old) which is very rich in inclusions, only one species of Meinertellidae was found (Sturm & Poinar 1997). Silvestri (1912) described 2 genera and 8 species of Machilidae or Machiloidea incertae sedis from Baltic amber (ca. 35–40 mill. years old). Remains of Archaeognatha which cannot be placed in Machiloidea came from the Triassic of the Ural (*Triassomachilis uralensis* Sharov, 1948) and from the Lower Carboniferous of Illinois (Kukalová-Peck 1991). *Triassomachilis u.* had apparently relatively small compound eyes which did not touch in the median plane and annulated abdominal stylets. Paclt (1972) assumes with reference to two drawings of Sharov (1948) that the separation of the eyes is an artefact, but he cannot offer convincing facts. Sharov (1966) characterizes the eyes as "situated apart and with few facettes". Kukalová-Peck (1991) interprets this finding as a mayfly nymph without any argumentation. A detailed description is lacking for the specimens from the Carboniferous with leg-like appendages on the abdominal segments. Fragments of insects which could be related to the Archaeognatha are known from the Middle Devonian of Gilboa, New York (Shear et al. 1984) and from the Lower Devonian of Quebec, Canada (Labandeira et al. 1988). So the species described here from Lebanese amber is the oldest fossil which can undoubtedly be classified within the Machiloidea. This species raises questions e.g. regarding the evolution and biogeography of the Meinertellidae, which will be discussed here.

Materials and methods

The single specimen is a moderately preserved male (body length 4.5 mm). The surrounding amber shows many thin parallel layers (thickness 0.1–1 mm). Between and within the layers are abundant fissures filled with gas. In addition, thin patches of a red material and fine detritus are included (Fig. 9). The longitudinal axis of the animal is oriented parallel to the stratification of the amber. Apparently the animal was damaged before being completely enclosed. A hole in the cuticle of the abdomen (near the 6th segment), disarticulated and transported parts of antennae, maxillary palps and legs, as well as many isolated setae and the absence of the distal parts of maxillary palps and caudal appendages show that there may have been interactions with other animals or objects. These irregularities and damages make a complete description difficult.

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The amber piece was reshaped in order to obtain parallel planes close to the animal. The connection between amber, slide and cover glass was made by Euparal (artificial resin) or benzylbenzoat. The latter substance has a refraction coefficient which is similar to that of amber and enters fairly well into open fissures. The piece was examined using illumination from above (fiber-optic lamp Schott KLM 1500) and from below. Microfotos: Minolta 700, microscope Olympus BH2.

***Cretaceomachilis* Sturm & Poinar gen. n.**

Type species: *C. libanensis* Sturm & Poinar

Etymology. The first part of the name alludes to the Cretaceous period and to the latin "*creta*"(chalk). "*Machilis*", the second part of the name refers to one of the first genera described within the Archaeognatha and in the suborder Machiloidea.

Description and diagnosis. (Figs 1, 3, 4, 11): Antennae without scales; article 2 of maxillary palp with a large, characteristic hook near the base of which is a small, oval sensory (?) field with fine characteristic parallel stripes; article 3 of labial palp not distinctly broadened; legs II and III with coxal stylets each, smaller on legs II; penis probably shorter than half the length of abdominal coxites IX.

Discussion. The habit and most of the characters of the specimen correspond largely to the general features of the extant Machiloidea, e.g. compound eyes, frons, antennae, maxillary and labial palps, legs, stylets and caudal appendages. However marked differences exist in the above mentioned older findings of Archaeognatha from the Triassic and Carboniferous periods. Thus the specimen described here is the oldest Archaeognatha which can be placed undoubtedly within the Machiloidea.

The main reasons for placing the fossil into the family Meinertellidae are the absence of scales on scapus, pedicellus and flagellum (a characteristic which occurs in all extant Meinertellidae and in the highly derived genus *Petrobiellus* [Petrobiinae: Machilidae]) and the presence of a characteristic hook near the distal end of article 2 of the male maxillary palp. The latter character occurs in the males of all species of Meinertellidae but is lacking in all species of Machilidae and Machiloidea incertae sedis (Sturm & Bach 1993). Unfortunately the abdominal sternites were not visible in the Lebanese specimen. In the Meinertellidae they should be distinctly smaller than in the other Machiloidea.

The specific features of the genus are mentioned in the diagnosis. Within the extant Meinertellidae, coxal stylets on the legs II + III – very probably a plesiomorphic character (Sturm & Bach 1993) – occur only within the *Machiloides*-group. This group includes four genera: *Machiloides* the genus with the highest number of plesiomorphic characters within this group(31 spp.) (Sturm & Bach 1993), *Nesomachilis* (11 spp.), *Allomachilis* (1 sp.) and *Kuschelochilis* (1 sp.).

The striated field near the base of the hook on the maxillary palps of *Cretaceomachilis* is probably a sensory structure which could be of importance during mating (cf. Sturm 1986). Comparable structures have not been found in other Archaeognatha. However, other strange and unique sensory organs, present only in males, were described (Sturm & Messnér 1992) for species of the probably ancient genus *Mesomachilis* (Machiloidea *incertae sedis*).

***Cretaceomachilis libanensis* Sturm & Poinar, sp. n.**

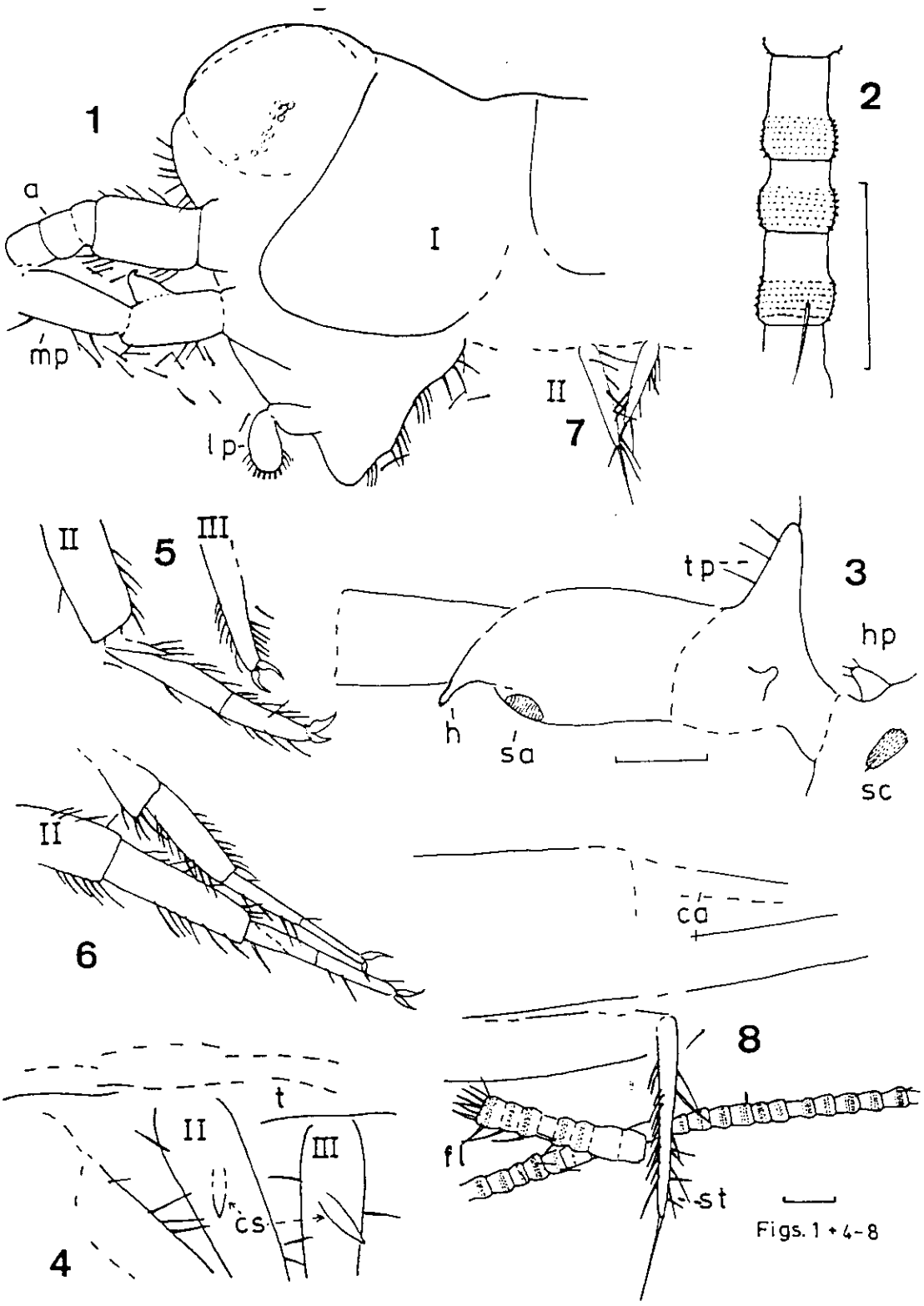
Holotype. Male, body length 4.5 mm, inclusion from Lebanese amber, exact locality unknown; collection Milki Nr. 194/35; deposited at the American University of Beirut, Beirut, Lebanon.

