AMERICAN MUSEUM
Novitates

PUBLISHED BY THE AMERICAN MUSEUM OF NATURAL HISTORY
CENTRAL PARK WEST AT 79TH STREET, NEW YORK, N.Y. 10024
Number 3097, 31 pp., 84 figures May 19, 1994

Electron Microscopic Studies of Mummified Tissues in Amber Fossils

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ABSTRACT

The degree and consistency of fine and ultrastructural preservation of primarily soft tissue remains preserved in amber fossils were examined, using scanning (SEM) and transmission electron microscopy (TEM). 16 insects of various taxa and structure were studied with the SEM, as well as 4 plant specimens, which were from two chemically distinct ambers: 25–30 million year old (myo) amber from the Dominican Republic, and ca. 40 myo amber from the Baltic region. A new technique was used for "exhuming" a specimen in order to examine the in-situ preservation of whole organs and tissues. Remarkable preservation found in both ambers confirmed earlier reports based on a few specimens. Preservation of all soft tissues in Dominican amber insects appears to be more consistent than in Baltic amber insects, several of which were virtually hollow "casts." Membranous structures preserved in the insects include air sacs, uncollapsed tracheae, and various portions of the gut, as well as the brain and bundles of muscle fibers in their original origins and insertions. Very little shrinkage was observed. Specialized pockets, the mycangia, in wood-boring beetles (Platypodidae) still possessed spores and conidiophores of their symbiotic fungus. For plants, columnar cells of leaf mesophyll were found in their original positions and sizes, pollen grains retained the exine sculpturing, and mats of epidermal cells were preserved in anthers.

At the ultrastructural level, both flight muscle and brain tissues show substantial degradation of cytoplasmic components due to dehydration. Although sarcomeres are easily identifiable in native muscle samples, the sarcomeric repeats disappear upon hydration, indicating that the repeated struc-

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ISSN 0003-0082 / Price $3.20

tures are probably composed of inorganic salts deposited on thick filaments during dehydration. Membranous structures are generally better preserved than proteinaceous ones. Flight muscle mitochondria are particularly well preserved with tracheoles and T tubules also identifiable. Axon tracts in the central nervous system can be distinguished from cytoplasmic regions, and parallel strips of membranes surrounding cytoplasm are abundant in rehydrated brain samples.

Mode of tissue preservation appears to be a rapid and thorough fixation and dehydration, sufficient for preserving DNA in amber more consistently than any other kind of fossil. Pollen was not found viable, but the possibility remains that viable bacterial and fungal spores occur in amber.

INTRODUCTION

In general reviews of taphonomy, reference is often made to Conservat Lagerstätten, or fossil deposits of exceptional preservation (Allison and Briggs, 1991; Briggs, 1991). These include, for example, the Eocene oil shale of Messel, Germany; the fine-grained Lower Cretaceous limestone of Ceará, Brazil (Grimaldi, 1990; Maisey, 1991; Martill, 1988); and the intricately preserved carbonaceous flowers from the Turonian (mid-Cretaceous) of New Jersey (Crepet et al., 1992). For deposits like the shale and limestone, impressions of soft tissues may remain ("extraordinary" preservation, sensu Briggs [1991]), but these are only low molecular weight residues and carbonized films. The flowers from the Raritan-Magothy Formation of New Jersey are actually charcoalified: turned to pure carbon after forest fires swept over them while buried in leaf litter. A sedimentary deposit with unique preservation are the arthropods from the middle Devonian shale of Gilboa, New York (ca. 378 million years old): sockets of setae, sensilla, and other microscopic details are preserved, apparently as the original cuticle (Shear et al., 1984; Schawaller et al., 1991).

Three rarely-discussed modes of preservation retain features of organisms more lifelike than any other kinds of fossils: freezing, dehydration, and preservation in amber. All apparently prevent hydrolysis of complex molecules, by either suspending water as a solid or removing it. Very often frozen organisms, such as the carcasses of mammals in cold deserts, are partially dehydrated, due to the sublimation of ice crystals. Frozen and dehydrated organisms rarely extend past the Holocene or Pleistocene; thus, their paleontological value is limited. Yet, no preservation is more celebrated than the highly ritualized burials of the ancient Egyptians, particularly those from the 21st dynasty (ca. 1050 BC)(David and Tapp, 1984), although exceptional mummification occurs in other ancient cultures around the world (Cockburn and Cockburn, 1980; Hansen et al., 1991). Egyptian cadavers were first eviscerated, then washed in wine, and the thoracic cavity stuffed with incense, cassia and other spices, and crushed myrrh (a fragrant, resinous gum from *Commiphora* [Burseraceae]). The cadavers were also stored for about 70 days in dry natron (hydrated sodium carbonate, $Na_2CO_2 \cdot 10H_2O$, a natural deposit of salt lakes). Linen wrappings around the body were likewise impregnated with resin, or with pulverized amber. The success of Egyptian mummification was revealed by histological (Sandison, 1955; Zimmerman, 1973) and ultrastructural studies (Curry et al., 1979; Lewin, 1967; Riddle, 1980). Cells, albeit shrunken, contained nuclei and nuclear membranes, although mitochondria were not readily observable (Lewin, 1967). DNA, too, has been extracted and sequenced from Egyptian mummies (Pääbo, 1985).

How the Egyptians began the use of amber for mummification is intriguing, especially since amber does not occur in Egypt. Amber is the highly polymerized, fossil form of tree resins; hundreds of deposits occur around the world, varying in age, botanical origin (Langenheim, 1969), and the kind and quality of small organismal inclusions in it. Very ancient amber (ca. 125 million years old) occurs in Lebanon, Jordan, and Israel (Schlee and Dietrich, 1970; Bandel and Vavra, 1981; Nissenbaum, 1975), but the early Egyptians probably traded for it with the Phoenicians, who may have acquired it from more extensive deposits in Sicily. Amber and resin were certainly not as important as preservative agents of the mummies as was the natron,

but their use was perhaps inspired by the remarkable external preservation of small organisms, like insects, that the Egyptians probably saw in the amber. They certainly were unaware of the great antiquity of amber, and of the preserved details of internal tissues. Here, we report the preservation of soft, internal insect and plant tissues fossilized in lower Tertiary ambers, with unexpectedly consistent, lifelike fidelity: a process of natural mummification, with finer preservation than is found in human mummies, and perhaps the ultimate in Conservat Lagerstätten.

Acknowledgments

It is a pleasure to acknowledge the help of several people who were instrumental in bringing this study to fruition. At the AMNH, Peling Melville provided a great deal of time and expertise to the scanning electron micrographs; and Jacquie Beckett produced prints from the SEM negatives. Jake Brodzinsky was a very helpful source of study specimens. We thank Drs. Rob DeSalle (AMNH), George Eickwort (Cornell University), and Jean Langenheim (University of California, Santa Cruz) for their critical review of the manuscript; and Drs. Thomas D. Pollard and David B. Weishample of the Johns Hopkins School of Medicine, who provided support and stimulating discussions.

Costs for the work at the AMNH were payed for by an NSF grant to Melanie Stiassny, which sponsored Elizabeth Bonwich in the REU program in Comparative and Evolutionary Biology. The personal generosity of Dr. Herbert Axelrod is gratefully appreciated, which allowed for the purchase of the specimens used here and numerous other rare ones which will never, ever be marred.

Abbreviations

an	antenna
as	air sacs
at	atrium (of spiracle)
b	brain
cm	cibarial muscle
cr	crop
ef	eye facets
es	esophagus
fl	flagellomere
ge	genitalia
gl	glossa
g	gut
h	head
lmd	longitudinal median dorsal muscle
my	mycangium
oc	ocelli
oim	oblique intersegmental muscle
olm	oblique lateral muscle
pe	pedicel
ph	phragma
pr	pyloric region
th	thorax
tr	trachea
ve	ventriculus

Previous Studies

There have been several reports on the preservation of tissues in amber fossil arthropods, each of which is based on only one or two specimens. Kornilowitsch (1903) was the first to report the fine structure of tissue preserved in amber fossil insects. He thin-sectioned the legs of Diptera and Neuroptera in Baltic amber and found remarkable striations in the muscles. Mierzejewski (1976a) was the first to apply electron microscopy to tissues "exhumed" from amber fossils. He reported "optic cells, pigment cells, or crystalline cones" in the facets of a dolichopodid fly preserved in Eocene Baltic amber, as well as the minute tracheae that deliver oxygen to these and other insect cells. (Unfortunately, the SEM in his plate II, fig. 2, offers little resolution; secondary iris cells in the middle of each optic cell do not appear to be preserved). Elsewhere (Mierzejewski, 1976b) illustrated with the SEM an entire book lung from a Baltic amber spider: these are an invaginated series of thin parallel plates with a thin cuticular covering. The reports by Poinar and Hess (1982, 1985) and Poinar (1992) are based on a single sample of tissue which adhered to the inner wall of the abdomen of a fungus gnat (Diptera: Mycetophilidae), also in Baltic amber. Using transmission electron microscopy (TEM), they reported nuclei, "lipid droplets" (histochemical tests for lipids were not done), mitochondria and cristae, and apparent endoplasmic reticulum in epidermal cells. Muscle banding was not well preserved. They considered such preservation to be anomalous, since it was believed that the resin would need to be in direct con-

tact with tissues, which might occur if the body wall was traumatically opened. The possibility was mentioned that "sugars and terpines [sic] present in the original resin" could have dehydrated the tissues. Poinar (1992) also presented a TEM of tissue from the abdomen of a braconid wasp in Canadian Cretaceous amber (ca. 80 myo), in which "folded membraneous [sic] structures adjacent to the vacuolated cytoplasm" (p. 270) were identified.

Schlüter (1989) presented SEMs of a termite and possible ant head preserved in Cenomanian amber from France (ca. 100 myo). Fine structure of the epicuticle that was observed included microtrichiae, and reticulations on the original cuticle. The preservation of soft tissues was not examined. He also found minute crystals of marcasite in some specimens, which is often associated with the iron-rich sediments of ancient deltas where amber deposits usually occur. Ground water with dissolved minerals seeps into fine cracks and permeates an inclusion where crystallization later occurs. In fact, Baroni-Urbani and Graeser (1987) described with SEMs a pyritized cast of an ant in Baltic amber. Enough detail was preserved to determine that the cuticular microsculpturing of this extinct species differed from that of any living species. The work of these authors implied, though, that electron microscopy of the cuticle of amber insects would depend on pyritization, since this mineral would conduct electrons well.

Studies by Henwood (1992a, b) used SEM and TEM on three insects preserved in Oligo-Miocene amber from the Dominican Republic. In the TEM study, the flight muscle of an empidid ("dance") fly was cross-sectioned, revealing the hexagonal ultrastructure of myofibrils and the mitochondria densely packed among them, even showing the intricate folding of the internal cristae. Longitudinal sections of the flight muscle, which could have shown additional ultrastructural detail, were not made. In the SEM study, Henwood studied two beetle specimens, a cantharid ("soldier beetle") and a nitidulid ("fungus beetle"). Nitidulids are as heavily sclerotized as most other beetles (the cantharid less so), and both these specimens were completely intact, indicating that the viscous resin does not need to contact the internal organs directly. Some tissues were found in their original positions, but shrunken to about 50% of the original size. Most interesting was the observation of a proventriculus in the nitidulid, which is posterior to the esophagus and is very slightly sclerotized (virtually membranous). Blunt, heavily sclerotized teeth lining the interior serve as a grinding mill and food filter, and the entire structure was remarkably intact. Unfortunately, Henwood's method of cutting entirely through the specimen with a saw abraded much of the internal remains, and she concluded by acknowledging the need for an alternative method of extraction.

The present study expands upon the previous ones by using many more specimens of insects of various taxa, sizes, internal anatomy, and degree of cuticle sclerotization as well as several plant specimens. The insects derive from two chemically distinct ambers, which allows additional taphonomic comparisons. This approach more fully addresses the question of consistency of soft tissue preservation in amber fossils. Also, we used a procedure for examining whole tissues and organs that was minimally destructive, leaving the internal structures more intact. The remarkable tissue preservation is apparently due (at least partially) to dehydration, which is an ideal condition as well for long-term storage of pollen (Shivanna et al., 1991). Thus, we examined possible viability of *Hymenaea* pollen in Dominican amber, which is the tree that gave rise to this amber (Langenheim, 1969; Hueber and Langenheim, 1986) and whose anthers are a relatively common inclusion.

