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## The extraction of fossil arthropods from Lower Eocene Cambay amber

NINA MAZUR, MICHAEL NAGEL, ULRICH LEPPIN, GABRIELE BIERBAUM, and JES RUST

**Fossil arthropods preserved in amber typically comprise only hollow spaces lined with a thin layer of diagenetically altered or decomposed cuticle. In contrast, the cuticle of inclusions from Lower Eocene Cambay amber (Gujarat, India) is commonly perfectly preserved and moreover allows the extraction of whole specimens. Here we report on the examination of several groups of solvents to determine their ability to dissolve Cambay amber. The amber dissolves well in toluene, xylene, dichloromethane and trichloromethane (chloroform). Even orange oil and turpentine oil dissolve Cambay amber quite well. The extraction of inclusions by dissolving the amber matrix enables various novel observations and further investigations with biological analytical methods, although the material is extremely fragile after extraction.**

### Introduction

Amber preserves a diverse array of organisms from different habitats in and close to the amber-producing forests. The detailed analysis and comparison of amber inclusions reveals the evolutionary history of these organisms, and is especially important in cases where the fossil record is poor or previously unknown. In addition, by studying the fossils of particular deposits, the composition, diversity, and ecology of former terrestrial ecosystems can be elucidated. Indian Cambay amber was deposited about 50–52 million years ago during the Early Eocene, at a time approaching the Indian subcontinent's collision with Asia. The amber bearing deposits are located in the Cambay shale of western Gujarat (Rust et al. 2010). The fossil resin from the Vastan and Tadkeshwar lignite mines has preserved a highly diverse flora and fauna, including 22 orders and more than 60 families of arthropods so far (Rust et al. 2010; Paul C. Nascimbene, personal communication 2012).

All natural tree resins, whether fossil or extant, are classified on the basis of their structural chemical characteristics (Anderson et al. 1992). The Cambay amber's chemistry, as well as the microanatomy of associated fossil wood and the recent recovery of fossil pollen, indicate that trees of the angiospermous family Dipterocarpaceae were the source of the fossil resin. Dipterocarps produce a Class II resin, also referred to as a dammar-like resin (Dutta et al. 2009). In contrast to the more typical Class I gymnosperm resins, Class II resins have a bicadinene-type sesquiterpenoid skeleton (Mallick et al. 2009) and will conse-

quently dissolve in organic solvents (van Aarssen et al. 1990). Terpenoids are structurally derived from isoprene. Whereas terpenes only contain hydrocarbons, terpenoids also contain functional groups. Younger resins have a high percentage of volatile and aromatic substances. Over time these substances volatilize as the resin polymerizes. The polymerization process is further enhanced by oxidation reactions, cross-linking and cyclization, leading to the hardening of the resin (Poinar 1992).

Despite its Lower Eocene age, the Class II Cambay amber is only weakly polymerized and crosslinked. During processing work in the laboratory, freshly polished surfaces become tacky and soften considerably. Most of the rough amber is dark red or russet to almost black in colour, and even the more transparent pieces often contain dark zones and impurities which hinder optical examination of fossil inclusions.

Early attempts to dissolve fossil resins and extract inclusions were made by Viehmeyer (1913), who was able to extract several ants from Sulawesi copal, which is probably only a few thousand years old. He used a mixture of absolute alcohol and sulphuric ether (diethylether). Galippe (1920) tried to dissolve a blend of different ambers, but was only partially successful, as not all samples reacted to the solvent. Later, Azar (1997) dissolved Early Cretaceous Lebanese amber, and reported that only chloroform gave satisfactory results. However, it must be emphasized that the amber (and any inclusions) broke apart and fragmented, and did not fully dissolve.

In the present study, we examined several groups of chemical solvents to determine their ability to dissolve pieces of Cambay amber. Given that the Class II Cambay amber is much less polymerized than the more prevalent and well-studied Class I resins, it stands to reason that its solubility should be more like that of copal, which is already known to dissolve in several organic solvents.