Etymology. The species-name refers to the country of origin.

Description. Head (Fig. 1). Orthognathous, eyes large, touching at the median plane; ocelli not recognizable; frons slightly protruding, with short setae. Antennae (Figs 1, 2, 8, 9, 10) longer than body, without scales; scapus about 2× longer than broad; jointlets of flagellum not always clearly discernible, chains of distal part of flagellum with about 10 articles; each article with at least one ring of setae. Mandible not visible. Maxillary palps (Figs 3, 11) with only 3 proximal articles preserved; on the dorsal basis a small horizontal process present; article 1 with triangular process and one clearly visible lateral hump; hook on article 2 clearly protruding, with broad basis, distal end curved mediadly. Article 3 of labial palps distally only slightly broadened (Fig. 1), distal end with sensory rods common in all extant Machiloidea.

Thorax: Tergites resemble those of the extant Machiloidea (Sturm & Messner 1995); spiniform setae on the lateral margins of the tergites not visible; legs II and III with coxal stylets (Fig. 4); all legs with some very long setae, tarsi with 3 articles, spiniform setae on the distal parts of the legs not discernible (Figs 5, 6).

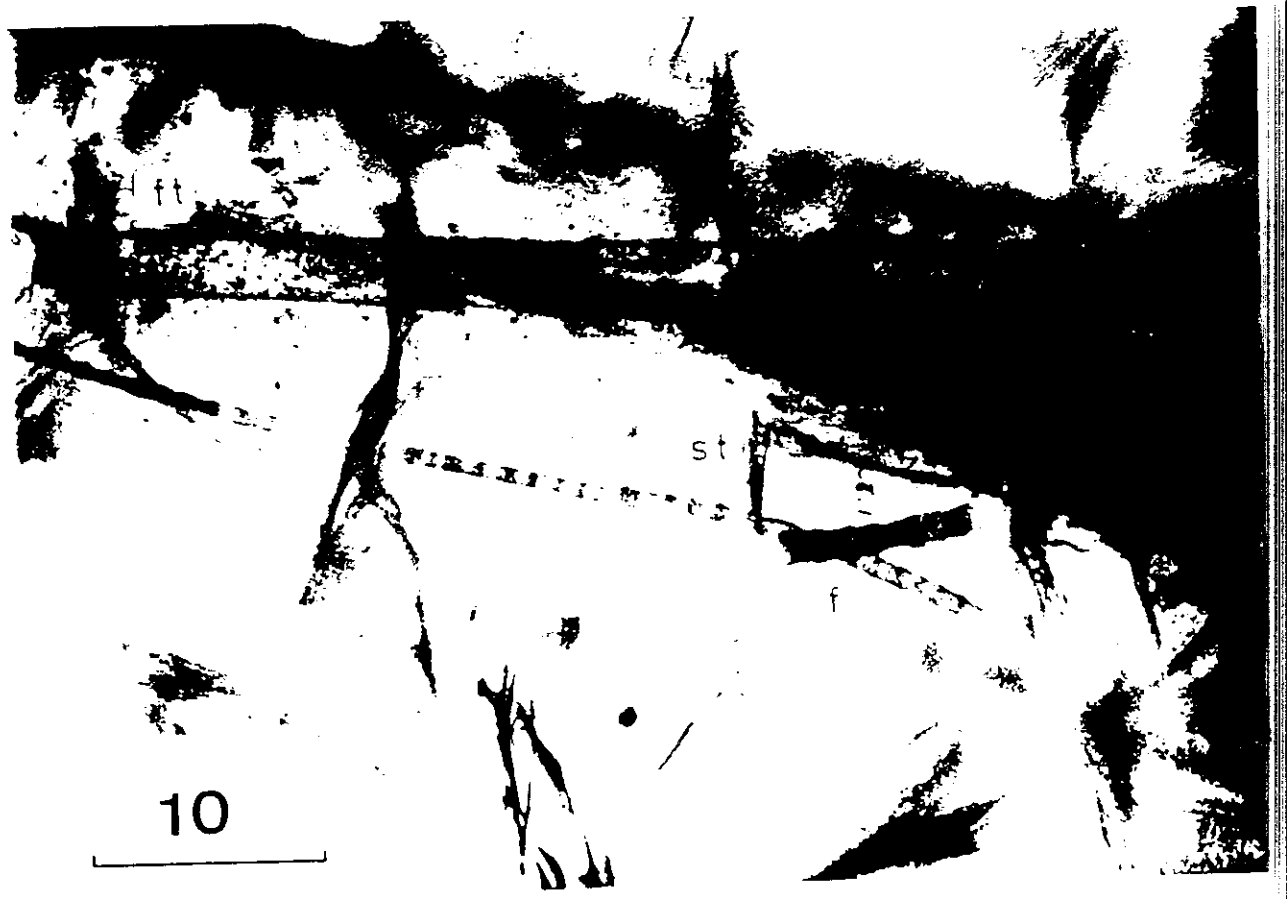
Abdomen: Form, size and structure of the sternocoxites not clearly visible, II–IX with 1 pair of

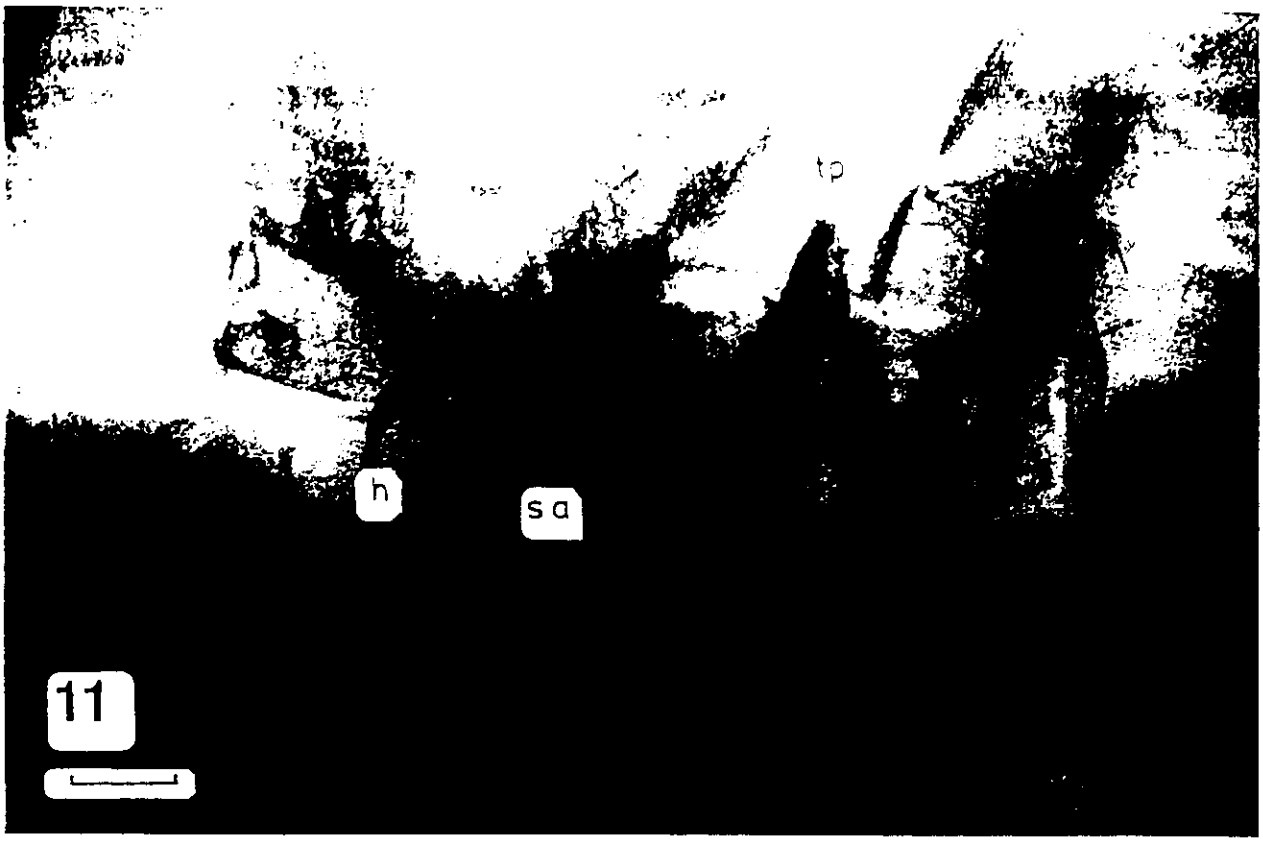


Figs 1–8. *Cretaceomachilis libanensis* gen. n. sp. n. Scale bar 0.1 mm. **1.** Head with basal parts of antenna (a), maxillary palp with hook on article 2 (mp) and articles 2+3 of labial palp (lp); lateral view. **2.** Three articles from the distal part of the antenna. **3.** Articles 1–3 of the maxillary palp, lateral view; h = hook, hp = horizontal process, sa = striated area, sc = scale, tp = triangular process. **4.** Coxae of legs I–III, ventral view; cs = coxal stylets, t = margins of tergites. **5., 6.** Distal parts of legs II + III; **5.** lateral view; **6.** ventral view. **7.** Abdominal stylets II+III, lateral view. **8.** Distal end of abdomen with basal parts of caudal appendages (ca), coxite IX with stylet (st) and parts of flagellum (fl.); lateral view.