MATERIALS AND METHODS

Amber fossilized insects and plant parts were acquired from several sources. Baltic amber was acquired from Jorge Wünderlich (Staußenhardt, Germany), and Dominican amber from Jacob Brodzinksy (Santo Domingo, Dominican Republic), Manuel Perez (Orland, FL), and several other dealers in Santo Domingo and Santiago. Specimens were selected according to several criteria, most important of which was an abundant supply of individuals. Particular effort was made to use only common species (e.g., where

a series of at least 20 specimens existed in the AMNH collection and which could be easily replaced). Secondly, selected specimens had no fine cracks between the surface and the inclusion, which might have allowed invasion of the amber seal by bacteria, moisture, or other elements of decomposition. Third, specimens represented a great taxonomic diversity, which generally corresponded with great variation in basic body form, habits, relative amount of muscle mass, etc. . . . Fourth, species of significantly different sizes were chosen, since the proportion of muscle mass to body surface area would apparently be a significant factor in mummification. Lastly, species were chosen based on differences in the degree of sclerotization and thickness of cuticle, beetles being the most heavily sclerotized and termites being the least.

The following specimens were examined: For Dominican amber—three stingless bees (*Proplebeia dominicana* [Wille], Apidae: Meliponini), two termites (*Reticulitermes* sp., Termitidae), three platypodid beetles, two fungus gnats (*Mycetophila* sp.: Mycetophilidae), two scuttle flies (*Megaselia* sp.: Phoridae), one leaflet of *Hymenaea* (Leguminosae), and seven anthers of this tree. For Baltic amber—two dolichopodid flies, and two mycetophilid fungus gnats.

Internal organs of the insect inclusions and the pollen contents of stamens were exposed using the following method. Each specimen was first photographed with light micrography using a Zeiss SV-8. Amber was ground to within 3–4 mm on all sides of the inclusion. A groove ca. 1.5 mm thick was circumscribed around the mid-sagittal line of the specimen (while observing under 10× magnification), using a 2 in. diameter emory wheel on a motorized flexible shaft (Dremel, Inc.). The groove formed a circle less than 1 mm from the inclusion. Fine powder from the cutting was blown away with compressed air. A sharp, pointed X-Acto blade was used to carefully score the internal edge of the groove, even closer to the inclusion, until slight leverage between the two halves of amber caused the piece to split, generally along the axis of the groove and through the middle of the inclusion. Very little particulate debris from the cutting contaminated the preparation and no abrasion of the internal parts occurred.

For observation with the SEM, the intact end of the amber piece was mounted to a stub using liberal amounts of silver paint. A 5Å coating of gold-palladium was applied. Amber has excellent insulative properties, as best seen when it gathers static charges upon rubbing (hence the Greek name for amber, *elektron*). Thorough grounding will reduce electron charging, although additional precautions were made using low voltage (2-3 kV). Use of a Zeiss DSM (Digital Scanning Microscope) 950 with electron-collection enhancement features allowed study at such low kVs with little loss of resolution.

To observe the ultrastructure of tissues and cells with the TEM, two specimens of the extinct Dominican amber bee, *Proplebeia dominicana*, were freshly opened (this is one of the most common inclusions in this amber). Bundles of the longitudinal median dorsal muscles were carefully lifted from the thorax using hand-sharpened watchmaker's forceps, with the tips first washed in alcohol and dried. Brain tissue was removed from the head in the same way. Tissue specimens were immediately stored in dry Eppendorf tubes and express shipped from New York to Baltimore (1 day delivery). They were not frozen or refrigerated. Numerous possible "controls" could be used to compare to the amber specimens: air dried and freshly fixed specimens; and specimens stored for varying amounts of time in tree saps, various fresh resins, Canada Balsam, etc. . . . That is a future project beyond the scope of the present one; it would address the chemical factors responsible for the preservation we report here.

Samples were prepared for transmission electron microscopy by two protocols. Some were embedded directly in LR White resin (Newman et al., 1983; Polysciences Inc., Warrington, PA) by immersing the tissue in unpolymerized LR White for at least 8 hours. A second set of tissue samples was rehydrated and dehydrated before embedding. These samples were rehydrated overnight at room temperature in Ruffer's solution (30% ethanol, 1% $NaCO_3$ in distilled water [Lewin, 1967]). Following rehydration, the samples were dehydrated by progressive 60 minute

incubations in 30, 50, 75, and 90% ethanol in distilled water. The final dehydration was carried out by three 20 minute incubations in 100% ethanol followed by one 20 minute incubation in 50% ethanol/50% LR White. The samples were then infiltrated in 100% LR White for at least 8 hours. Each set of embedded samples was polymerized at 50°C overnight in fresh resin in gelatin capsules. Ultrathin (90 nm) sections were cut from the polymerized blocks on a Reichert-Jung Ultracut E microtome using a Diatome 35° angle compression-free knife, then transferred to Formvar-coated EM grids and allowed to air dry. The sections were stained with 1% OsO_4 and uranyl acetate. All samples were viewed and photographed on a Zeiss TEM 10A transmission electron microscope at 60 kV.

To test for pollen viability we attempted to induce pollen tube growth on the following medium: 1 ml. of "mother" solution (0.1 mg/ml boric acid, 0.3 mg/ml $CaNO_3$, 0.2 mg/ml $MgSO_4$, 0.1 mg/ml KNO_3 dissolved in sdH_2O), which was added to: 9 ml sdH_2O, 9 g sucrose, 0.8 g gelatin. Pollen controls were from fresh petunia flowers. Negative controls were made by microwaving the petunia pollen on high for 2 minutes, which rendered the pollen inviable. Four anthers of *Hymenaea* in Dominican amber were cut open and the tissues scraped out using an ethanol-cleaned pin. Controls and experimental samples were incubated on the culture medium for 72 hours at room temperature and examined for pollen tube growth. Experimental samples (from amber) were tested in an area of the lab separate from the controls, to reduce any possible contamination.

Mounted specimens were retained on SEM stubs and are deposited in the amber fossil collection of the AMNH, should future examination be of interest. Each has a catalog number, to which we refer in the text and the figures. The following references were consulted for interpreting morphological and cytological structures; terminology derives from these references: Bold (1973); Chapman (1982); Smith (1968); Snodgrass (1925, 1935). The SEM plates are arranged according to the specimen and type of organism, even though the following discussion is presented by types of tissues. TEM plates are presented last, but ultrastructure is discussed under sections concerning the appropriate tissues.

THE AMBERS

Amber from the Dominican Republic and the Baltic were chosen because of the differences in age and chemistry, the latter feature of which is well documented. Dominican amber was unquestionably formed from an extinct species of the living genus *Hymenaea* (Leguminosae) (Langenheim, 1969; Hueber and Langenheim, 1986). Although cited often by some as being Eocene in age (e.g., Poinar, 1992), the available evidence on stratigraphy suggests an origin around the Oligo-Miocene boundary for the oldest deposits, and Henwood (1992b) cautioned against an Eocene age. *Hymenaea* trees today produce copious amounts of resin, containing arrays of sesquiterpene hydrocarbons and diterpenoid resin acids (Langenheim, 1981; 1990).

Baltic amber, by contrast, derives from a conifer, possibly a pine (*Pinus*) or close relative, but probably an araucarian (Gough and Mills, 1972; Mills et al., 1984). It has a particularly high concentration of resin acids, such as succinic and communic acids (Gough and Mills, 1972). The chemical differences between Dominican and Baltic amber are readily observable via the external preservation of the insects in them. Insects in Dominican amber are typically perfectly preserved, whereas Baltic amber specimens often have a milky coating on the body. This milkiness is due to a froth of microscopic bubbles (Mierzejewski, 1978), presumably the products of microbial decomposition and/or autolysis of internal tissues.

Much older amber fossils from the Turonian (mid-Cretaceous, ca. 90–94 myo) of New Jersey and the Neocomian of Lebanon (ca. 125 myo) are in the AMNH collections, but were not used for these tissue studies. The paleontological value of this material is greater than that of the Tertiary material, and it is difficult (or impossible) to replace. Any studies causing partial or entire destruction of a Cretaceous specimen should be done only after exhaustive morphological study on a large series of specimens of one species.

INSECT PRESERVATION

Cuticular Structures. Preservation of cuticular structures (external as well as invaginated into the body) would not seem surprising in amber fossil insects, given the intricately preserved external detail of insects. The natural positions of structures, and the detail of their preservation, however, were unexpected. In the stingless bee (specimen B1), the row of imbricate plates on the glossa (or tongue) was found, with a fringe of fine hairs along its left edge—the hairs function in imbibing nectar (fig. 6). Also seen in the stingless bees (B1, B3) was a curious detail of the mesothoracic phragma (a paddle-shaped apodeme). It possessed a geometric pattern of eight cells, each having a finer pattern of roughly hexagonal cells on it (figs. 17, 18). These must be imprints of the muscle bundles, fibers, and myofibrils. Similar geometric patterns are observed on insect egg chorions, which are the imprints of the follicle cells (Hinton, 1981); and the epicuticle of various insects bears the hexagonal imprints of epidermal cells. It has always been assumed that the original cuticle was intact in amber fossil insects, but the degree of preserved detail and molecular composition has not been examined. In one of the scuttlefly specimens, the reverse and obverse images of the antennae showed that actual sensilla and setulae can be preserved not just as imprints (figs. 26, 27). This specimen clearly shows how the actual material is separated from and smaller than the amber "cast." Since it is unlikely that the cuticular surface would shrink, even during dehydration, it is more likely that the cast surface represents expansion of the amber, probably due to polymerization of the original resin. How much original chitin remains in the "amberized" insect cuticle has yet to be determined. Insect cuticle is composed of 25–40% chitin, which is a β-pleated polysaccharide sheet intercalated with proteins. Miller et al. (1993) found that the cuticular remains of Pleistocene beetles buried in sediments (ca. 15,000 years old) contained half the amount of chitin that would be expected from fresh material.

Fine structural details of the original cuticle can also be seen by the microtrichiae preserved on the wing membrane of a dolichopodid fly in Baltic amber (fig. 56). A row of six minute campaniform sensilla occur on the wing vein of a mycetophilid midge, also in Baltic amber (fig. 61).

Musculature. Insects have a complex musculature, possessing approximately twice the number of muscles as do mammals (Snodgrass, 1935). Much of this musculature is in the thorax and serves to power the legs and wings. Commonly seen was preservation of the longitudinal median dorsal muscles of the thorax; in stingless bee specimen B1 (fig. 4) it is seen as closely joined bundles forming a large sheet occupying most of the thorax. This muscle mass is completely intact. Smaller muscles are also well preserved, with their insertions and origins intact. A bundle of 10–11 muscles is attached to the mesothoracic phragma and postnotal wall of stingless bee B1 (fig. 10). In B3, the slender oblique laterals, oblique intersegmentals, and portions of longitudinal median dorsal muscles were seen (fig. 17). Small muscle fibers attached to the membranous sucking pump (pharynx) of stingless bee B1 showed transverse striations at higher magnification (fig. 8). A compact bundle of small muscles was found dislodged from the mostly empty thorax of a mycetophilid midge in Baltic amber (fig. 55). Not all muscles appeared so intact: some thoracic muscles of platypodid beetle P1 were very fibrous and shredded. Shrinkage of muscles was not discerned in any specimen that possessed them, although the muscle and other soft tissue was always a very dark red or tar black. Causes of the discoloration are unknown.

At the ultrastructural level, native *Proplebeia dominicana* flight muscle is easily identifiable, with striking patterns of repeated sarcomeres present in longitudinal sections of all myofibrils (fig. 69). The Z-and M-lines are present as spaces between the electron-dense remains of the thick filaments in the A-band. Higher magnification reveals almost no fibrillar appearance to the A-bands, suggesting that the electron-dense material present there is composed primarily of inorganic salts deposited on thick filaments during dehydration (figs. 70–71). Strips of electron-dense mitochondria separate the myofibrils. The

mitochondria have well preserved, densely packed cristae characteristic of insect flight muscle (fig. 72).

Membranous structures in flight muscle are generally better preserved than proteinaceous ones. In addition to mitochondria, tracheoles are present, and the membranes of epithelial cells lining these tracheoles are apparent (fig. 73). In some sections, T-tubules are preserved (fig. 74). Extensive membranes are often found superficial to the muscle cells themselves (fig. 75).

Samples that were rehydrated prior to embedding are essentially composed of strips of mitochondria, separated by spaces with almost no electron-dense material (fig. 76). The material composing the sarcomeres is almost completely extracted by the rehydration step. We suspect that the extracted material is composed of inorganic salts, with perhaps some remaining proteins. It is possible that fixation with glutaraldehyde during rehydration might preserve some proteinaceous structures like sarcomeres while extracting inorganic salts.

Several flight muscle samples dissected from *Proplebeia dominicana* had an additional tissue structure attached to the surface of the muscle bundle itself (fig. 77). We suspect this tissue to have connected the flight muscle to the axillary sclerites of the wing, since cuticle is often found attached (fig. 78). This tissue consists primarily of amorphous fibrillar material (probably collagen fibers) interposed with small electron-dense particles (figs. 79–80). We do not know the origin of these particles, which may be nuclei, cells that have been extensively distorted by the rehydration process, or precipitated inorganic salts. In any case, the fibrillar matrix is apparently quite well preserved, and is an attractive candidate tissue for isolation of fossil polypeptides.