Dissolving amber provides new and promising opportunities to study inclusions in greater detail (Figs. 1–3), especially with respect to subcellular or other microstructures. Otherwise, studies are restricted to traditional optical examinations which, as already mentioned above, are sometimes particularly difficult in case of the Indian amber. Dissolving the amber and extracting inclusions also enhances and improves the examination of any associated micro organisms, and allows biochemical as well as geochemical analyses of the inclusions.

Besides solving ability, it was important to find a solvent that provides minimal adverse health risks, and which dissolves

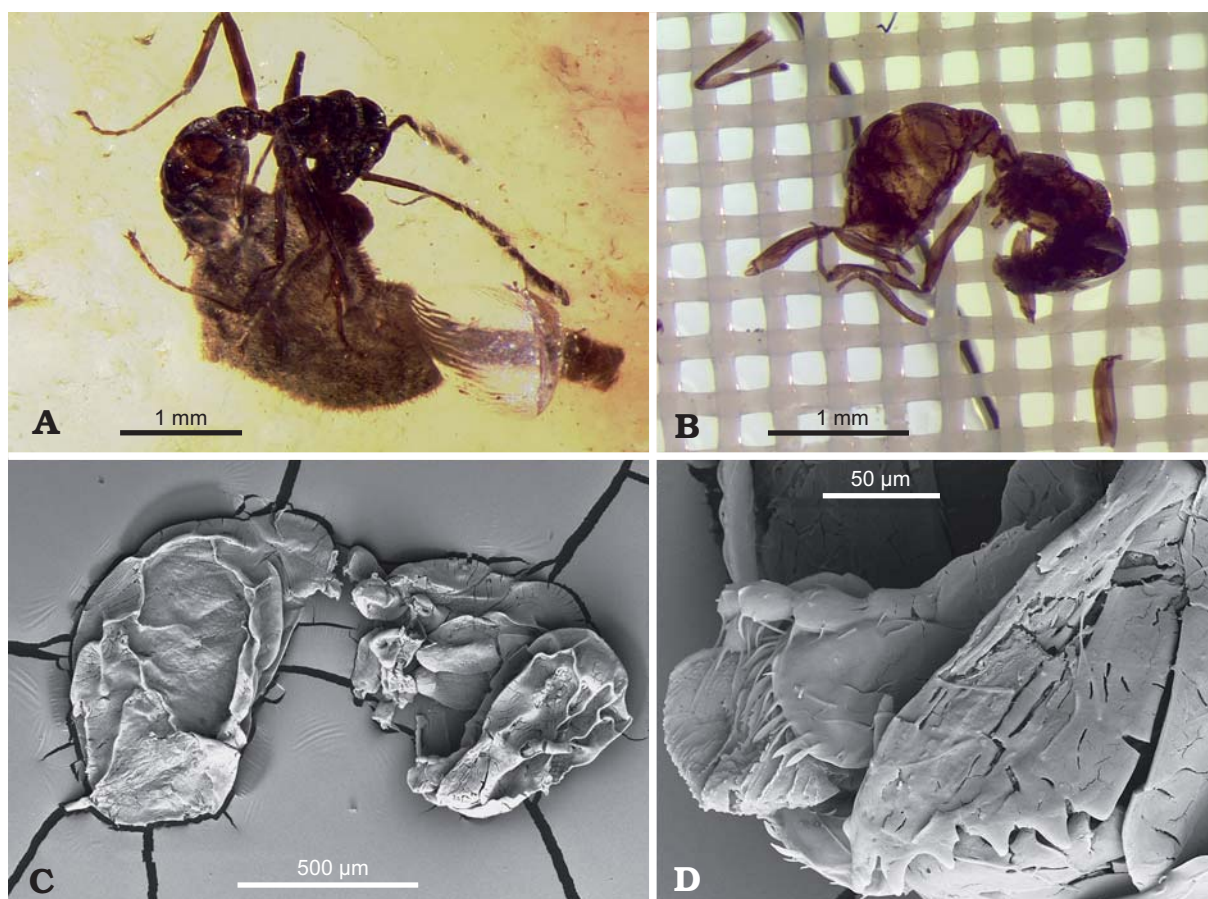


Fig. 1. Extraction of an ant (STB-314-T'09) from Lower Eocene Cambay amber, Tadkeshwar lignite mines, India. **A.** Ant and plant remain in Indian amber. **B.** Isolated ant specimen after dissolution of the amber matrix with orange oil, legs disarticulated. **C.** Scanning electron micrograph of the ant. **D.** Detail of mouthparts with mandibles, galea with strongly developed galeal comb, labial palpus, and glossa.

the amber quickly without destroying or changing the chemical structure of the embedded fossils.

