Part II - Parasite Remains Preserved in Various Materials and Techniques in Microscopy and Molecular Diagnosis

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Arthropods and Parasites Found in Amber

Reginaldo Peçanha Brazil • José Dilermando Andrade Filho

Amber is a fossilized resin resulting from the transformation of resins produced by various plant species that existed in ancient times. Amber occurs in various parts of the world.

Amber generally consists of small deposits with no commercial importance but of great scientific relevance. Amber has been known since prehistoric times, used as an amulet or object of worship. Over the centuries, humans have used amber as a jewel, objects of art, or even objects of daily use. Various amber products were also used in the past in making varnish, soap, bath salts, or medicinal products.

The oldest indication of the use of amber by humans is a piece of amber found in a cave in the Pyrenees in France, plus pieces found elsewhere in Europe, with no sign of having been carved.

Carved amber beads date to the Paleolithic (9,000 BC), found in a cave in the South of England. Beginning in the Neolithic, in the Bronze Age (4,000-1,000 BC) there was a major expansion in the trade and distribution of Baltic amber. The amber trade routes were established during this time.

During the Iron Age, carved amber flourished in quality, and pieces discovered in archaeological sites vary from sculpted figures to veritable jewels that remain stunning to this day.

Phoenicians, Etruscans, Greeks, and Romans used amber both for trade and to make objects of art. During the Medieval Period, amber reappeared when the Teutonic Knights, in 1283, became absolute lords of Prussia and used their monopoly over amber production on the Samland Peninsula to produce religious articles, especially Paternoster beads. The knights kept an iron grip on the amber industry, to the point of prohibiting amber combing on the Baltic beaches, subject to the death penalty. In the 14th century, Bruges became the largest amber manufacturing center in Europe, with more than 300 registered amber craftsmen.

Although amber is not a mineral, many consider it a semiprecious stone, especially due to the ease in carving it. Its color ranges from yellow to orange, becoming transparent after polishing and prized as a jewel. The original resin served to protect plants from microorganisms and even from insects attempting to penetrate the wood of trees. Resin

can seep from any wound to a tree, and even a simple insect attack is sufficient to form it. Resin protects the tree by acting as a healing agent, and its antiseptic properties also protect against microorganisms.

Different types of trees produce different types and amounts of resin. The conifers were always the main producers of resin, but other plants can also exude this substance. At present, only two types of trees can produce stable resin that would eventually fossilize into amber over time. They are the Kauri pines (*Agathis australis*) of New Zealand (Figure 1) and some species of *Hymenaea* legumes from East Africa and South and Central America.

Figure 1 – Kauri pines (*Agathis australis*) of New Zealand

The transformation of resin into amber depends on various factors. When resin seeps from the tree, the hardening process begins, with the loss of volatile products. The resin contains substances like oils, acids, alcohols, and aromatic compounds responsible for the 'resinous' smell. The process is also known as polymerization: organic molecules cluster together to form larger molecules called polymers. This process takes 2 to 10 million years until the resin now

called amber is completely polymerized, without any volatile substance. The intermediate product is copal, which is also hardened resin, but which has still not lost all its volatile substances. Although copal is a relatively old resin, it is much younger than amber and is thus known as a sub-fossil resin. As mentioned above, fossilization of resin into amber takes some 2 to 10 million years, although many factors are involved in estimating its formation with certainty. Copal is often confused with amber, but when exposed to light and air it forms polygonal cracks on its surface, reacts to alcohol, and has a low melting point when compared to amber.

From the paleontological point of view, amber with plant or animal inclusions is extremely valuable for studying organisms and environments from 15 to 150 million years ago.

AMBER DEPOSITS

Resin production by plants dates as far back as the Paleozoic. However, more abundant amounts of amber are only found since the Triassic. The Cretaceous was extremely important in the history of the Earth, since flowering plants appeared and replaced the old conifers and ferns and became the dominant plants.

Ambers with inclusions are found mainly in the Baltic Region, Dominican Republic, Mexico, Burma (Myanmar), Lebanon, and New Jersey, USA. Among the ambers with inclusions, those from the Baltic and the Dominican Republic are the most common. Baltic amber is of marine origin and dates to the Upper Eocene or Lower Oligocene. Meanwhile, amber from the Dominican Republic can vary from the Upper Eocene to the Middle Miocene and is found in mines in the interior of the island.

BALTIC AMBER

Baltic amber is one of the world's most famous ambers and is found in the countries around the Baltic Sea. It represents some 80% of all amber found in the world. The largest deposits are on the Samland Peninsula, part of Russia between Poland and Lithuania.