Nervous Tissue. Nervous tissue is notoriously difficult to preserve well. It was very surprising, therefore, to find several specimens with the brain (protocerebrum) intact (stingless bee B1 [figs. 5, 9]; phorid fly [fig. 25], platypodid beetle, P1 [fig. 30]), and mycetophilid midges [fig. 59]). In the stingless bee and platypodid the tissue was loosely fibrous, indicating loss of some interstitial material, although there was virtually no shrinkage of the entire structure. In the other specimens this structure was dense and amorphous.

Brain tissue removed from a specimen of *Proplebeia dominicana* was embedded and sectioned for TEM and was rather poorly preserved, as expected. Some histological features are still observable, however. Most areas of the sections are covered with a dense outer layer of electron-dense salts (fig. 81). Notable exceptions to this are regions of convoluted membranes, which we suspect to be the remains of the major axon tracts of the central nervous system (fig. 81).

In brain tissue that was rehydrated before embedding, some other interesting details became apparent after extraction of the inorganic salts. In some areas, extensive tracts of parallel membranes surround patches of electron-dense cytoplasmic remains (figs. 82, 83). We believe these to be membranes of oligodendrocytes wrapping large axons. Another possibility is that these membranes are artifacts occasionally seen in TEM of necrotic tissue (commonly called "myelin figures"), caused by extraction of lipids from dying cells. Cytoplasm of neural cells is generally poorly preserved, with almost no cytoplasmic details. The putative axon tracts observed in brain sections are also preserved after rehydration, although the membranes appear swollen and distended compared to native samples (fig. 84).

Membranous Structures. These include portions or most of the digestive, excretory, and reproductive systems, as well as the dorsal aorta. In several specimens, but seen best in B1 (figs. 11, 12), were the folds of fine membranous air sacs, which occupy the posterodorsal half of the thorax in bees. Unlike the other parts of the respiratory system (tracheae and tracheoles), these membranes have no trusswork of minute, chitinous rings (taenidia). Excellent examples of preserved tracheae are seen in a Baltic amber mycetophilid (figs. 67, 68). The tracheae are hardly collapsed. Cross sections of tracheoles were seen in TEM thin sections of *Proplebeia* bee flight muscle from Dominican amber (fig. 73).

Portions of digestive tracts of several specimens were remarkably intact. In one mycetophilid the ventriculus possessed a geometric pattern of folds on the outer surface,

each fold forming a cavity with a papilla in it (figs. 21, 22). The papilla is probably a crypt, perhaps of regenerative cells. A portion of the thin esophagus was seen in one termite (specimen T1, not figured); and a more extensive portion of the foregut, including the crop, was found in stingless bee B2 (figs. 16, 17). In one platypodid beetle, an extensive portion of the gut in the abdomen was observed, which included the entire midgut and hindgut (fig. 38). The region where the fine malpighian tubules occur had an odd preservation, which was an amorphous arrangement of stacked mounds; holes; and flat, hexagonal crystals (fig. 41). Other parts of the midgut showed unusual formations of deep, fine pleats (fig. 39). The detail of membranous tissue preservation is best seen from an unidentified structure from a termite thorax (fig. 24). The tissue is very thin with one margin frayed, showing loose fibers.

Other organisms. Intimately associated with arthropods in amber are a host of organisms, such as parasites and symbiotic microbes, as well as evidence of other ecological relationships. It is not uncommon, for example, to find stingless bees in amber with clumps of pollen adhering to the hairs on the hind legs and abdomen. One bee specimen (B1) was examined that had a clump of pollen on the ventral side of the abdomen. The exine was intact enough to observe that two distinctly different pollen types were collected (fig. 13), indicating that the bee had been visiting two kinds of flowers. The exine of the most common pollen type is very finely pitted and quite similar to that of living *Hymenaea* (Langenheim and Lee, 1974) as well as to the amber *Hymenaea* (fig. 48); the other type has coarse sculpturing (fig. 14).

Some wood-boring beetles feed not on the wood, but on the fungus cultures that they inoculate in the galleries. Ambrosia beetles (families Scolytidae and Platypodidae) are quite common in the Dominican amber; no doubt they were living in the amber tree itself, since amber pieces are occasionally found with beetle galleries in them. The beetles transmit the fungus via specialized structures, the mycangia (Batra, 1963). Mycangia are pockets of invaginated cuticle that occur in various insects, mostly beetles, that harbor inoculum of symbiotic fungi. The beetles feed on the fungi and also serve as the main dispersal agent. Mycangia vary tremendously in the Coleoptera, with structures occurring on the mandibles, head, elytra, abdomen, and thorax (Crowson, 1981). Perhaps no fungal-beetle symbiotic relationship is more specialized and intimate than that of ambrosia beetles and their ambrosia fungus (subclass Hemiascomycetidae). The various ambrosia fungi are known only from the beetle's mycangia and galleries and are specific to the species of beetle, not the host tree (Batra, 1963).

Specificity of the ambrosia fungus to the beetle may be due to the apparent glandular nourishment that the fungus receives in the mycangium. In fact, the shape and location of saclike mycangia in the Scolytidae and Platypodidae are often species-specific (Batra, 1963; Francke-Grosmann, 1956). Batra classified five categories of mycangia in these beetles based on their location on the body.

In an unidentified platypodid beetle (P2), two large ventral mycangia were found on the insect's left side (mycangia occur in pairs; see fig. 33). The anterior one was largest, approximately 200 μm long, drop-shaped, and was lying between the meso- and metathorax (fig. 34). The posterior mycangium was round and lying between the metathorax and abdominal sternite 1 (figs. 35). The size and general location correspond with other reports based on living species (cf. figs. 5, 16, and 17 in Batra [1963]). Both mycangia were replete with spores and conidiophores (fig. 36). Viability of the spores has not been tested.

PLANT PRESERVATION

Leaves typically have a heavily cutinized (waxy) epidermis, beneath which there is a layer of mesophyll. The mesophyll is the photosynthetic layer of cells, and comprises of a spongy layer of squamous cells and a palisade layer of columnar cells. In a small leaf of *Hymenaea* in Dominican amber, an entire surface was exposed, which was charcoal-black, finely cracked, and crumbling (fig. 44). Close examination, however, revealed that the palisade layer of mesophyll was intact in places: columnar cells were easily seen and showed no distortion (fig. 45).

The anthers of *Hymenaea* are, not surpisingly, rather common in Dominican amber. According to Jean Langenheim (personal commun.), during the pollination period the area beneath a *Hymenaea* tree is covered with dehisced stamens (the anthers do not fall from the filaments). Seven specimens were exhumed: four for testing pollen viability, three for study under the SEM. In five of the anthers the material inside had a black, gummy appearance, but was actually brittle and very dry (e.g., figs. 46, 47). Under the SEM this material was amorphous, and presumably is a dried film lying over pollen and tissue. In anther specimen A1 most of the pollen was not clearly visible because it was obscured by this apparent film. Some pollen grains that were dispersed into the amber were found to have the exine less obscured (this anther was chosen for study, in fact, because it was dehiscent and apparently mature). The exine of the pollen of the extinct Dominican amber tree, *Hymenaea protera*, has a surface of dense, fine pits very similar to that of *Hymenaea courbaril* and *H. verrucosum* (fig. 48; cf. fig. 4 in Langenheim and Lee [1974]). This is also very similar to the pollen found on stingless bee specimen B-1 (fig. 14). In the other two anthers (1 dehiscent, the other not; see figs. 49–51) there was completely different preservation. Internal tissues were not a tarry black, but chalky and brown. Extensive mats of dense, fine, hairlike structures filled most of the anther, but no pollen grains were apparent. These structures are probably columnar epithelial cells. In the test for viability, pollen tubes grew on all the positive control samples. As expected, no tubes were found in the negative controls or in the experimental samples removed from the *Hymenaea* anthers in Dominican amber. Despite the dehydration properties of amber, enzymes and other labile molecules in the pollen probably undergo autolysis.

CONCLUSIONS

The observations reported here and elsewhere reveal tissues, cells, and cellular ultrastructure in amber insects and plants, with a startling lifelike fidelity. In two of the four small (< 4 mm body length) Baltic amber flies that were examined here, the thoracic and abdominal cavities were largely vacant of tissue, but internal tissues and organs of the other Baltic amber flies were as intact as is typically found in the Dominican amber insects. Thus, the preservative qualities of ambers are not equivalent, although the tissue which did remain in the Baltic amber flies was as well preserved under the SEM as that in Dominican amber flies (TEM of Baltic amber fly muscle, for example, was not done in our study). In the insects with preserved tissues, the organs showed little or no shrinkage, contrary to the observations of Henwood (1992b) who reported up to 50% shrinkage of muscle from a Dominican amber fly. The general lack of shrinkage or autolysis, and preservation of such delicate structures as air sac membranes and brain tissue, indicate a very rapid mummification, which tempts explanations on the possible chemical process involved.

The best scenario we can provide to explain such apparently rapid and thorough cellular fixation is that the most volatile, low molecular weight fractions (mono- and sesquiterpenes) in the original resin readily diffused through intact body walls and perfused the tissues. For insects, the intersegmental membranes would be an important area of diffusion (even though they are thinly cuticular and possess a waxy layer, the intersegmental membranes are the thinnest parts on an arthropod). These volatile fractions must have replaced the cellular water. This can be seen in many amber fossilized insects: often there is a transparent, light brown "halo" around them, which must be aqueous components of the body fluid sequestered by the surrounding resin. Monosaccharides, alcohols, aldehydes, and esters also occur in resins (Langenheim, 1990), and a few investigators have implicated a role for at least some of these in "amberization." Currently, studies have begun to examine the infiltration of the volatile sesquiterpene hydrocarbons and some oxygenated forms into insect tissue. The role of monosaccharides and other compounds is probably much less significant than that of terpenes, because they occur in much lower concentrations, and (with the exception of alcohols) would perfuse tissues more slowly than volatile terpenes. Henwood (1992a), for example, mocked a sap flux with maple

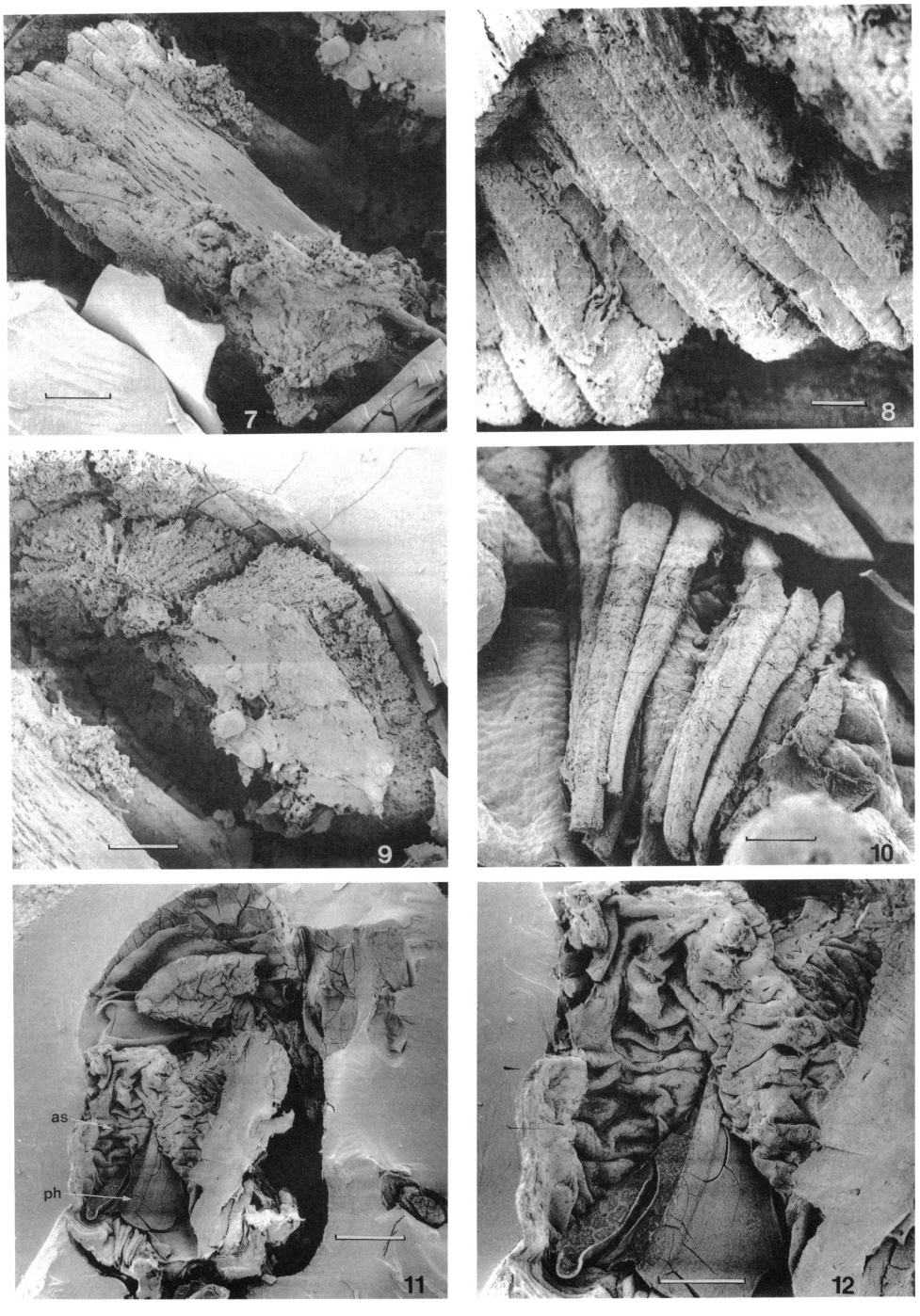
syrup, in which flies were preserved and their muscles later thin sectioned. Muscles in the syrup-preserved flies were not nearly as well preserved as the muscles of amber insects reported here and by Henwood, and the sugar concentration of the syrup is much higher than any found in natural plant exudates, let alone in resins.