*Institutional abbreviations.*—STB, Steinmann Institute, University of Bonn, Germany.

## Material and methods

Pieces of amber were selected at random, the only criterion being an approximately similar weight, which varied from 0.28 to 0.50 g, with an average of 0.34 g. Pieces containing inclusions were not selected, since the effect of the solvents remained unclear before the experiments. After weighing, the amber pieces were placed in sterile test tubes with gasketed caps, which were necessary to prevent evaporation of volatile solvents. The following solvents were evaluated under a foam hood:

- Acetone is the simplest ketone, and has a broad variety of possible applications, but is primarily used as a polar aprotic solvent.
- Dichloromethane and Trichloromethane (Chloroform) belong to the halogenated hydrocarbons. Halogenated alkanes have a widespread use in commerce as insecticides, herbicides and, of course, as solvents. Halogenalkanes are better solvents than the corresponding alkanes, because of their increased

polarity due to the highly negative charge of the halogen. Both chemicals are toxic. Dichloromethane is highly volatile and can dissolve a wide range of organic compounds. It has a high evaporation time. Chloroform (Trichloromethane) also has a broad range of applications, although concerns about its health effects have led to a search for alternatives. It has a very high evaporation time.

- Dimethylformamide is a commonly used aprotic organic solvent with a low evaporation rate. It is classified as an amide, which derives from ammonia. It is also harmful to humans.
- Formaldehyde (37%) also called methanal, is the most simple aldehyde. It is the most reactive of all aldehydes and is quite harmful to humans.
- Orange oil is derived from the peel of orange fruit. It is composed of mostly d-limonene, a cyclic monoterpene. Other components include long chain aliphatic hydrocarbon alcohols and aldehydes like citral B. For several reasons, the precise composition of orange oil can vary, due to its natural origin. In contrast to the listed aromatic hydrocarbons, there is no evidence for carcinogenicity or genotoxicity in humans.
- Petroleum ether is a mixture of alkanes of different chain length. Through fractional distillation, petrol ether is available with different flash points ranging from 30–40°C up to 100–120°C. Apart from its danger for the environment, it is