stylets which are not ringed; terminal spines of stylets long (Figs 7, 8), sometimes longer than 0.5× stylet-length. Penis not discernible, probably shorter than 0.5× length of coxite IX; caudal appendages broken, remaining parts oriented nearly parallel to the longitudinal axis of the body.


h
9


ft
st
f
10



Figs 9–11. *Cretaceomachilis libanensis* gen. n. sp. n. Photos, lateral view; scale bar: 0.5 mm for 9. – 10.; 0.1 mm for 10. **9.** Whole animal, head to the right (h). The irregularities of the amber and the stratifications are obvious. **10.** Dista. end of body with broken filum terminale (ft), broken parts of flagellum (f) and abdominal stylet (st). **11.** Maxillary palp (isolated from body) article 1 with triangular process (tp), article 3 with hook (h) and striated area (sa).

Diagnosis: See diagnosis of the genus.

Discussion. Due to the poor state of preservation, the absence of a female and irregularities of the surrounding amber the description cannot be complete.

From a phylogenetic and biogeographic point of view, the fossil is of considerable interest. In the Mediteranean area the Meinertellidae are represented only by the genera *Machiloides* and *Machilinus*. Whilst *Machilinus* is a strongly derived genus which shows little morphological similarity with *Cretaceomachilis* i. *Machiloides* and especially the only mediterranean species (*Machiloides tenuicornis* Stach, 1930: Spanish Pyrenees) show a striking accumulation of plesiomorphic characteristics within the Meinertellidae (Sturm & Bach 1993): e.g. coxal stylets on legs II + III, frons moderately protruded, ocelli sole-shaped, article 3 of labial palp not distinctly broadened, tarsi with 3 tarsomeres, sexual dimorphic characteristics of maxillary palps weakly developed, mating behaviour compared to other Meinertellidae little differenciated (Sturm 1986). The species described here is very likely near to the genus *Machiloides*.

The greater part of fossil Meinertellidae was found during the last 30 years. Wygodzinsky (1971) describes a ?*Neomachilellus* sp. (female, body length 6.5 mm) from the Miocenic amber of Chiapas (Mexico). A second inclusion from the same amber exists in the collection of the American Museum of Natural History (= AMNH: Chiapas No. 21). It was determined by the authors of this article as a male ?*Neomachilellus* sp. In every case the two specimens are highly derived Meinertellidae. More than 90 specimens of the Dominican amber (ca. 25 mill. years old) can be placed with certainity in a new subgenus and species of the genus *Neomachilellus* (Sturm & Poinar 1997). Another member of the family (male, 4.2 mm body length, AMNH, NJ 621e) comes from the New Jersey amber (USA, Cretaceous: Turonian, ca. 90–94 mill. years old). The finding of *Cretaceomachilis* indicates that during the Lower Cretaceous Archaeognatha existed which had a high number of characteristics typical for the extant Meinertellidae. This means that the separation of the Meinertellidae from the other representatives of the Archaeognatha was completed or nearly completed at that time. This does not exclude a long

period of coexistence between original and derived forms, comparable perhaps with the existence of the extant Machiloidea incertae sedis.

The finding of Miocenic Meinertellidae, especially from the Dominican amber, indicates that during this epoch the separation into the extant genera was largely completed, since the genus *Neomachilellus* Wygodzinsky, 1952 and its sister genus *Meinertellus* Silvestri, 1905 constitute one of the most derived genus groups (Sturm & Bach 1993).

The similarity between *Cretaceomachilis* and *Machiloides* is also of biogeographical interest. *Machiloides* has the most extended distribution within the Archaeognatha. Its species are present in the following regions (numbers indicate the number of species in each region): E USA (2), S South America (5), N Spain (1) E and S Africa (22), Madagascar (5), N and E Australia (5), Tasmania (1). Possibly the separation of the Meinertellidae from the rest of the Archaeognatha took place on the plates of the recent northern hemisphere. The following facts could support this hypothesis: (a) The presence of 3 extant *Machiloides* species in the northern hemisphere. These species are distintly less derived than the majority of the southern species and perhaps "relics". (b) The findings of a Cretaceous species of Meinertellidae in the USA (New Jersey) and of *Cretaceomachilis* which is near to *Machiloides* in the Lebanese amber. (c) The occurrence of the extant Machilidae and Machiloidea incertae sedis is nearly restricted to the northern hemisphere. Only in Africa and Indochina do 2 genera extend about 5° latitude south of the equator. This suggests that the separation of the Meinertellidae from the rest of the Archaeognatha took place on the plates of the actual northern hemispere. The worldwide distribution of the genus *Machiloides* was probably facilitated by the closer arrangement of the continents during the Mesozoic.

Aknowledgements

The authors are indebted to Dr. Milki (American University of Beirut) for the loan of the *Cretaceomachilis* inclusion and to Dr. D. Grimaldi (American Museum of Natural History, New York) for the loan of the *?Neomachilis* specimen from the Mexican amber and the Meinertellid specimen of the New Jersey amber. We are also obliged to Dr. R. Willmann for his critical and helpful comments.

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APPENDIX III

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May 13, 1996

Dr. Raif Milki
Department of Health Services
American University of Beirut
Beirut, Lebanon

Dear Dr. Milki

I would like to invite you to visit our Department at Oregon State University this summer in order that we can continue our collaborative project on the study of the Milki Collection of Lebanese Amber.

We would be happy to receive you sometime after the 12th of September this year.

Our discussions should include the following topics:

1. Identification of amber fossils in the Milki Collection.
2. Description of the amber sites in Lebanon.
3. Preparation of a book on this subject.
4. Preparing photographs and drawings to illustrate the book.
5. Discussing DNA studies with material from the Milki Collection.

We look forward to your arrival and fruitful discussion.

With kind regards.

Sincerely,
George Poinar, Jr.
Paleobiology

APPENDIX IV

Lebanese amber

G. O. Poinar, Jr., M. Prentice

and R. Milki

Contents

Introduction

Amber from Lebanon contains some of the oldest known arthropods and plants found in fossilized resin, dating from the Early Cretaceous. The significance of Lebanese amber is its great age. Many of the insect groups were diverging and adapting to conditions in a sub-tropical rain forest that contained many new angiosperms taxa, as well as the fully established gymnosperms. Today all are extinct at the species level, most are extinct at the genus level and a fair number are extinct at the family level. By this Era, the "modern" insect orders had already appeared but family divergence was still in an active state of evolution.

Geological setting

Lebanese amber was formed during the Aptian period of the early Cretaceous, which is roughly 125-130 million years ago. Some of these primary deposits subsequently have been eroded and re-deposited. These secondary deposits, which date from 105-115 million years are found in sandstone and limestone sedimentary layers, as well as in lignitic beds among the sedimentary layers (Schlee & Dietrich, 1970).

Location

Lebanese amber actually is spread over a wide expanse that includes both Palestine and Jordan, showing that the original forest with three resiniferous trees was indeed quite extensive (Poinar, 1992). However, the majority of this "near-East amber" that has been collected was recovered in Lebanon near the towns of Jezzine, Dahr Al Baydar, Ain Al Suha and Meyrouba.

Plant source

Studies with nuclear magnetic spectroscopy have indicated that Lebanese amber was derived from trees of the family Araucariaceae, probably an extinct species of Agathis (Lambert et al., 1996). This genus of trees, commonly known as kauri pines, is absent from the northern

hemisphere today, but back in the Mesozoic, when the Lebanese amber was being formed, it was one of the major trees throughout the region. Just when and why it became extinct in the northern hemisphere, and why it still exists in the southern hemisphere in Austral-Asia (Australia, New Zealand, New Caledonia, Fiji and other oceanic islands), is not known.