The ultrastructural results prompt questions as to the actual molecular preservation that has occurred. What proteins, if any, are present? In what state might they be? Immunological evidence suggests preservation of protein tertiary structure in brachipod shells that are up to 4 million years old (but no older) (Collins et al., 1991), and glycoproteins from an 80 million year old mollusk shell were reported (Weiner et al., 1976). The density of mollusk and brachiopod shells may uniquely provide for the preservation of such macromolecules, particularly since the mode of fossilization in marine sediments would seem less than ideal for protein preservation. Contrary to this are the plant fossils from the fine, dense, anoxic clays of the mid-Miocene of Clarkia, Idaho. Chloroplast preservation is reported from leaves found there (Niklas et al., 1985), as well as two cases of DNA, from a magnolia (Golenberg et al. 1990; Golenberg, 1991) and a bald cypress (*Taxodium*; see Soltis et al., 1992). However, Logan et al. (1993) found that preservation of biomolecules is highly selective in Clarkia fossils, with no evidence of polysaccharides, polyesters, or proteins, but only lignins and an aliphatic biopolymer present. Logan et al. questioned the DNA results from the Clarkia plant fossils and, indeed, DNA preservation in them is hardly consistent: among "hundreds" of extractions, these two published examples and several unpublished ones are the only successes (P. and D. Soltis, personal commun., 1992; E. Golenberg, personal commun. 1993). Quality of protein preservation is dependent on how one analyzes the molecule. Amino acid sequencing might reveal short chains of α-amino acid units, a result of peptide bond hydrolysis, and it would be useful only if hydrolysis occurs preferentially in some bonds. Or, side groups such as amino and carboxyl groups could be lost. If analytical methods are dependent on tertiary structure for detecting protein preservation, one would also be measuring the degree to which H bonding and crosslinking are still intact (which varies with the kind of protein). Ambler and Daniel (1991) reviewed successful extractions of protein from ancient materials.

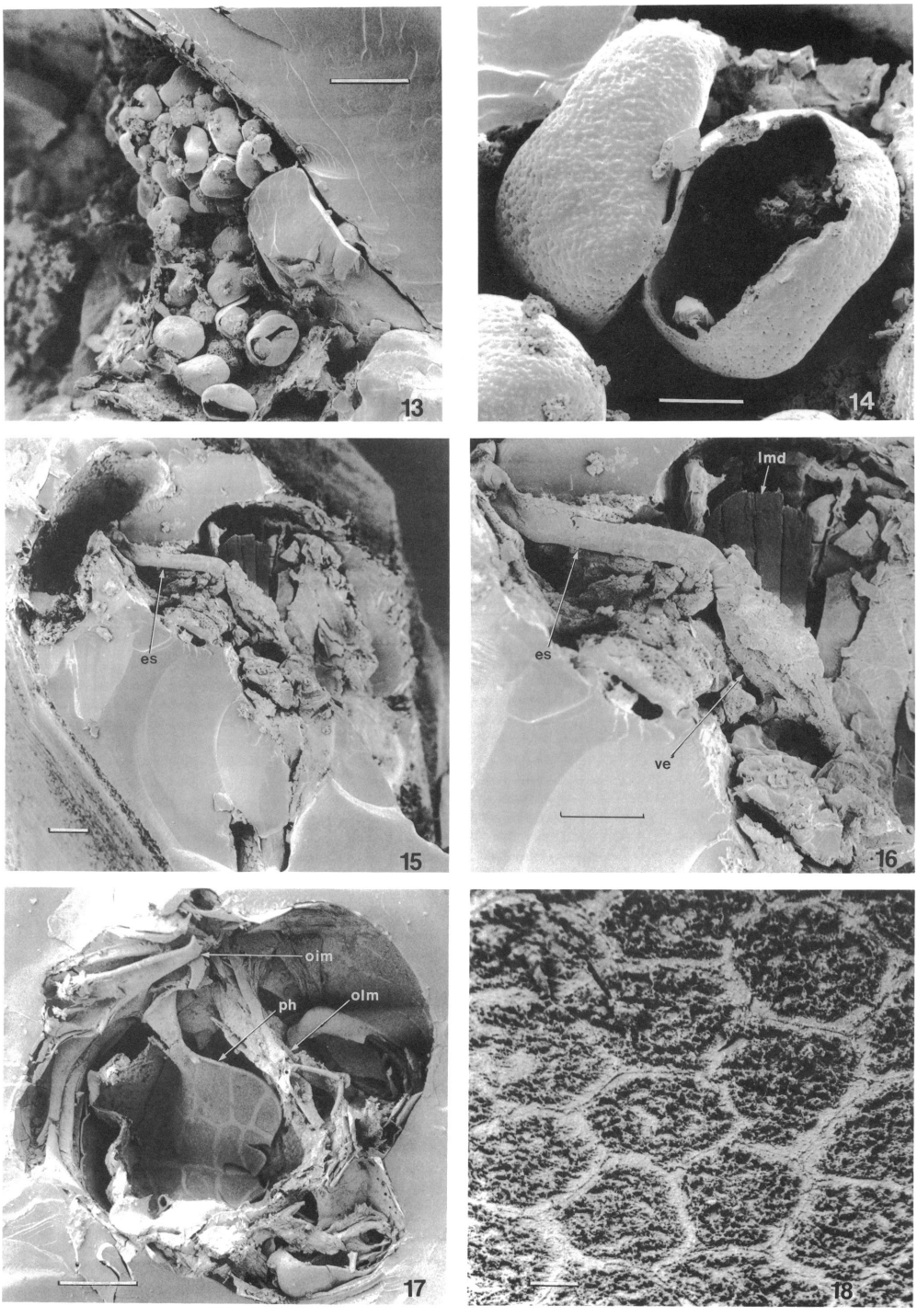
There is little doubt that amber will preserve DNA more consistently than any other kind of fossil. Four examples of DNA from insect and plant tissues in amber have been published, three of them from Dominican amber: 1. A large, primitive termite, *Mastotermes electrodominicus* (DeSalle et al., 1992); 2. A stingless bee, *Proplebeia dominicana* (Cano et al., 1992); 3. A leaf of the amber tree, *Hymenaea protera* (Cano et al., 1993b: an unrefereed report in which sequences were not presented); and 4. A nemonychid weevil in 125 million year old Lebanese amber (Cano et al., 1993a). Three other, unpublished successes are known thus far, all concerning Dominican amber: a drosophilid fruit fly (Y. Shirota, Hirosaki University); an anisopodid woodgnat, *Valeseguya disjuncta* (DeSalle and Grimaldi, unpubl.: AMNH); and several chrysomelid beetles (B. Farrell, personal commun.: Univ. Colorado). Such consistent preservation of DNA must be attributable to the unique chemistry of resins. The fact that the primary structure of DNA remains reasonably intact in amber fossils (approximately 250 base pair segments on average), indicates an ability of the amber to preserve the phosphate-ester bond between the deoxyribose sugar units. This bond is the one most susceptible to hydrolysis (Eglinton and Logan, 1991; Lindahl, 1993), and points mostly to the dehydration properties of the resin.

What proteins remain, and in what state, is the subject for future studies. Regardless of this, all evidence still indicates that amber very consistently and exquisitely mummified the small organisms that became entombed in the ancient resin.

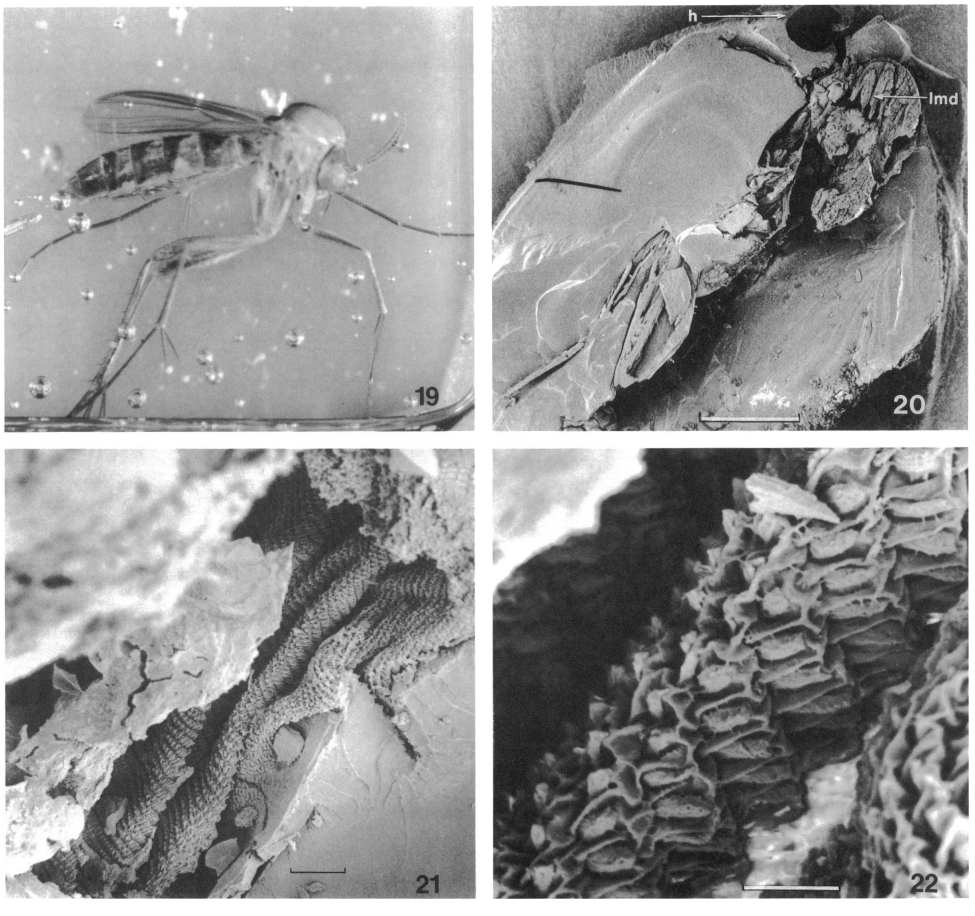
Figs. 1–6. Stingless bee, *Proplebeia dominicana* (B1) in Dominican amber. **1**: Intact specimen, showing cut circumscribed around mid-saggital line. **2**: Freshly opened specimen (light micrograph). **3, 4**: Opposite halves of entire bee. **5**: Detail of head. **6**: Detail of glossa. Scales: 3,4: 500 μm; 5: 100 μm; 6: 20 μm.