This amber comes from underwater deposits in the Baltic Sea and washes up on the coast after the frequent storms that release the pieces. Despite some doubts about the type of tree that produced the Baltic amber, plants and insects preserved inside it indicate that it originated from different types of trees (Pinaceae and Araucariceae) that would have been common in a subtropical forest. The forest apparently occupied a large area of what is now Europe. Amber can now be found in Poland, Germany, Lithuania, Estonia, Denmark, Sweden, Netherlands, and even Great Britain.

Baltic amber is usually lemon-yellow or orange and can be opaque or slightly transparent. The presence of large amounts of succinic acid (3-8%) gave this amber its other name, succinite. The more transparent form usually presents inclusions with insects. Baltic amber was extensively studied for its entomological fauna, of which more than five thousand species have been described. Amber from the Dominican Republic, with a typically tropical fauna, has fewer species described.

AMBER FROM THE DOMINICAN REPUBLIC

Dominican amber (referring to the Dominican Republic) comes from the island of Hispaniola in the Caribbean. Although known since Columbus discovered America, it has only been explored since 1960, in various mines.

There are numerous mines on the island, where Dominican amber is marketed freely and sold as a semiprecious stone or amber with inclusions (Figure 2, plate). It is generally light-colored, but with a wide variety of hues. Most pieces range from yellow to orange, some with different tones in the same piece.

Figure 2 – Amber with insects, Dominican Republic

1 – Ceratopogonidae (biting midge).

2, 3, 4, 5, 6 – Phlebotominae (sand fly).

Photographs: Reginaldo Pecanha Brazil and José Dilermando Andrade Filho, 2008.

The mines in the Dominican Republic are located on the higher parts of the island, known as the Cordillera Septentrional. The mines are actually narrow tunnels dug by the local inhabitants to find the amber. The pieces are taken to the cities of Santiago and Santo Domingo, where they are polished and sold. The age of Dominican amber varies from the Lower Eocene to the Middle Miocene. Chemically, Dominican amber is similar to East African copal, having been produced by the same type of trees from genus *Hymenaea*. In the Dominican Republic, the plant that produced the resin for amber (*H. protera*, now extinct) is different from that existing today.

Dominican amber presents a variety of inclusions. The insects are widely diversified and are preserved better than in other ambers. The presence of numerous tropical insects in Dominican amber indicates that the island of Hispaniola consisted of a tropical forest not very different than at present.

INCLUSIONS IN AMBER

Different ambers accurately reflect the types of resin that originally seeped from the respective plants. These resins vary chemically according to their origin. They consist of different terpenes at various concentrations, determining the preservation of organisms in the amber. As mentioned above, insects found in Dominican amber are better preserved than in amber from the Baltic or elsewhere (Mexico and Myanmar).

Animals and plants are not the only inclusions found in amber. Air bubbles and water droplets are also common. However, inclusions of plants and animals are highly important for studying the flora and fauna of the past and even the relations between them.

Size is a question that should be considered in amber inclusions, since animals larger than 2 cm such as tree frogs, scorpions, spiders, and large insects are generally strong enough to crawl out of the resin before it hardens.

It is common to find insect legs or wings or spider legs that broke off when these animals tried to escape the resin. Some animal products are also observed in amber: feces from small animals, hairs, feathers, insect molting, etc.

Plants

Plants are quite common in amber, although not all plant inclusions are easily identifiable. Baltic amber commonly contains plant fragments, especially bark and bristles from oak blossoms, probably from the trees that produced the amber. Pollen and spores occur frequently but can only be observed under the microscope. The flora from Baltic amber has been classified much more extensively than that of Dominican amber.

Fossil plants in amber cover a wide variety. Plant specimens in Dominican amber are mainly angiosperms (flowering plants), while Baltic amber contains both angiosperms and gymnosperms (Poinar Jr. & Poinar, 1999).

Arthropods

Amber is extremely important for identifying fossils of terrestrial animals, mainly small insects that are not normally well-preserved in sedimentary rocks. Together with the sedimentary rocks that are effective at preserving large insects such as dragonflies (Odonoptera) and grasshoppers and locusts (Orthoptera), but which are not observed frequently in amber, we can reconstruct the forests of the past. As for other arthropods, biological vectors of parasites, the findings are also scarce, although some groups are more frequent. The following important groups of arthropods have been found preserved in amber:

TRIATOMINAE – Triatomines are insects belonging to the order Hemiptera, suborder Heteroptera. The family, Reduviidae, is much older, and the presence of fossil forms suggests that the predators originated in the Late Permian and Early Triassic (245-290 million years ago). Hematophagous hemipters probably emerged soon after the appearance of the first mammals. Although various species of predators from the Reduviidae family have been found in amber, the same is not true for the subfamily Triatominae, in which thus far only one triatomine species is known, *Triatoma*

dominicana Poinar Jr., 2005. This fossil is included in amber from the Dominican Republic, and the species was described based on the molting from the fifth larval instar of a nymph. Since it is only the molting, and also since some structures were lost (e.g., rostrum), the author that described it included it in this genus with doubts, and new records are necessary to confirm the presence of this genus in the Miocene. When it was trapped in the amber, the insect released a droplet of feces, which contains a trypanosomatid, described as *Trypanosoma antiquus* Poinar Jr., 2005. Poinar Jr. (2005a) suggests that this kissing bug fed on an infected bat, since there are bat hairs close to the insect.