The ancient kauri forest in Lebanon was probably very similar in many aspects to the present day kauri forest of *Agathis australis* trees in northern New Zealand. Many of these trees are giants, ranging from 500 to 1000 years of age (Poinar & Poinar, 1994). Their branches form a canopy and shade a thick understory of smaller trees, shrubs, vines, ferns, and smaller plants. As these trees grew, the resin that they produced accumulated on the ground and was eventually covered up with leaves and other debris, eventually becoming covered with soil. Later the land where ancient forest once existed became inundated with sea water and the fossilized resin became integrated into sedimentary rock layers. As the resin aged, it passed through a copal or semi-fossilized stage into one of true amber. The layers that held the amber were then uplifted into mountains and became exposed to the weathering processes, resulting in the amber being washed out of the eroding rocks. Today in Lebanon, it is possible to search over this area and pick up the amber pieces from the surface of the ground or chisel it out of exposed sedimentary rocks.

In the areas where these trees occur today there are large deposits of resin in the ground varying in age from several years to forty thousand years.

Nature of Lebanese amber

During the 125 million years Lebanese amber has been in the earth, the sedimentary layers in which it lay have been subjected to various stresses as a result of normal earth moving forces. This, coupled with the regular daily and seasonal fluctuations in temperature which would affect the surface layers, has had its effect on the amber. Thus it is not surprising that this aged amber is highly fractured. These fractures causes the amber to fall apart as it is removed from the earth and makes it difficult to study. Any small force could crack it further and the numerous fractures makes it difficult to clearly see many of the inclusions. Thus studying and photographing specimens in Lebanese amber is quite a challenge and preserving

the amber after it is removed is another challenge. Preserving the amber can be done by embedding the pieces in liquid plastic. The plastic enters the cracks and helps to clarify the pieces as well as protecting them from further environmental effects. One of the problems, however, is that when the plastic makes contact with the membranous surface of the insect wings, it can obscure the fine venation, which may be an important character for taxonomic purposes. However once the plastic has hardened, it is possible to re-polish it without fear of it fracturing further.

Lebanese amber varies in color from light yellow to dark brown and even black. While the light yellow pieces appear to have few inclusions and the dark pieces usually contain unidentifiable plant material, it is the middle hews that appear to be most fossiliferous.

Types of inclusions in Lebanese amber

In discussing the various fossils that have been found in Lebanese amber, they will be discussed under their appropriate taxonomic group, with notes about their possible habitat including likely animal and plant associates.

Arachnida

Mites.

Erythraeoidea. The presence of 6-legged larvae of erythraeid mites indicates a parasitic relationship with insects since these larval mites are known currently to attack and suck the blood from various arthropods, especially insects. After crawling on various organs of their host (legs, wings, abdomen, thorax), the mites puncture the cuticle with their mouthparts and slowly imbibe the hemolymph. Discovering unattached erythraeid larvae in amber indicates that it was in the process of searching for a host or had just finished feeding on a host and was preparing to molt to the next (nymphal) stage, when it would then assume the role of a predator. (P)

Spiders

Spiders are an ancient group and their presence reveals the presence of various arthropod prey in the original environment.

Hexapoda

Collembola

Springtails are the oldest Hexapoda known, dating back to the Devonian Period, some 350 million years ago. They occur in freshwater and terrestrial habitats where they can be seen crawling or jumping with the aid of their fulcrum. Springtails feed on fungi and other agents of decay, including fungal spores and even nematodes. They also occur under the bark of trees and are common amber inclusions. Those in Lebanese amber have not yet been identified.

Archeognatha

Members of this order were originally included in the Thysanura and included the bristle-tails and silverfish. Lebanese amber contains the oldest known representative of the family Meinertellidae, namely *Cretaceomachilis libanensis* Sturm & Poinar (1998). These creatures possess styli on their ventral abdominal segments as well as three long tail filaments. Their bodies are covered with scales and they prefer concealed habitats such as beneath bark and stones. These primitive insects feed on decaying organic matter and escape their prey by rapid movements and squeezing in narrow surfaces.

Blattaria

Cockroaches are an ancient insect group that has existed since the Carboniferous. They prefer warm climates and those that do exist in northern climates often invade domiciles in the winter. Their presence in Lebanese amber indicates a warm climate at the time of resin deposition. Cockroaches are scavengers and feed on a variety of plant and animal material. Many live under the bark of trees which would bring them into contact with resin deposits. Although they are protected by a shiny hard exoskeleton, deposit their eggs in a tough capsule and possess a strong internal defense system, they are preyed upon by a range of predators and parasites, including wasps which also have been recovered from Lebanese amber.

Gryllidae

Crickets may extend back to the Carboniferous, where they developed into terrestrial and arboreal forms. All crickets are basically plant feeders, although many will attack and devour smaller arthropods. Unidentified forms occur in Lebanese amber (Whalley, 1981).

Psocoptera

Bark lice are another ancient group dating back to at least the Jurassic. These small insects demonstrate a high level of variability regarding size, antennal structure, and behavior. As their name implies, these insects live on and under the bark of trees where the majority feed on fungi, plant secretions and debris. Bark lice are usually one of the commonest groups in amber due to their habits of living on the trunks of trees. Whalley (1981) noted that about 5% of the insects in Lebanese amber was composed of psocids or bark lice.

Homoptera

Aleyrodidae

Whiteflies are tiny homopterans that are characterized by powdery scales on their wings and body. They suck juices of various plants so the Lebanese amber fossils could have been feeding on nearby plants as well as on the resin producing tree. From these deposits were described the extinct genera *Bernaea* and *Heidea* (Schlee, 1970), which are the oldest known whiteflies (check).

Psyllidae

Psyllids are called jumping plant lice due to their ability to rapidly leap from one location to another. They are normally quite small with long antennae and two pairs of membranous wings. The Lebanese forms are the oldest known psyllids (check)

Fulgoroidea

Planthoppers are a varied group, ranging widely in form and size. They are especially abundant in warm climates. Many are also capable of jumping although as a group they are strong fliers. A cixiid planthopper has been reported in Lebanese amber (Whalley, 1981).

Hemiptera

Members of this order are known as true bugs that have the outer pair of wings thickened more than the inner pair. The earliest fossils of this order are members of the Dipsocoridae, Thaumastelidae and Anthocoridae in Lebanese amber (Whalley, 1981).

Enicocephalidae

This family is represented in Lebanese amber (Grimaldi et al., 1993).

Coleoptera,

Archostimata

This group represents the most primitive group of the beetles which includes the Micromalthidae with the single genus Micromalthus, a first instar larva of which has been noted in Lebanese amber (Whalley, 1981).

Colydiidae

The earliest known members of these cylindrical bark beetles occur in Lebanese amber (Whalley, 1981). These small beetles occur under bark where they feed on a range of fungi and lichens.

Elateridae

Click beetles are widespread and some occur under bark. In these forms, the larvae usually occur in decaying wood. They have been reported in Lebanese amber (Whalley, 1981).

Dermestidae

Members of this family of beetles, commonly known for their habits as scavengers, have been reported from Lebanese amber (Whalley, 1981).

Nemonychidae

The only beetle yet described from Lebanese amber is the small nemonychid weevil, *Libanorhinus succinus* Kushel & Poinar (1993). Many present day members of this family develop in the male strobili of members of the Araucariaceae. Pinaceae and Podocarpaceae.

Thysanoptera

These small insects possess small elongate wings, often with a fringe of hairs on their outer margin. The biology of most species is unknown however in general they feed on plant parts (pollen and spores) while some are predatory on smaller mites and springtails. They occur in Lebanese amber and five species, *Exitelothrips mesozoicus, Scudderothrips sucinus, Jezzinothrips cretacicus, Neocomothrips hennigianus, Rhetinothrips elegans, Progonothrips horridus* and *Scaphothrips antennatus* have been described by zur Strassen (1973).

Diptera

Chironomidae

Midges are small, dainty flies whose larvae are often aquatic, feeding on decaying vegetable matter in ding or running water. Many probably also bred in tree holes, possible in the resin producing tree. The oldest midge is *Libanochlites neocomicus* Brundin (1976) from Lebanese amber.

Ceratopogonidae

The biting midges represent one of the most common group of insects in Lebanese amber (Whalley, 1981). The larvae live in wet terrestrial and aquatic habitats and are associated with decaying bark. The adults often take blood from both vertebrates and invertebrates (often from the wing veins of larger insects).

Scatopsidae

The larvae of scavenger flies are detritivores and can feed on both decaying animal and plant matter. Those found in Lebanese amber may have bred under bark.

Trichoceridae

Members of this family have been reported from Lebanese amber (Whalley, 1981).