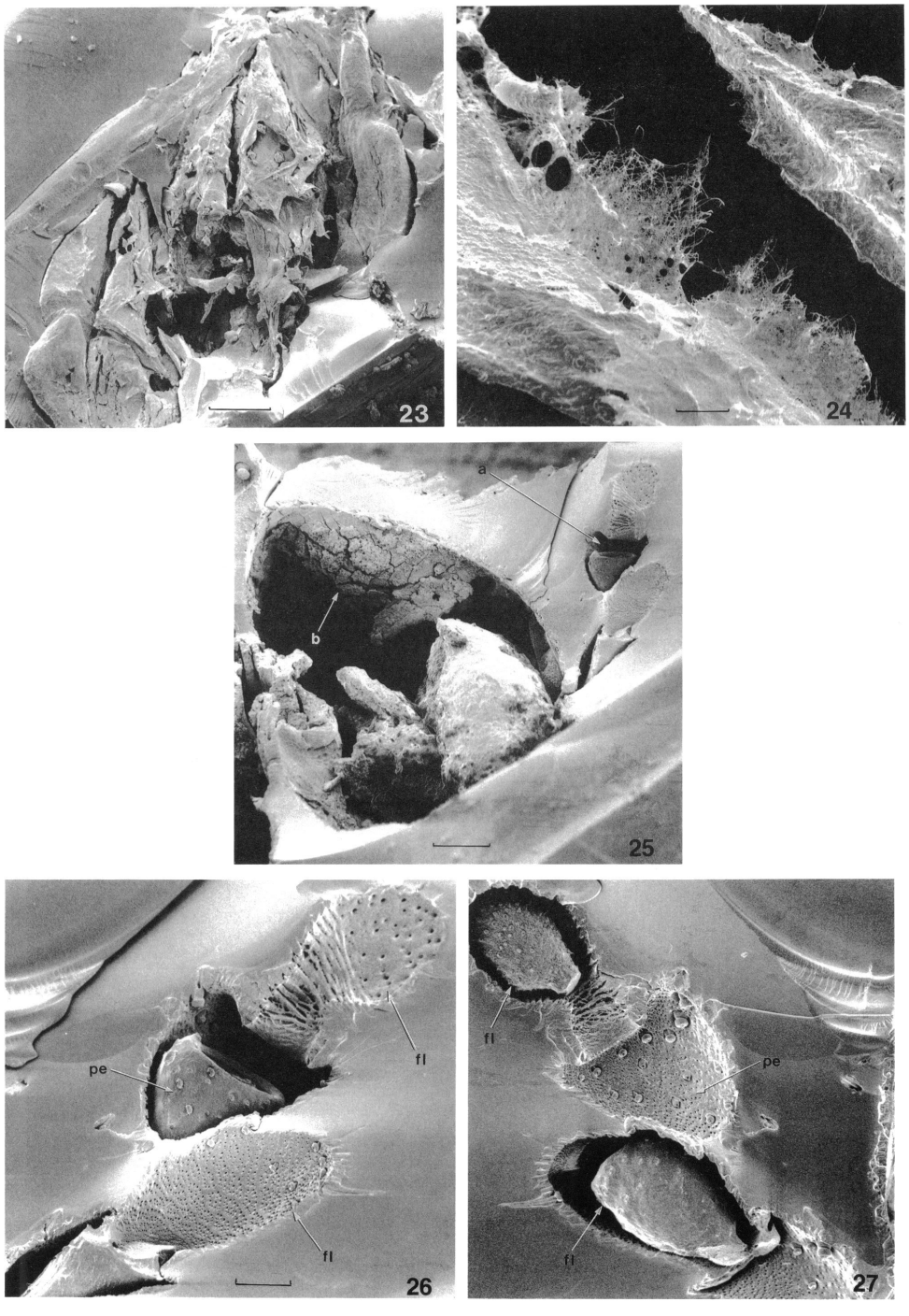
Figs. 7–12. Details of stingless bee (B1). **7**: Sucking pump with attached muscles. **8**: Detail of muscle (note transverse striae). **9**: Protocerebrum (brain). **10**: Bundle of small muscles in thorax. **11**: Thorax. **12**: Detail of thorax, showing membranous air sacs. Scales: 7: 50 μm; 8: 10 μm; 9: 50 μm; 10: 20 μm; 11: 200 μm; 12: 100 μm.



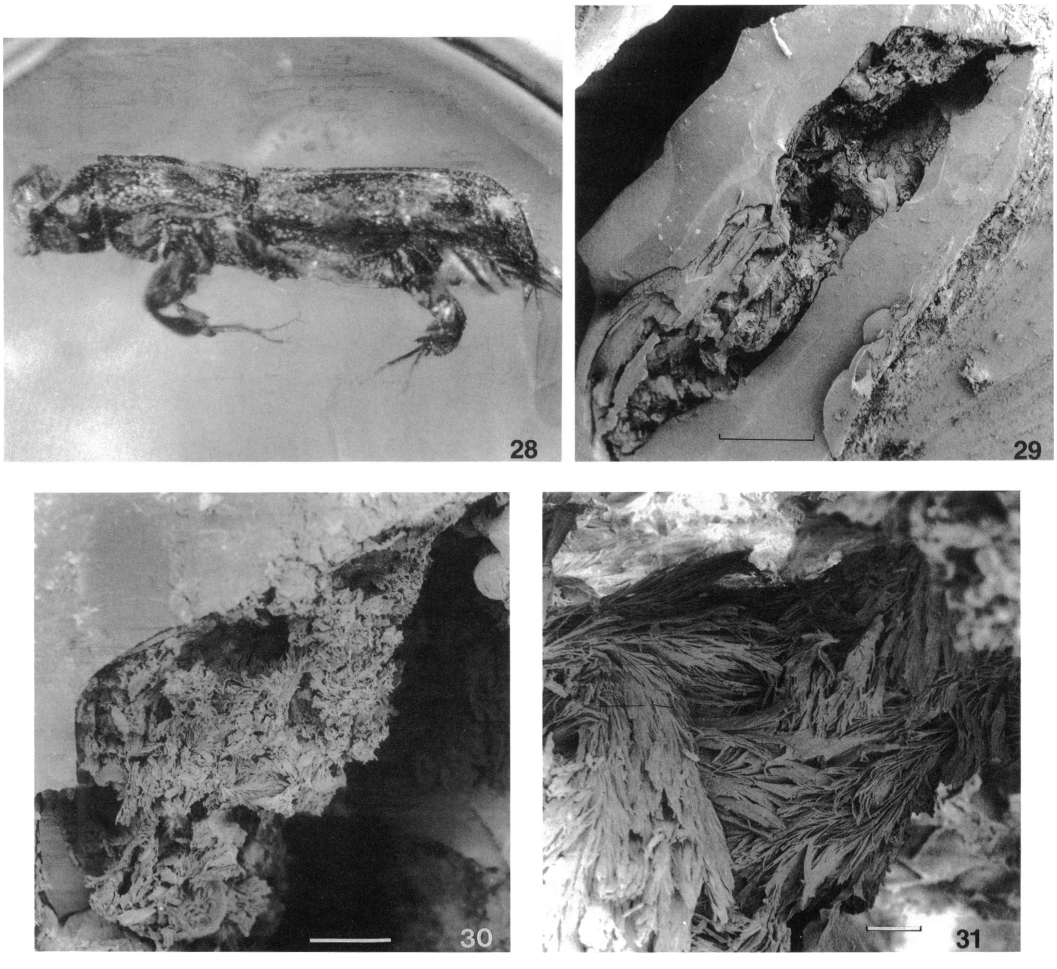
Figs. 13–18. Stingless bees, *P. dominicana*, in Dominican amber. **13**: Clump of pollen from abdomen of B1. **14**: Detail of one pollen grain. 15, 16: Specimen B2. **15**: Head and thorax, lateral view. **16**: Detail of esophagus and crop seen in 15. 17, 18: Specimen B3. **17**: Transverse section through thorax. **18**: Cell imprints in cuticle of phragma seen in 17. Scales: 16, 17: 200 μm; 18: 5 μm.



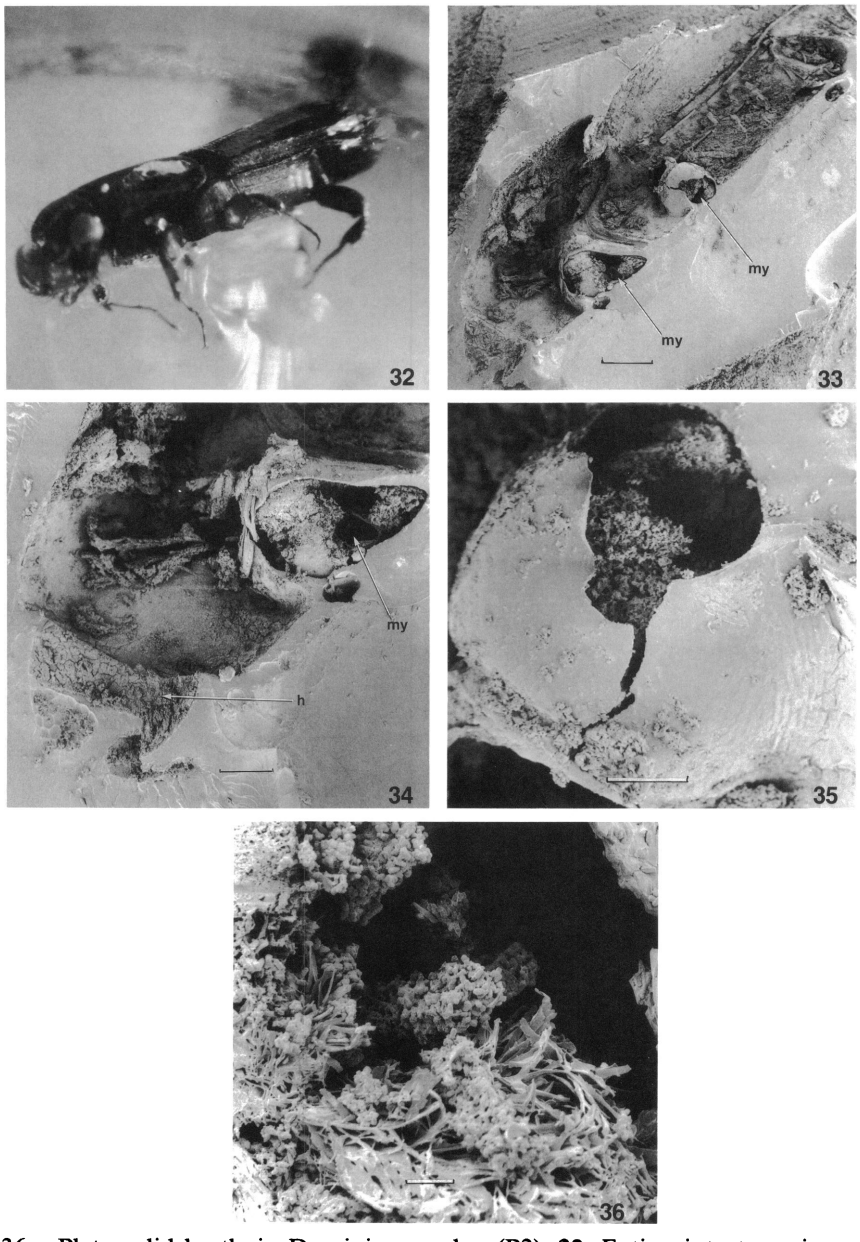
Figs. 19–22. Female fungus gnat (*Mycetophila* sp.: Mycetophilidae) in Dominican amber (M1). **19**: Habitus of intact specimen. **20**: Lateral view of entire, opened specimen. **21**: Portion of the membranous ventriculus. **22**: Detail of ventriculus. Scales: 20: 500 μm; 21: 20 μm; 22: 5 μm.