PHTHIRAPTERA – The fossil record for lice is quite fragmented and controversial. *Saurodectes vrsanskyi* Rasnitsyn & Zherikhin 1999 was described as the first fossil biting louse, found in Russia (Lower Cretaceous) and included in a new family (Saurodectidae). It has been suggested that this louse was a parasite of pterosaurs (Kumar, 2004). Although thus far there is no evidence of the presence of lice from this order in amber, adult winged specimens from the order Psocoptera, family Liposcelididae, have been found in amber from the Cretaceous in Myanmar (*Cretoscelis burmica*) and from the Miocene in the Dominican Republic (*Belaphopsocus dominicus*). Genus *Cretoscelis* is an extinct group of Liposcelidae, a family of free-living lice, considered a sibling group of true lice (Phthiraptera), ectoparasites of birds and mammals. Nits on hairs conserved in amber from the Baltic prove the presence of ectoparasites on small mammals from the Eocene (Galati, 2003; Grimaldi & Engel, 2006).

SIPHONAPTERA – As with lice, fossil fleas are quite uncommon. After all, these insects have a very close relationship with their hosts. Thus, the odds that they would become buried or trapped in resin are virtually zero. Fleas appeared some 140 million years ago, in parallel with their mammalian hosts, and thus far only five fossil species have been identified: three from Baltic amber (*Palaeopsylla baltica*, *Palaeopsylla dissimilis*, and *Palaeopsylla klebsiana*) and two from Dominican amber (*Pulex larimerius* and a species of *Rhopalopsyllus* still not described). These fleas probably tormented rodents and bats in the Eocene and Miocene.

IXODIDAE AND ARGASIDAE – Ticks have been found in amber from the Cretaceous to the Miocene. Despite diverse speculations on the origin of the ticks, the oldest fossil found thus far dates to the Cretaceous (90–44 million years ago). Some ticks described in amber are from genera like *Amblyomma*, but most belong to extinct genera.

CULICIDAE – Culicids or mosquitos present a cosmopolitan distribution. Females of the species are blood-feeding and serve as the vectors for a huge variety of parasites. Fossil species are not common, much less in amber inclusions. The species described belong to amber from the Eocene/Oligocene in the Baltic and from the Miocene in the Dominican Republic. Four species of *Aedes* have been identified in amber from the Baltic: *A. damzeni* Szadziewski, 1998, *A. hofferinsorum* Szadziewski, 1998, *A. serafini* Szadziewski, 1998, and *A. perkunas* Podenas, 1999.

Until recently it was believed that anopheline mosquitos only appeared in the New World in the late Tertiary. However, the description of *Anopheles* (*Nyssorhynchus*) *dominicanus* Zavortink, T. J. & Poinar Jr., 2000 in Dominican amber reinforces their earlier presence.

Other extinct genera of mosquitos have been found in amber from both Myanmar and the Dominican Republic. In genus *Culex*, a recent description was *C. malariager* Poinar Jr., 2005, which fed on birds. An interesting detail with this specimen is that it was infected with a species of protozoan, described as *Plasmodium domiminicana* Poinar Jr., 2005, as will be discussed later.

CERATOPOGONIDAE – Ceratopogonidae have been found in resins from the Cretaceous in various regions of the world and in amber from the Tertiary in Mexico and the Dominican Republic. The family includes some 253 fossil species. The most well-known fauna is from the Baltic (Eocene). Importantly, a ceratopogonid (probably from the

extinct genus *Protoculicoides*) was found in Burmese amber, naturally infected with what the authors identified as a monogenetic trypanosomatid.

PHLEBOTOMINAE – The subfamily was created by Rondani in 1840. For many years it was thought to include only a single genus: *Phlebotomus*. In 1948, Theodor proposed that the subfamily be divided into four genera: *Phlebotomus* (Rondoni) and *Sergentomyia* França for the Old World species, *Brumptomyia* França & Parrot and *Lutzomyia* França for the New World species. The subfamily Phlebotominae includes the majority of the blood-feeding Psychodidae, including some disease vector species. This subfamily is found on practically all of the continents with a long evolutionary history, having appeared in the Late Paleozoic or Early Mesozoic (Andrade Filho & Brazil, 2003; Azar & Nel, 2003).