Asilidae

Adult robber flies are medium to large insects, with legs often armed with long setae and spines in order to hold their victims as they suck out their body contents with a short proboscis. Both adults and larvae of robber flies are predatory on other invertebrates. They are rare in all amber so their occurrence in Lebanese amber is of interest (Whalley, 1981).

Tipulidae

Craneflies are medium to large sized insects with large, this legs, small heads, and usually elongate narrow bodies. The wings are usually elongated and slender. The larvae live in wet or damp soil or in standing water. All stages are vegetarians, and while some adults have mouth parts with a proboscis, they are used to obtain nectar from flowers. They have been reported from Lebanese amber (Whalley, 1981).

Empedidae

In 1970 Hennig described *Trichinites cretaceus* and *Microphorites extinctus* from Lebanese amber. These flies are predaceous and usually catch prey while on wing.

Psychodidae

In 1972, Hennig described *Phlebotomites brevifilis* and *P. longifilis* from Lebanese amber. These insects are blood suckers.

Lepidoptera

Micropterigidae

Whalley (1978) described a primitive moth in the extinct genus *Parasabatinca* in this family. This form belongs to a group now restricted to Australia-new Zealand and Africa.

Neuroptera

Several of these net-winged insects have been described from Lebanese amber. They include species in the extinct genera *Glaesoconis*, *Banoberotha enigmatica* Whalley and *Paraberotha* , all in the Coniopterygidae and a species of ant lion (Myrmeleontidae) (Whalley, 1980).

Hymenoptera

Representatives of this large and diverse order have also been reported from Lebanese amber. Whalley (1981) reports representatives of the families Chalcididae, Proctotrupidae and Gasteruptiidae and *Libanobythus milkii* was described in the Scolobythidae by Prentice et al., 1996.

Discussion

APPENDIX V

Identifications from the Milki Collection

The series S-1 to S-25 consists of insect fragments or particles for DNA studies.

The I series are specimens brought to California by R. Milki in 1993.

The I94 series are specimens brought to California by R. Milki in 1994.

I-1
I-2
I-3
I-4
I-5
I-6
I-7
I-8
I-9
I-10
I-11
I-12
I-13
I-14
I-15
I-16
I-17
I-18
I-19
I-20-ceratopogonid-P
I-21
I-22-small larva-neuropteran?
I-23-ceratopogonid-partial
I-24-psyllid
I-25-cricket?
I-26
I-27-aculeate wasp,12 seg. antennae, 1 marg. cell-P
I-28-ceratopogonid-P
I-29-?
I-30-ceratopogonid-partial
I-31-aculeate?
I-32-wasp-parasitica-unknown to Bob Zuparko
I-33-wasp head
I-34-Platygasterid?-unknown to Bob Zuparko
I-35
I-36-part of antenna
I-37-seed or beetle
I-38-?
I-39-ceratopogonid and leg

I-40
I-41
I-42-fungus
I-43-homopteran
I-44-spider-P
I-45-scelionid?, psyillid-scelionid according to Bob Zuparko
I-46
I-47-ceratopogonid-poor
I-48-ceratopogonid and collembola-P
I-49-beetle-P
I-50-Evaniidae
I-51-2 midges
I-52-large leg
I-53-body or shed skin of fly
I-54-fulgorid
I-55-?
I-56-small insect
I-57
I-58
I-59-ceratopogonid
I-60-?
I-61- cockroach abdomen-P
I-62-?
I-63-ceratopogonid-partial
I-64-chalcidoid-unknown to Bob Zuparko
I-65-ceratopogonid
I-66-homopteran
I-67-ceratopogonid-P
I-68-chalcicoid-Scelionidae according to Bob Zuparko
I-69-?
I-70-partial ceratopogonid
I-71
I-72-insect part
I-73
I-74-ceratopogonid and insect fecaes
I-75
I-76
I-77
I-78-legs only
I-79
I-80
I-141-small damaged insect

I94-1- Plant part, leaf?-P
I94-2- scatopsid (JB)
I94-3- thread-like object-Plant fibers?
I94-4- Nematode-like object
I94-5-
I94-6-midge
I94-7-possible nematode-P
I94-8-Eriophyllid mite-P
I94-9-large cockroach-covered with whitish deposit-P
I94-10-Trichocerid (JB)-P
I94-11a-Bark louse
I94-11b-Asilid type (JB)
I94-11c-?
I94-11d-chironomid (JB)
I94-11e-elaterid beetle-P
I94- 11f-?
I94-12a-chironomid (JB)-P
I94-12b-nematoceran legs
I94-13
I94-14-ceratogonid & homopteran-P
I94-15-empedid & tipulid (JB)
I94-16-lower brachyceran (JB)-P
I94-17-seed or artifact
I94-18-chironomid, larva, thrips, cockroach legs-P
I94-19-?
I94-20-partial ceratopogonid
I94-21-wasp & plant rootlets-P-wasp seems a Dryinidae according to Bob Zuparko
I94-22-ceratopogonid
I94-23
I94-24-Chrysididae
I94-25
I94-26-homopteran
I94-27-long-legged ceratopogonid
I94-28-partial fly
I94-29-scolytid?
I94-30-hymenopteran-unknown to Bob Zuparko
I94-31-nematoceran
I94-32-small piece-unknown(JB): medium-ceratopogonid(JB): large-nematoceran(JB)
I94-33-incomplete insect
I94-34-ceratopogonid
I94-35-beetle- hard to see
I94-36-psyllid, fly, leaves-P
I94-37-ceratopogonid-exposed
I94-38-partial nematoceran
I94-39-male fly-partial
I94-40
I94-41 -nematoceran because of branched R-sector and cubital(JB)
I94-42-small fly

36-cecidiomyidae-P
37-larva-?
38-?
39-diptera-P
40-spider leg
41-thrips-?
42- cecidiomyidae
43-diptera
44-insect legs
45-plant hair-P
46-small bubble
47-nematoceran
48-debris
49-seed -P- or oil globule
51-egg or oil globule
52-strange big-eyed roach
54- roach eggs-goes with 52?
55- small bubbles-P
56- various objects-?
57- ?
58- ?
59- ?

Specimens brought by Dr. Milki in September 10-16, 1995 to Corvallis. These were all embedded in plastic in small magnification boxes.

1-95-small fly
2-95- ?
3-95-ceratopogonid
4-95-?
5-95-?
6-95-?
7-95-chironomid
8-95-?
9-95-?
10-95-fly head? bryophyte stems?
11-95-?
12-95-
13-95-nematoceran
14-95-?
15-95-orthopteroid remains
16-95-part thysanuran
17-95-nematoceran
18-95-Large fly (P-Milki)
19-95-bug and spider (P-Milki)
20-95-Dolochopodid?
21-95-scelionid? (Photo-Milki)

I94-43-ceratopogonid-P
I94-44-two nematocerans
I94-45-psyllid-P
I94-46-lower brachyceran(JB)-P
I94-47-mite
I94-48-Empedidae(JB)

Notes
Bob Zuparko examined I-32, I-34, I-64, and I94-30. The one that has a long petiole and some wing venation looks like a Chalcioidea or Scelionidae.

Wood slide box (most of these are recent forms that have been mounted on glass slides with balsam; a few are amber; these are listed below).

67-homopteran
72-Phlebotomus
73-homopteran
75-Cecidiomyidae
77-Thysanopteran

Plastic slide box (only the amber ones are listed)

1-Hymenoptera - Parasitica P
3-plant?
4-dipteran
5-bubbles, globs
6-Diptera?
7-spider P
9-hemipteran
10-Diptera?
11-unknown
12-unknown
14- homopteran
17-cockroach - P
18-ceratopogonid-P
19-ceratopogonid-P
23-insect
24-ceratopogonid-P plus spider
28- insect
29-Thysanoptera-P plus spider in part
30-insect
31-mite and spider-P
32-cecidiomyidae-P
34-part fly
35-cockroach-D

22-95-ceratopogonid
23-95-small strand-hair?
24-95-nematoceran and fly with long legs

Rn2- scelionid
Ns 10-nematoceran

APPENDIX VI

MEDICAL INTELLIGENCE

CURRENT CONCEPTS

JANE F. DESFORGES, M.D., *Editor*

THE POLYMERASE CHAIN REACTION

A New Method of Using Molecular Genetics for Medical Diagnosis

BARRY I. EISENSTEIN, M.D.

THE development of molecular genetics, both as a self-contained field and as a body of techniques broadly useful in biologic investigation, has had a profound influence on medical research. The beneficiaries include every discipline in basic science and, at least indirectly, most clinical and applied medical disciplines. Certain technical milestones can be identified over the past several decades that have been particularly important in the progress of the field. One is the discovery of restriction endonucleases, which together with the development of DNA ligation and transformation procedures, led to the ability to clone and thus propagate genes of any organism in laboratory-maintained bacteria. Another, equally important breakthrough was the discovery of a relatively simple way to sequence the DNA of cloned genes.