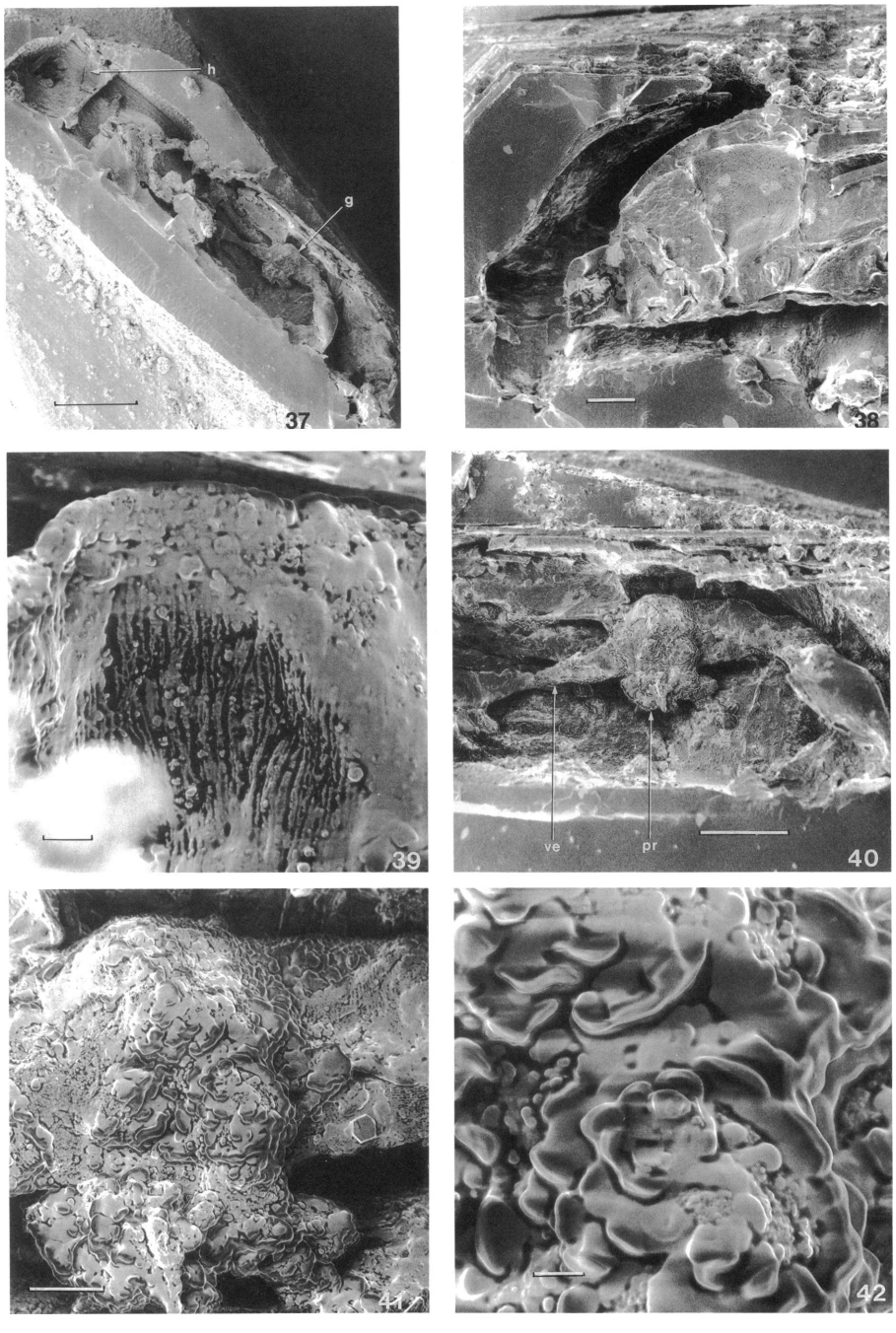
Figs. 23–27. Insects in Dominican amber. 23, 24: Isoptera (termite), *Reticulitermes* sp. (T1). **23**: Thorax, showing unidentifiable muscle bundles. **24**: Detail of the edge of a sheet of membrane. 25–27: Fly, *Megaselia* sp. (Phoridae). **25**: Head. **26, 27**: Antenna, opposite halves. Note the actual structures and corresponding impressions. Scales: 23: 200 μm; 24: 10 μm; 25: 50 μm; 26, 27: 20 μm.



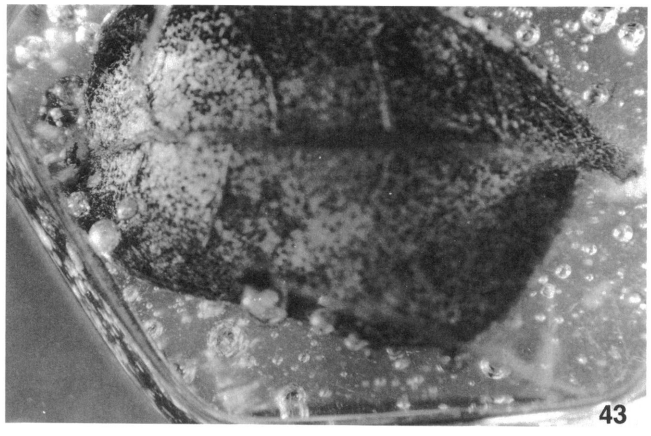
Figs. 28–31. Platypodid beetle in Dominican amber (P1). **28**: Entire, intact specimen. **29**: Entire specimen, opened. **30**: Protocerebrum (brain). **31**: Detail of fibrous tissue in or on brain. Identity of the tissue is uncertain–it does not resemble nervous tissue. Scales: 29: 500 μm; 30: 100 μm; 31: 20 μm.

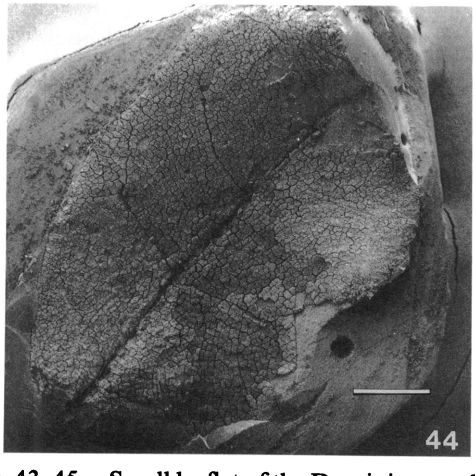


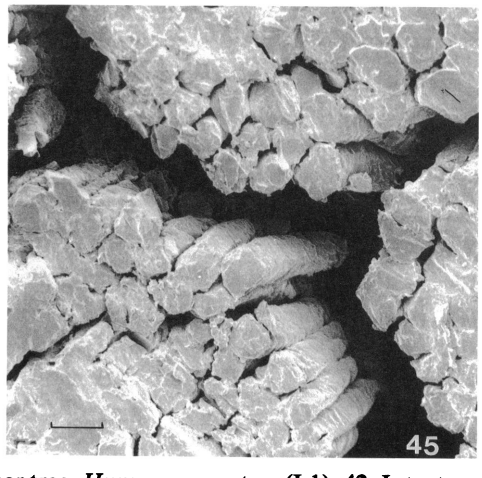
Figs. 32–36. Platypodid beetle in Dominican amber (P2). **32**: Entire, intact specimen. **33**: Entire specimen, opened. **34**: Detail of head and part of thorax, showing anterior mycangium. **35**: Detail of posterior mycangium. **36**: Detail of fungal spores and conidia in mycangium. Scales: 33: 200 μm; 34: 100 μm; 35: 50 μm; 36: 10 μm.



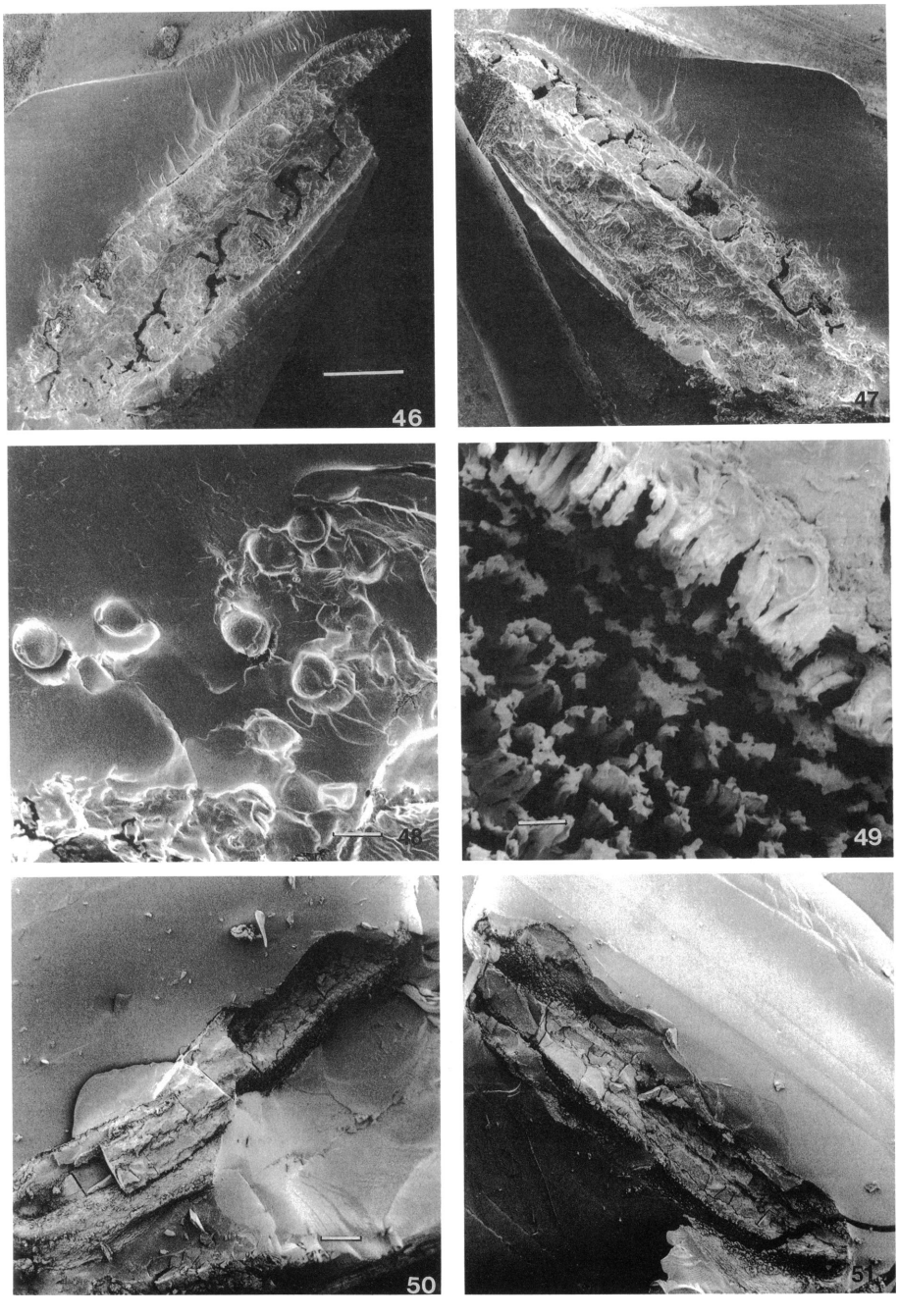
Figs. 37–42: Platypodid beetle in Dominican amber (P3). **37**: Entire beetle (lateral), opened. **38**: Apex of abdomen separated from amber cast. **39**: Wall of ventriculus, showing group of fine, deep pleats. **40**: Gut. **41**: Area near pylorus, showing amorphous structures and some crystals, but not malpighian tubules. **42**: Detail of amorphous structures near pylorus. Scales: 37: 500 μm; 38: 100 μm; 39: 10 μm; 40: 200 μm; 41: 50 μm; 42: 10 μm.