The genus *Pintomyia* was created by Costa Lima in 1932 as a subgenus of *Phlebotomus* and raised to the genus category by Galati in 1995. The latter author divides *Pintomyia* into two subgenera: *Pintomyia* and *Pifanomyia*. Both include living and extinct species. The living species are found mainly in the Andes Region and Central America and are usually associated with woods or forests. Speciation in this group with such wide distribution is the result of biogeographic and climatic events that affected the region's flora and fauna in the last 15 million years.

With the growing interest in amber with insect inclusions, various fossil Phlebotominae have been studied. Our group described a new species based on the first description of a sand fly from Dominican amber (Brazil & Andrade Filho, 2002; Andrade Filho, Galati & Falcão, 2006). The majority of the fossil species described in amber from the Dominican Republic belong to genus *Pintomyia*, subgenus *Pifanomyia*, while only one sand fly species (*Trichopygomyia killickorum*) from the Dominican Republic falls outside this group (Andrade Filho, Falcão & Brazil, 2004). The other three species already described by our team belong to this group of species.

Peñalver & Grimaldi (2005) recently described five fossil sand fly species from the Dominican Republic. The authors only fit one species inside *Pifanomyia* [as the *verrucarum* group of Young & Duncan (1994)]; however, the other four species apparently also belong to this group. A detailed study of the description of these sand flies or a study conducted directly on the fossils themselves is necessary to elucidate this problem.

Most modern-day species in subgenus *Pifanomyia* are found in Central America, including the Caribbean islands, mainly in forest areas. This may indicate that the extinct species also lived in this type of habitat, and that this was the predominant vegetation in the Dominican Republic 20 million years ago.

In addition to adult sand flies, other interesting discoveries have been made. Poinar Jr. (2007) found two sand fly larvae some 100 million years old in amber from Myanmar. Found on one larva was a fungus that could have been serving as food when the insect was trapped by the sap. As it struggled to free itself from the sap, the larva excreted a bit of feces containing a trypanosomatid, probably an ancestor of the digenetics. A primitive genus of trypanosomatid parasite of the sand fly was also described by Poinar Jr. & Poinar (2005) in Burmese amber, leading the authors to the hypothesis that *Paleomyia burnitis* Poinar, 2004, a primitive sand fly, transmitted *Paleoleishmania proterus* to vertebrates in the Cretaceous.

Parasites of arthropods

Fossil arthropods parasitized by fungi, bacteria, protozoa, or nematodes are quite rare, but not impossible to find. Entomofungi are apparently more common, since the fact that these fungi are on an arthropod's exoskeleton when it is trapped in amber makes their identification easier than for an internal parasite. Various types of ectoparasitic fungi have been observed in amber from both the Dominican Republic and Myanmar (Poinar Jr. & Poinar, 2005).

The feces of *Triatoma dominicana*, a triatomine from some 20 million years ago, contained trypomastigotes of *Trypanosoma antiquus* – according to Poinar Jr. (2005a), a parasite of bats in the forest of the Dominican Republic. Other trypanosomatids also appear in Ceratopogonidae and sand flies. In Ceratopogonidae (*Protoculicoides*) discovered in amber from Myanmar (from some 100 million years ago), a monogenetic trypanosomatid was identified, and the promastigote forms found in the sand fly *Palaeomyia burmitis* Poinar, 2004 were considered a genus of the primitive leishmania *Paleoleishmania proterus* (Poinar Jr. & Poinar, 2004).

As mentioned above, Poinar Jr. (2007) found two sand fly larvae from some 100 million years ago in amber from Myanmar. Found on one larva was a fungus that could have been serving as food when the insect was trapped by the sap. As it struggled to free itself from the sap, the larva excreted a bit of feces containing a trypanosomatid, probably an ancestor of the digenetics.

In Dominican amber, the same author describes the species *Culex malariager* (Poinar Jr., 2005b). Birds were probably the food source for the specimens belonging to this species. An interesting detail in this specimen is that it was infected with a species of protozoa, *Plasmodium dominicana* (Poinar Jr., 2005c). The description of this *Plasmodium* species is based on oocysts, sporozoites, ookinetes, and microgametocytes found in the body cavity of the female *Culex malariager*. According to the author, this protozoan was probably responsible for avian malaria and would have been involved in malaria of primates. In this case, it would have originated in the Americas (Poinar Jr., 2005c).

Fossil nematodes are rare, and most of those found in amber are indicative of a phoretic or parastitic association with insects or arthropods (Poinar & Buckley, 2006).

Other important discoveries have been made in addition to adult sand flies. A primitive genus of a trypanosomatid parasite of sand flies was also described by Poinar Jr. & Poinar (2005) in Burmese amber, leading the authors to suggest that *Paleomyia burnitis* Poinar, 2004, a primitive sand fly, transmitted the species *Paleoleishmania proterus* to vertebrates during the Cretaceous.

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