The recent development of the polymerase chain reaction,[1-6] although not of the same fundamental importance as cloning and sequencing DNA, is clearly one of the most substantial technical advances in molecular genetics in the past decade. A recent citation analysis found that the use of the polymerase chain reaction was reported in 353 journal articles in 1988; only the subject of high-temperature superconductors had greater immediacy.[7] The procedure's importance lies in its ability to amplify impure DNA, either fragmented or intact, by the simple chemical (i.e., in vitro) rather than biologic proliferation of a predetermined stretch of DNA. It is possible to amplify specific DNA sequences — from as short as 50 base pairs to over 2000 base pairs in length — more than a millionfold in only the few hours needed to complete the automated procedure. Moreover, the ability to propagate crude DNA from amounts too minute for standard amplification (i.e., cloning) gives the method such extraordinary power and sensitivity that the DNA in fixed pathological specimens,[8-10] buccal cells from mouth washes,[11] human hairs,[12] a single lymphoid[13] or sperm cell,[14] or ancient mummies[15,16] can now be analyzed.

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THE METHOD

The polymerase-chain-reaction method, developed by a team of workers at Cetus Corporation, is based on the repetitive cycling of three simple reactions, the conditions of which vary only in the temperature of incubation. All three reactions occur in the same small tube and with temperature-stable reagents; the repetitive cycling is therefore self-contained and fully automated. In addition to the target DNA to be amplified, the important reagents are two single-stranded oligonucleotides ("primers"), synthesized to be complementary to known sequences of the target DNA, larger amounts of the four deoxyribonucleoside triphosphates, and the heat-stable *Taq* DNA polymerase, isolated from the thermophilic bacterium *Thermus aquaticus*. Earlier forms of the polymerase-chain-reaction procedure, which used a heat-labile DNA polymerase, required the manual addition of fresh polymerase during each of the 30 or so cycles in a typical run. The substitution of *Taq* polymerase has allowed the automation of the procedure, encouraging the explosive growth in its use.

The first step in the procedure (Fig. 1) is the heat denaturation of native double-stranded DNA, which can be used virtually straight from any clinical, laboratory, forensic, or archeological specimen. The target DNA melts at temperatures high enough to break the hydrogen bonds holding the strands together, thus liberating single strands of DNA, which can subsequently reanneal to any other DNA that has complementary sequences. A great advantage of the polymerase-chain-reaction method is that this target DNA does not need to be particularly pure or plentiful; it can be a minor species in a complex mixture of other DNA and other substances. Recent experience suggests that amplification may begin in a sample containing only a single target molecule of DNA,[5] making the polymerase chain reaction the most sensitive detection technique for specific DNA sequences.

In the second step of the cycle, performed at reduced temperatures, two short DNA primers are annealed to complementary sequences on opposite strands of the target DNA (Fig. 1). These primers are chosen to encompass the desired genetic material; they define the two ends of the amplified stretch of DNA. The two primers must not anneal to each other, and their sites of annealing must be sufficiently distant from one another to allow the subsequent synthesis of new products. The specificity of the polymerase-chain-reaction method derives from the precision, even with short primers, of this DNA–DNA annealing reaction.

The cycle's third step is the actual synthesis of a complementary second strand of new DNA, which

in virtually unlimited amounts, a property it shares with standard gene cloning, is the great power of the polymerase chain reaction. The advantage of gene cloning is that it does not require the synthesis of primers the polymerase chain reaction needs, and it therefore remains the method of choice for many experiments, particularly those in which the sequence of the target DNA is unknown. On the other hand, in the ever-increasing number of cases in which appropriate primers can be synthesized, the polymerase chain reaction far surpasses standard cloning in its simplicity, speed, and ability to amplify a vanishingly small amount of impure starting material.

Research Applications

The polymerase chain reaction is potentially useful in any situation that requires the examination of DNA. Predictably, it is being used extensively in every research field involving genetic analysis. Fields such as archeology and forensic pathology that often avoided DNA analysis in the past because of its technical difficulties are also beginning to use the polymerase chain reaction. By amplifying the target DNA, the polymerase chain reaction can supplement or replace many standard cloning methods.[17] Examples include the isolation and labeling of DNA fragments, site-specific mutagenesis,[18,19] complementary DNA and genomic cloning,[20] analysis of protein–DNA interaction,[21] DNA and RNA sequencing,[22-25] and gene-therapy manipulations.[26]

Perhaps even more exciting to the investigator is the ability to perform DNA manipulations with the polymerase chain reaction that would be very difficult or impossible with standard methods. Among the best examples of this is the ability to alter the amplified sequence to incorporate site-directed mutations into the DNA[18,19] or to add known restriction sites to the ends of the amplified DNA to facilitate subsequent standard cloning. Another method, the inverse polymerase chain reaction, permits the amplification of sequences that flank the known sequence, thus expanding the frontier of explored DNA in the genome under investigation.[27,28] With the progressive reiteration of this procedure, long stretches of chromosome can be mapped. Alternatively, the procedure can be used to facilitate the analysis of genetic regulatory elements, which typically lie outside and adjacent to the genes that are being regulated.

Specific Applications in Medicine

In its most commonly applied form, the polymerase chain reaction is best suited to help answer the often critical diagnostic question: Does a given sequence of DNA exist in a given clinical specimen? As previously mentioned, the polymerase chain reaction is used extensively for this purpose in forensic pathology — to identify the source of blood stains, hair,[12] or sperm,[14] for example. Likewise, since infectious and genetic diseases are characterized by the presence of either foreign or aberrant DNA,[29] the medical fields concerned with such diseases will profit greatly from the diagnostic use of the polymerase chain reaction. There has already been progress in the rapid diagnosis of infectious diseases,[30-32] determining the sex of human embryos before in vitro fertilization,[33] the prenatal diagnosis of genetic disorders,[1,23,34-37] and HLA analysis and tissue typing for organ transplantation.[3,17,25] An excellent example of the use of the polymerase chain reaction for evaluative purposes is in the detection of the human immunodeficiency virus (HIV) genome in people who cannot be determined to be HIV-positive by conventional means, such as infants born to HIV-infected mothers[38] and seronegative people at high risk for the acquired immunodeficiency syndrome.[39,40]

Apart from its value in rapid diagnosis, the polymerase chain reaction has proved highly useful in studying the pathogenesis of disease because of its ability to detect specific DNA sequences that can be correlated with defined pathologic conditions. It has been instrumental in a number of medical discoveries, including the identification of autoimmune-linked HLA alleles in patients with diabetes mellitus[20,41] and pemphigus vulgaris[42,43] and the identification of papillomavirus sequences in malignant conjunctival and corneal tissues.[44] In these cases, the great sensitivity of the polymerase-chain-reaction method and its ease of use with multiple patient samples allowed the identification of critical human or viral DNA sequences that might have been extraordinarily difficult if not impossible to detect by other means. Likewise, in another example of an association with a previously unrecognized infectious disease,[45] the polymerase chain reaction was essential in the identification of a highly unusual form of silent HIV infection in which the patient has reverted to a seronegative status but still carries genetic evidence of the latent virus.[46] Other practical applications of the polymerase chain reaction in diag-

Table 1. Practical Medical Applications of the Polymerase Chain Reaction in Infectious Disease.

Diagnosis*
HIV[38-40,46-49]
HIV-1 and HIV-2 double infection[50]
HTLV-I and myelopathy[51]
HTLV-I and tropical spastic paraparesis[52]
Cytomegalovirus[53-55]
Hepadnavirus[56]
Papillomavirus in urine[57]
Cutaneous herpes simplex virus[58]
Human parvovirus B19[59]
Hepatitis B virus in serum[60]
Slow viruses in brain (BK and JC)[61]
Enterotoxigenic *Escherichia coli*[62]
Legionella pneumophila[63]
Trypanosoma cruzi[64]
Toxoplasma gondii[65]
Viral associations with cancer
HTLV-I and leukemia[66-68]
HTLV-II[69]
Papillomavirus and cervical cancer[9,66-71]
Papillomavirus and corneal lesions[44]

*HIV-1 and HIV-2 denote human immunodeficiency virus types 1 and 2, and HTLV-I and HTLV-II human T-cell lymphotropic virus Types I and II.