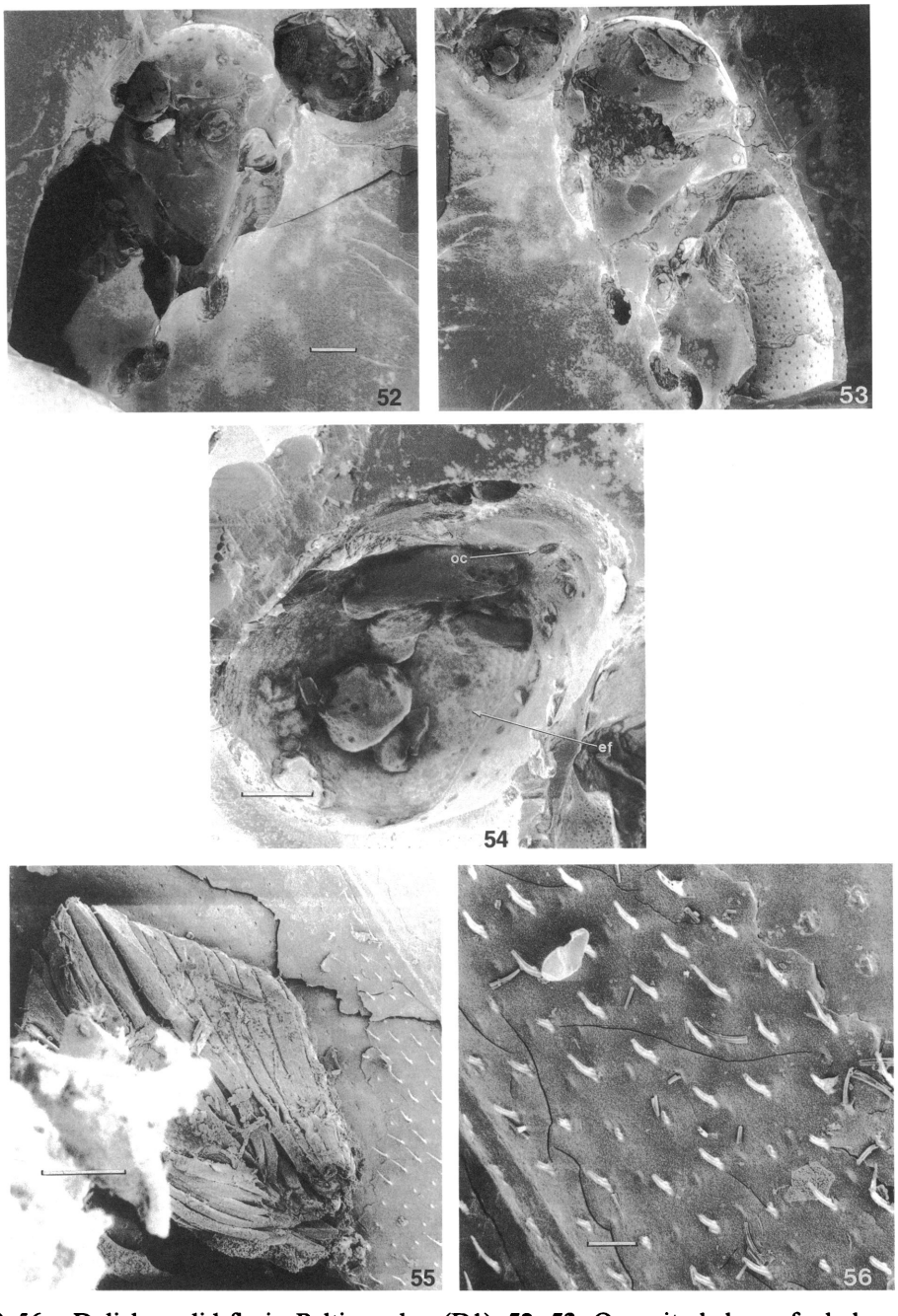




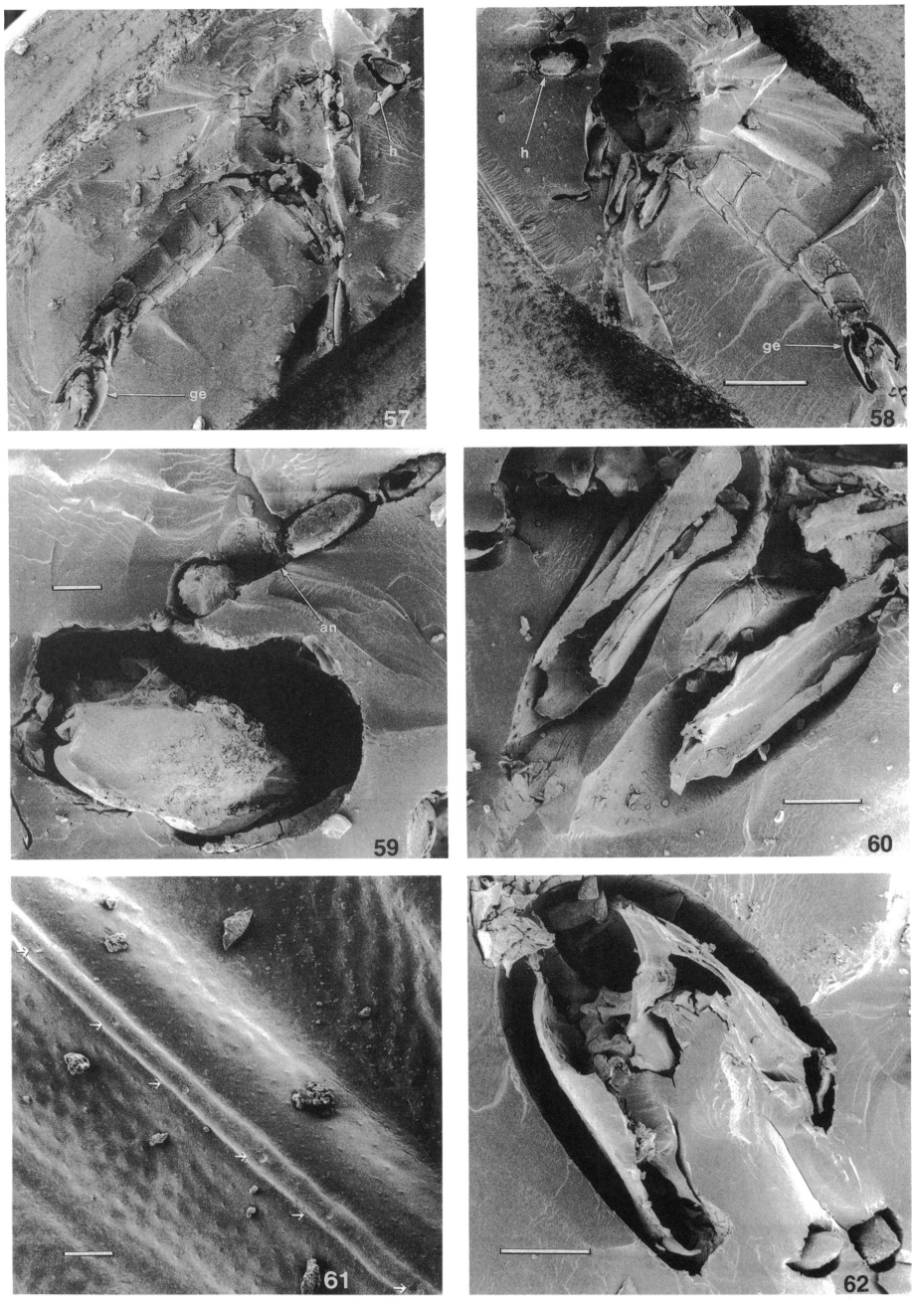
Figs. 43–45. Small leaflet of the Dominican amber tree, *Hymenaea protera* (L1). **43**: Intact specimen. **44**: Opened specimen (SEM). **45**: Group of columnar palisade cells from 44. Scales: 44: 1 mm; 45: 10 μm.



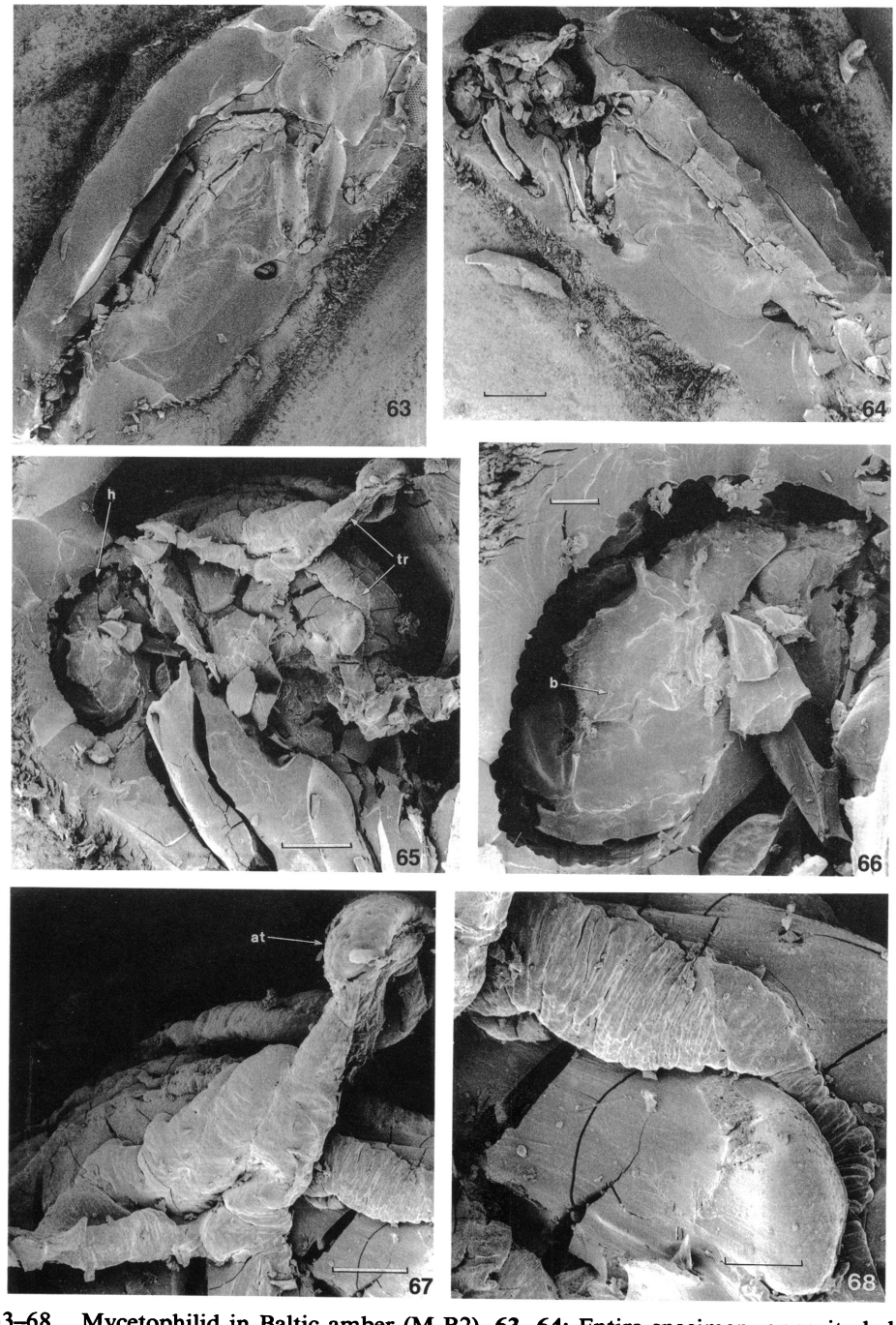
Figs. 46–51. Anthers of *Hymenaea protera* in Dominican amber. **46, 47**: Opposite halves of specimen A1. This specimen was clearly dehiscent and dispersing its pollen. **48**: Detail of pollen released near edge of anther. The pollen was not viable (see text). **49**: Portion of extensive matting from specimen A2, which was an immature anther. **50, 51**: opposite halves of anther A2. Scales: 46, 47: 500; 48: μm; 49: 10 μm; 50, 51: 200 μm.



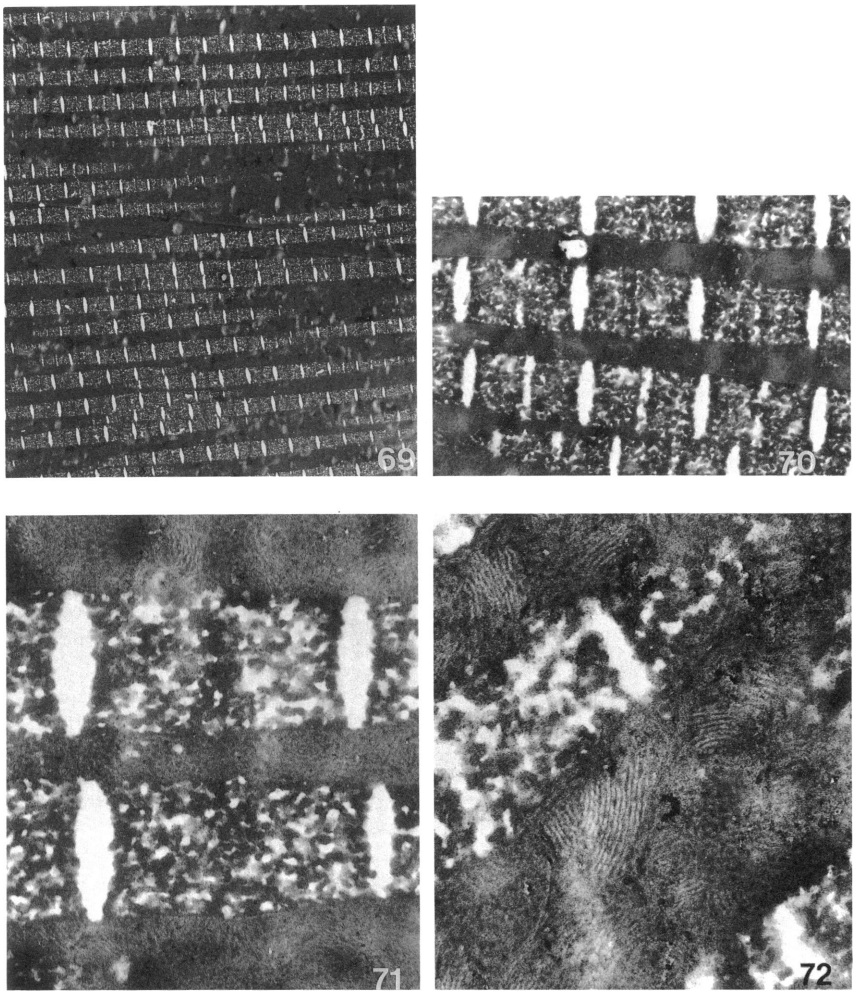
Figs. 52–56. Dolichopodid fly in Baltic amber (D1). **52, 53**: Opposite halves of whole specimen, opened. The bright area at the ventral part of the thorax is the milky impurity, which is due to numerous fine bubbles. The thorax was largely empty. **54**: Head, partly exposed showing original cuticle, and globule within (presumably the dried remains of liquified decomposition). **55**: Bundle of fine muscles dislodged from the thorax. **56**: Detail of original microtrichiae on wing. Scales: 52, 53: 200 μm; 54: 100 μm; 55: 50 μm; 56: 10 μm.