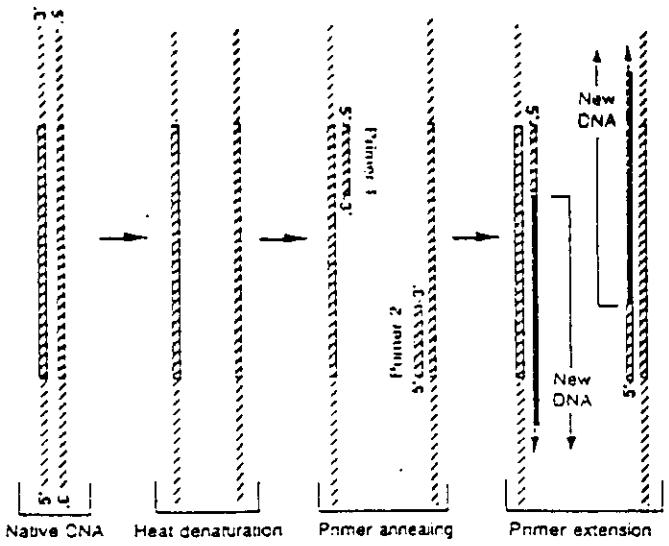

Figure 1. First Round of the Polymerase Chain Reaction.

The basic polymerase-chain-reaction cycle consists of three steps performed in the same closed container but at different temperatures. The elevated temperature in the first step melts the double-stranded DNA into single strands. As the temperature is lowered for the second step, the two oppositely directed oligonucleotide primers anneal to complementary sequences on the target DNA, which acts as a template. During the third step, also performed at a lower temperature, the *Taq* polymerase enzymatically extends the primers covalently in the presence of excess deoxyribonucleoside triphosphates, the building blocks of new DNA synthesis. The native DNA target sequences, which will be massively amplified as "short products" in the ensuing cycles, are boxed. The vector of action of the DNA polymerase is denoted by the arrows projecting from the newly synthesized DNA, indicated by the dark bars.

occurs through the extension of each annealed primer by *Taq* polymerase in the presence of excess deoxyribonucleoside- triphosphates (Fig. 1). A new single strand of DNA is synthesized for each annealed primer. Each new strand consists of the primer at its 5′ end trailed by a string of linked nucleotides that are complementary to those of the corresponding template. An essential feature of the polymerase-chain-reaction procedure is that all previously synthesized products act as templates for new primer-extension reactions (i.e., DNA synthesis) in each ensuing cycle. The result of this aptly named "chain reaction" is the geometric amplification of new DNA products. Since the primers form the kernels of all new DNA strands, each of the two different primers, as well as the four deoxyribonucleoside triphosphates (the building blocks of the DNA-polymer), must initially be present in massive amounts relative to the quantity of target (substrate) DNA. The exquisite specificity of the annealing reaction (second step) and the geometric amplification of the polymerase-chain-reaction products (third step) together give the method its extraordinary sensitivity in detecting the target DNA.

After extension of the primers the cycle is repeated, first by raising the temperature so that all double-stranded DNA is converted to single-stranded DNA, thus aborting any ongoing polymerization, and then by lowering the temperature to allow the steps of annealing and extension. The automation of the polymerase chain reaction depends on the heat stability of all its components: polymerase, primers, and deoxyribonucleoside triphosphates. The procedure also requires enough knowledge of the sequence of the target DNA, at least at the site of primer annealing, to allow the synthesis of appropriate complementary primers. Synthesizing the oligonucleotide primers is itself an automated procedure and relatively inexpensive to perform.

As the steps in the reaction advance, typically going through about 30 full cycles, the primers and deoxyribonucleoside triphosphates are progressively depleted, and the number of new DNA strands expands (Fig. 2). Critical to the method's success, but not obvious in the results of the first cycle, is the geometric expansion in the number of "short products" — identical double strands of DNA, the ends of which are precisely bounded by the two primers and their respective complementary sequences. These short products do not appear until the end of the third cycle (Fig. 2); their number then expands geometrically. In sharp contrast, the number of single-stranded "long products," which are the primer-extension products of the annealing to the original target DNA templates and which contain 3′ ends of various lengths, expands only arithmetically. The consequence of this differential synthesis of well-defined short segments of DNA is that when the polymerase-chain-reaction cycles are completed, the reaction mixture consists almost entirely of short products.

The ability to synthesize defined fragments of DNA

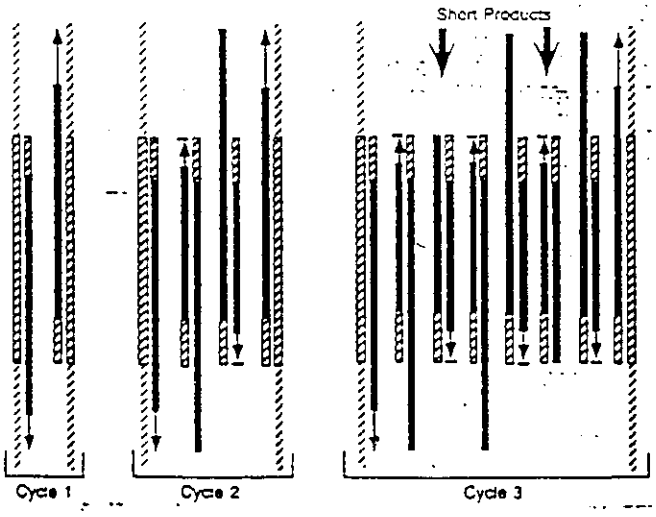

Figure 2. Products at the End of the Initial Polymerase-Chain-Reaction Cycles.

A key element of the polymerase chain reaction is the repetitive thermocycling of the steps shown in Figure 1. At each cycle the dark bars denote the accumulated DNA that has already been synthesized, and the currently synthesized DNA is indicated by the arrows that project in the direction of active DNA polymerization. Since the synthesized products of all previous cycles act as templates for all ensuing cycles, the number of short products increases geometrically at the completion of each cycle (the first three are shown). After the completion of the 30 or so cycles typical of the method, the ratio of short products to other DNA entities becomes so large that they appear to be the only detectable DNA in the reaction mixture.

Table 2. Practical Medical Applications of the Polymerase Chain Reaction in Human Genetics.

Diagnosis
Sickle cell anemia[1-4,11]
β-thalassemia and hemoglobin H disease[12,13,77,78]
Phenylketonuria[13]
Diabetes (insulin-gene mutation)[75]
Cystic fibrosis (allele linked)[17,76,77]
Hemophilia A (allele linked)[24]
Hemophilia B (gene mutation)[78]
Hemophilia B (allele linked)[79]
Cloning factor VIII mutation[80]
α_1-Antitrypsin deficiency (allele linked)[81]
Leber's hereditary optic neuropathy (mitochondrial mutation)[82,83]
Apolipoprotein mutations[84,85]
Duchenne's muscular dystrophy[86]
Lesch–Nyhan syndrome[87]
Huntington's disease (allele linked)[88]
Residual leukemia (Philadelphia chromosome)[89]
Lymphoma dissemination[90]
Pathogenesis (allele-linked diseases)
Diabetes mellitus[20,91]
Pemphigus vulgaris[42,43]
Myasthenia gravis and multiple sclerosis[91]
Oncogene-linked cancers[92-96]

nosis and the investigation of pathogenesis are shown in Table 1 (infectious diseases) and Table 2 (genetic disorders).

Interestingly, one of the great virtues of the polymerase chain reaction, its extraordinary sensitivity, can also be one of its greatest problems in medical diagnosis. False positive reactions from carry-overs of previously amplified DNA and from cross-contamination with true positive samples are major complications.[95-97] The use of the polymerase chain reaction to identify HIV DNA in clinical samples illustrates the point. Since the polymerase chain reaction can find as few as one specific DNA molecule in a sample and since earlier polymerase-chain-reaction amplifications of HIV DNA from previously examined true positive samples will undoubtedly have been performed in the same diagnostic laboratory, the likelihood that future polymerase-chain-reaction assays will be contaminated with HIV DNA is high. To avoid these artifacts, laboratories must not only pay careful attention to routine good practice, such as the extensive use of carefully chosen positive and negative controls, but must also implement special procedures, such as the use of positive-displacement pipettes and the physical separation of reactions occurring before and after the polymerase chain reaction.[17,98] Regardless of the precautions, until the reliability of such tests is rigorously determined, it will be necessary to confirm many diagnoses by other methods.

On the other hand, the ability to identify DNA with such exquisite sensitivity means that the need for radiolabeling for detection can be eliminated.[34,36,99] Because of the need for specially designed synthetic primers, lower cost may not always be an advantage of the polymerase chain reaction. But when speed is an important concern, and as costs decline with increasing volume — particularly if the primer does not need to be custom designed — the polymerase chain reaction should have a major influence on rapid medical diagnosis.[100]

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...viduals. These defects are nonspecific and unrelated to smoking or the presence of a lung tumour. In addition, identification of dynein arms, particularly inner dynein arms, and radial spokes in their entirety is extremely difficult; much depends on fixation, section quality, and thickness.[19,20] In a study by Rutland et al[21] nasal brushings from patients with primary ciliary dyskinesia and normal individuals were examined. The patients showed a greater percentage of microtubular abnormalities, fewer dynein arms, and a lower CBF. There was a wide overlap with the control group, but these workers concluded that "in each of the patients there was an unequivocal abnormality of at least one facet of ciliary motility or structure". Most cases have more than 50% of dynein arms missing and a CBF below 10 Hz.