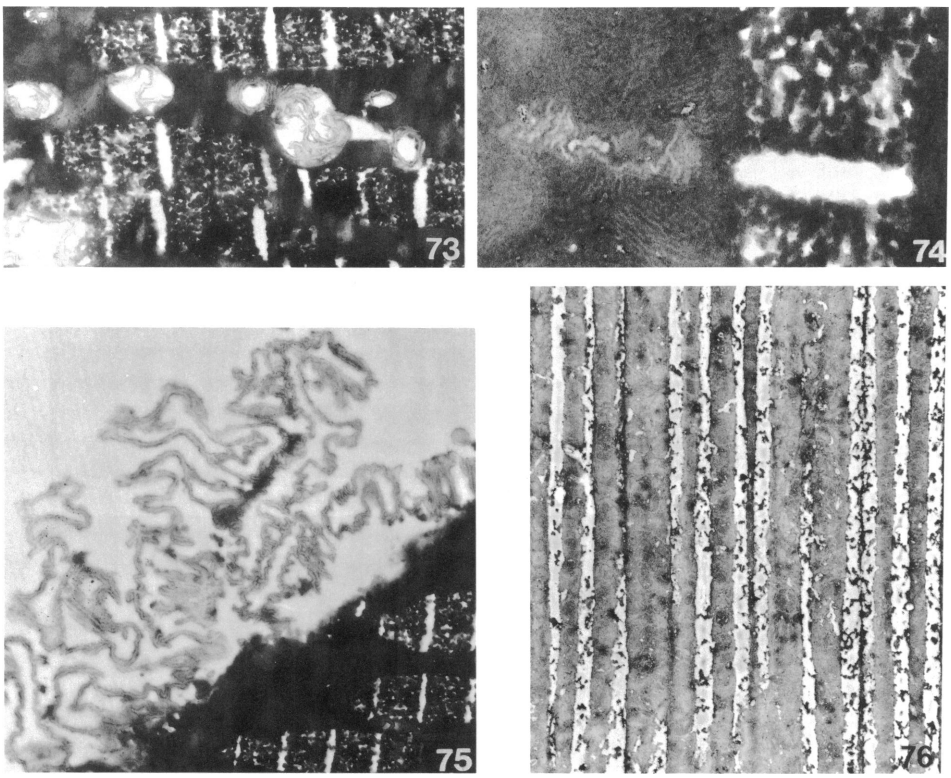
Figs. 57–62. Mycetophilid fungus gnat (*Mycetophila* sp.) in Baltic amber (M-B1). **57**, **58**: Opposite halves of entire, opened specimen. The thorax is largely hollow. **59**: Detail of head. **60**: Detail of bases of mid and hind legs. Original cuticle is present, but no remnants of soft tissue remain. **61**: Detail of wing membrane pressed into surface of amber, showing line of sensilla on vein (arrows). **62**: Male genitalia, showing internal sclerites and apodemes, but not muscle bundles. Scales: 57, 58: 500 μm; 59: 50 μm; 60: 100 μm; 61: 20 μm; 62: 100 μm.



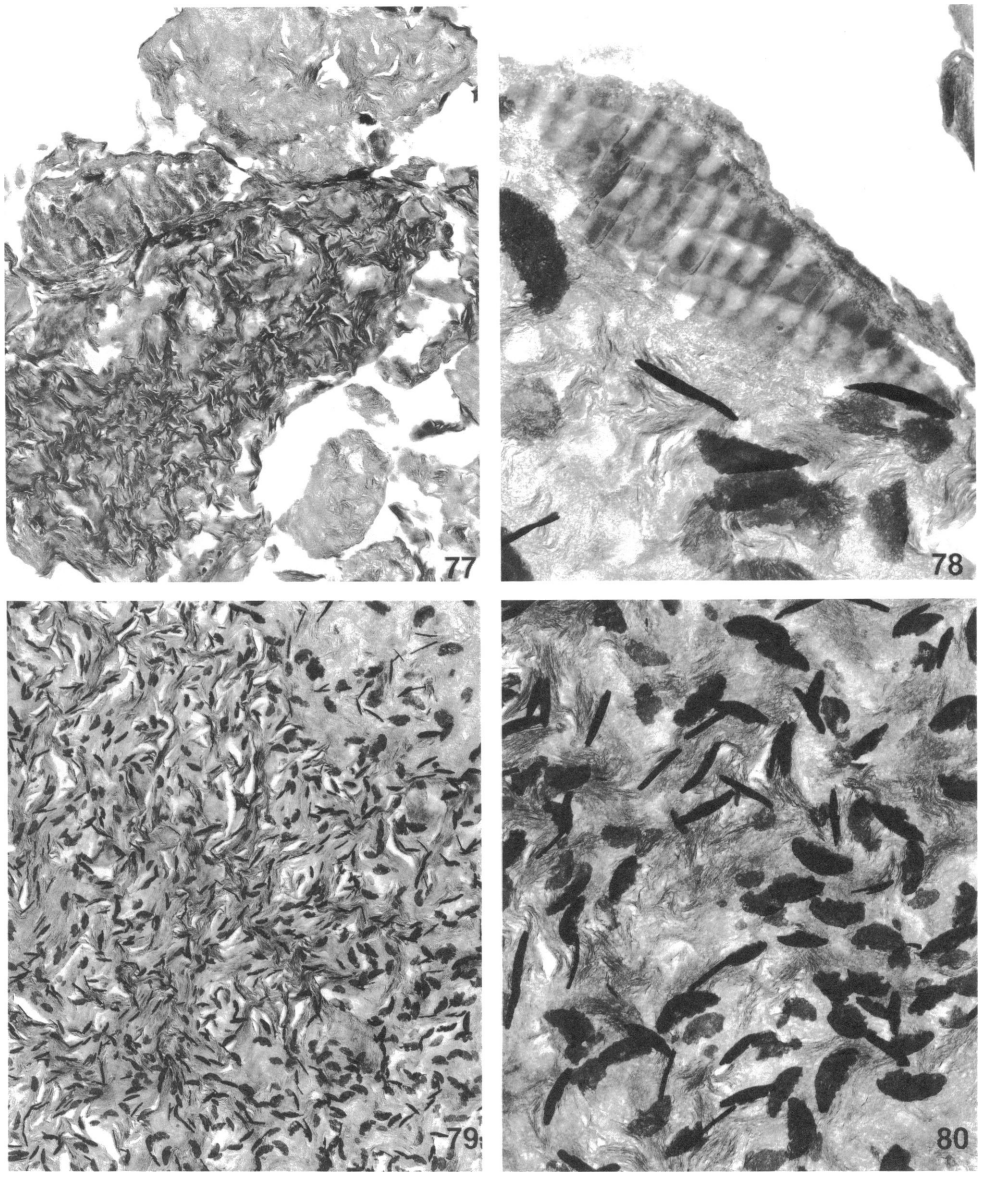
Figs. 63–68. Mycetophilid in Baltic amber (M-B2). **63**, **64**: Entire specimen, opposite halves. **65**: Detail of thorax. **66**: Detail of head. **67**: Detail of thorax, showing tracheae and atrium. **68**: Detail of another trachea. Scales: 63, 64: 500 μm; 65: 200 μm; 66: 50 μm; 67: 100 μm; 68: 50 μm.



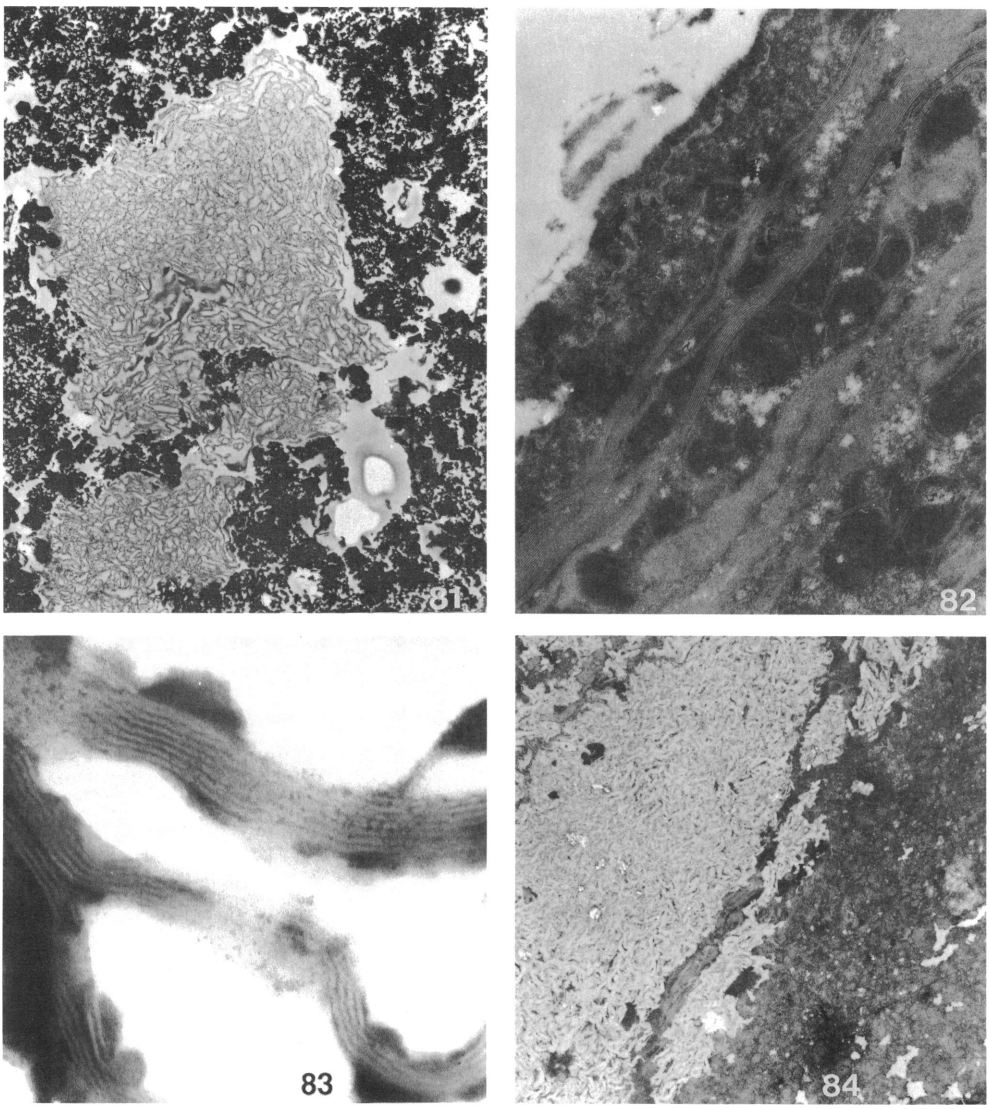
Figs. 69–72. TEM thin sections of *Proplebeia* flight muscle from Dominican amber. **69**: Myofibrils with sarcomeric repeats (2200×). **70, 71**: Detail of fig. 69. (70: 16,000×; 71: 35,000×). There is a lack of fibrillar material in the dark banded areas, suggesting replacement by inorganic salts. **72**: Detail of mitochondria tightly packed among myofibrils. Finger print patterns are the internal cristae of the mitochondria.



Figs. 73–76. TEM thin sections of *Proplebeia* flight muscle and associated tissue in Dominican amber. **73**: Tracheoles and associated epithelial cells lying between myofibrils (2500×). **74**: T-tubules (11,000×). **75**: Extensively folded membranes superficial to muscle cells (10,000×). **76**: Rehydrated muscle tissue (52,000×). Note that the sarcomeres are almost completely extracted, suggesting these are preserved mostly as inorganic salts.



Figs. 77–80. TEM thin sections of a connective tissue attached to flight muscle of *Proplebeia* in Dominican amber. **77**: Whole section (10,000×). **78**: Portion with cuticle attached (32,000×). **79**, **80**: Details of tissue, with amorphous fibrillar material (collagen fibers?) and electron-dense structures, which are possibly distorted cells, nuclei, or salt crystals (79: 12,000×; 80: 30,000×).



Figs. 81–84. TEM thin sections of brain tissue from *Proplebeia* in Dominican amber. **81**: Whole section, showing nervous tissue embedded in a thick layer of electron-dense salts (3500×). 82–84: Brain tissue after rehydration, which extracts the inorganic salts. **82, 83**: Extensively laminated membranes, perhaps of oligodendrocytes surrounding large axons (82: 22,000×; 83: 80,000×). **84**: Swollen membranes surrounding putative axon tracts (3500×).

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