The most serious consequence of the generalised ciliary defect is the possibility of recurrent lower respiratory tract infections progressing to bronchiectasis; early diagnosis will maximise therapy and may delay or prevent the onset of bronchiectasis. Buchdahl et al[22] have lately suggested that primary ciliary dyskinesia is not uncommon in children with otherwise unexplained chronic respiratory tract disease (prevalence 11%) and that wider facilities should be available for investigating suspected cases.

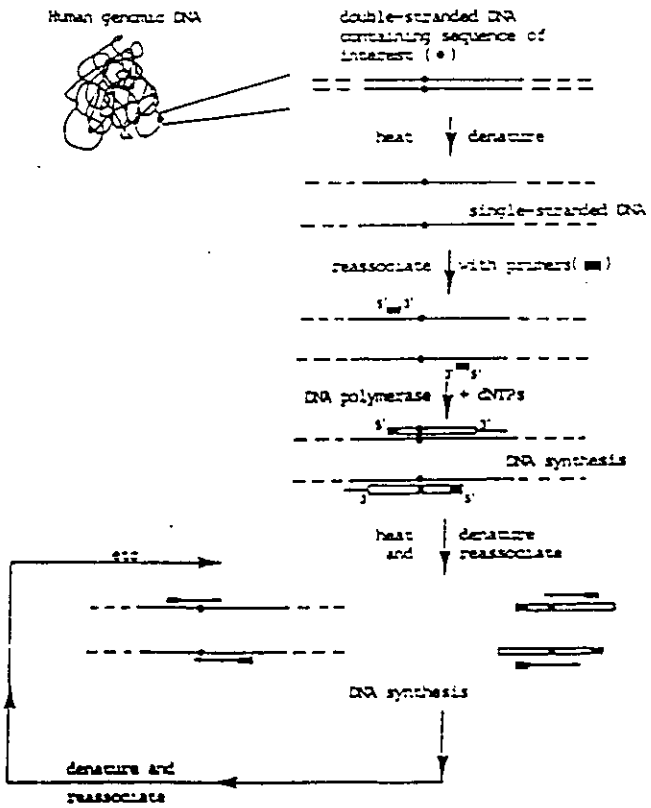
Ciliary dyskinesia may also be secondary to the damaging effect of either pathogenic or host factors, including bacterial products and leucocyte elastase.[23-25] Infection can cause transient dyskinesia, which is more often associated with microtubular abnormalities than with loss of dynein arms; these defects return to normal with time and appropriate treatment.[26]

DNA DIAGNOSIS AND THE POLYMERASE CHAIN REACTION

ADVANCES in DNA recombinant technology have made possible the molecular analysis and prenatal prediction of several human genetic disorders and the diagnosis of infectious diseases. These methods depend upon standard Southern blotting and the use of radioactive probes. A new method, known as the polymerase chain reaction (PCR), and developed by geneticists at Cetus Corporation, is set to revolutionise this technology by making it several times more sensitive and rapid.[1] An example of its application to the analysis of buccal epithelial cells obtained by mouthwash is presented in this issue p 1356.

In PCR, sequences of DNA are amplified over a million times in a few hours. The principle of the method is illustrated below. A pair of oligonucleotide primers, complementary to sequences flanking a particular region of interest, is used to direct DNA synthesis in opposite and overlapping directions. Single-stranded primers anneal to the denatured DNA sample and DNA synthesis proceeds in the 5' to 3' direction with DNA polymerase I after the addition of deoxyribonucleotide triphosphates (dNTPs). After heat denaturation, the newly synthesised molecules as well as the original DNA can reassociate with the primer and act as templates for further rounds of DNA synthesis; theoretically, amplification will increase exponentially to 2^n, where n is the number of cycles. Amplification more than a million times can routinely be obtained. A thermostable *Taq* (isolated from *Thermus aquaticus*) polymerase has lately been used, this being not much affected by the denaturation temperature so avoiding the need for replenishment every cycle.[2] PCR with this enzyme allows automation of the process, permitting the diagnosis of samples in less than a day.

PCR has many applications in clinical medicine. It has already been applied to the diagnosis of genetic disorders.[1,3,4] One advantage is that non-radioactive detection systems can



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be used, making the method accessible to third-world countries. If enough genomic DNA is available, the diagnosis can be made by direct visualisation of the amplified sequences after staining of the gel with ethidium bromide.[4] In view of its high sensitivity, the technique has been applied to single hairs[5] and now, as Mr Lench and his colleagues indicate, to samples obtained by mouthwash. This latter variation has enormous potential in population screening for single gene disorders such as cystic fibrosis.

PCR is very specific, permitting the detection of viral pathogens even when there is a large excess of host nucleic acid. It has been applied to the detection of human immunodeficiency virus[6] and human papillomavirus.[7] PCR analyses can be successfully carried out on formalin-fixed, paraffin-embedded tissues because the reaction is not dependent on high-molecular-weight DNA.[8] Thus analysis at the DNA level can be correlated with histological and clinical information.

PCR amplification is already playing a major role in many laboratories. Automation of the reaction promises the development of rapid, low cost, non-radioactive detection methods for an enormous number and variety of clinical applications.

CRITICAL QUESTIONS; CRITICAL INCIDENTS; CRITICAL ANSWERS

> "It may be difficult for the junior student to realise that all he learns in the earlier courses is necessary to equip him for the work of a doctor in general practice; but he may rest assured that it is. . . . Nothing has been omitted that is necessary, and nothing has been included that is superfluous."—A. MILES; *A guide to the study of medicine;* 1925.[1]

WHAT should medical students be taught? Readers of our correspondence columns (April 16, p 889; May 7, p 1054) will have already noted some debate about the obstetric input. The Hippocratic aphorism, *ars longa, vita brevis*, becomes ever more difficult as the medical art lengthens exponentially while student life remains fixed at five years. The traditional (and still prevalent) solution teaches everything a student might ever conceivably need, and produces a curriculum overflowing with biochemical pathways, physiological niceties, psychological details, and a host of obscure diseases. An alternative solution teaches those things that are especially needed. But what are they? Answers given by medical school committees typically reflect medical school power politics rather than practitioners' actual needs.

The problem of defining course content has now been approached by Waterston,[2] who revives the critical incident technique,[3] a method developed by Flanagan, an occupational psychologist, and described as the most successful method for developing taxonomies of clinical competence.[4] The starting point is actual events in medical practice, reported by practitioners, nurses, patients, or other interested parties. A critical incident is defined as: "any episode of patient care in which . . . specific actions by a physician had . . . specific beneficial or detrimental effects on a patient. The term 'critical' simply means that, very likely, the physician's actions were directly responsible for the effects observed in the patient."[5] A particular, clearly defined piece of knowledge, or an action, skill, or attitude, has therefore indisputably resulted in good or bad practice. Several hundred or several thousand such incidents are classified by several assessors, until the final distillate isolates the skills actually needed for the job, rather than those that are merely desirable in an ideal world. The authors of the most influential of such studies, used to revise the American National Board of Examiners' part III examination, commented that: ". . . these [critical incident] records provide a most interesting—and also a rather disturbing—description of what actually goes on during internship".[6]

Incidents in child health in primary care[2] emphasise the difference between necessary knowledge and knowledge that is only potentially useful. From the massive diagnostic panoply of paediatric neurology, Waterston[2] isolates for special emphasis the differential diagnosis of recurrent convulsions and of a large head in infancy, and the recognition and early diagnosis of meningitis and meningococcal septicaemia, Reye's syndrome, herpes encephalitis, and muscular dystrophy. Here one has a rational, if controversial, basis for curriculum development and assessment, and a technique for defining the core curriculum.[7]

The critical incident technique is sometimes so modified as to lose its special features,[8] so that, like Molière's Monsieur Jordain, who was surprised to find that he had spoken prose all his life, one suspects oneself of always having used the method without awareness. There are no formal assessments of either validity[9,10] or reliability (although critical incidents are the basis of the very reliable situational interview[11]); nonetheless, the technique undoubtedly elicits the key components of a skill, and helps formulate precise teaching objectives in undergraduate and postgraduate medical education. In 1968, the American Board of Orthopedic Surgery classified 1700 critical incidents contributed by 1100 orthopaedic surgeons, into nine major categories and ninety-four sub-categories which formed standardised material for the oral certification examination.[12] Much more specifically, the method elucidated sixty-two separate important skills for administering epidural anaesthetics.[13] It has also been used in creating a medical school curriculum,[14] assessing doctor-

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