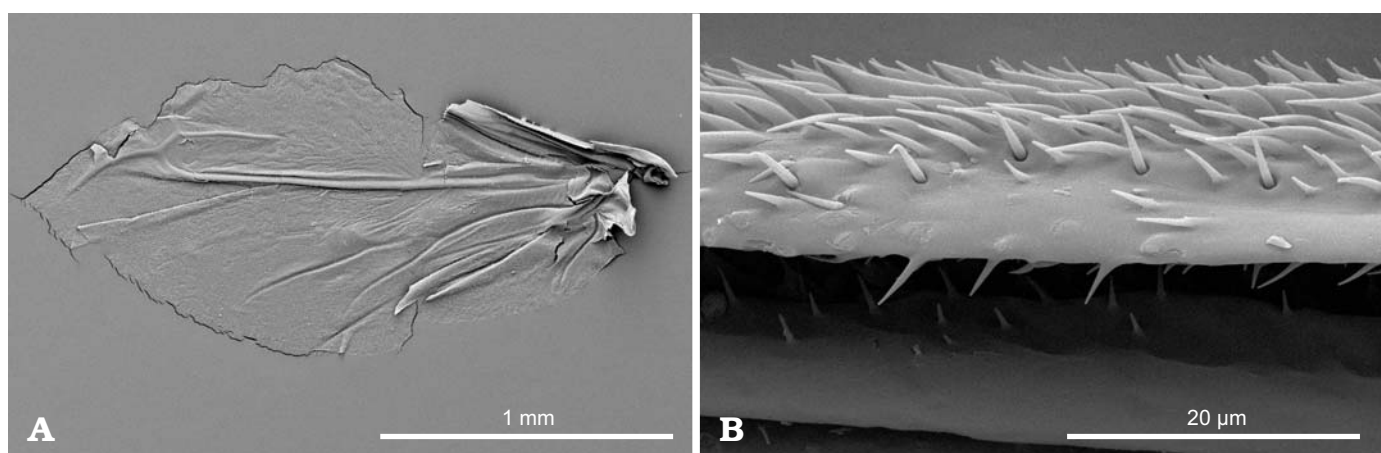


Fig. 2. Scanning electron micrographs of a dipteran wing (STB-315-T'09) extracted from Lower Eocene Cambay amber, Tadkeshwar lignite mines, India. **A.** Almost complete isolated wing. **B.** Detail of the folded wing membrane covered with setae in the basal part of the front edge of the wing.

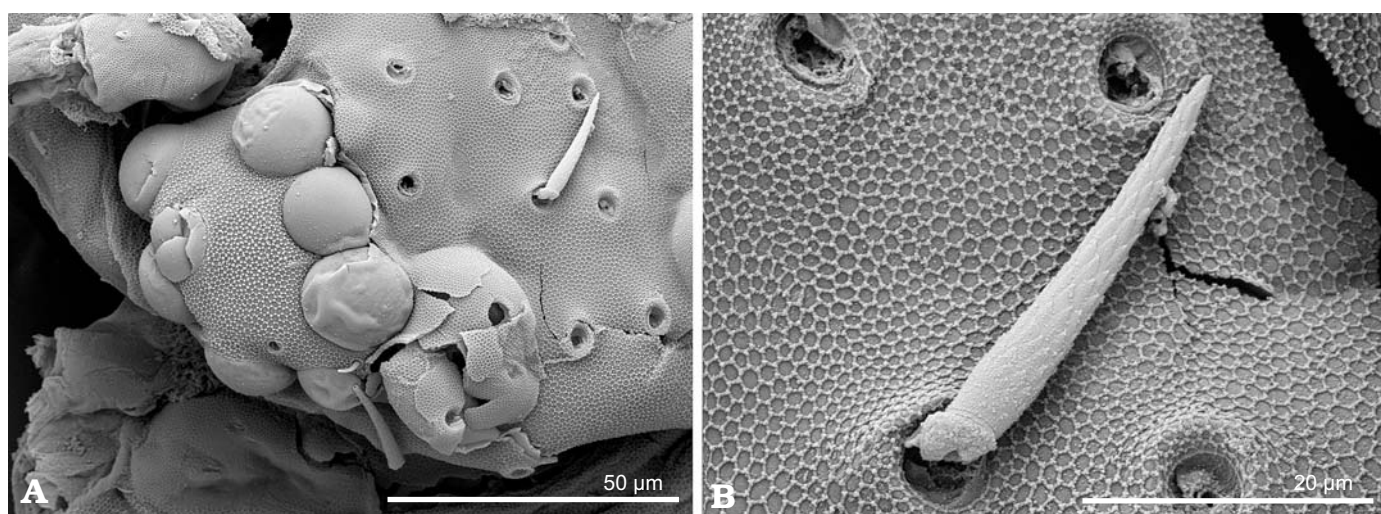


Fig. 3 Scanning electron micrographs of a Putoidea larva (STB-316-T'09) from Lower Eocene Cambay amber, Tadkeshwar lignite mines, India. **A.** Annular arrangement of the ommatidia of the eye with antenna base. **B.** Detail of a sensory bristle.

harmful for humans. For the experiments, we used pure petrol ether (Benzinum medicinale), as well as petroleum ether with a flash points of 40°C and 60–80°C.

- Turpentine oil is the distillate of natural resin, mainly from pine trees. This type of resin basically contains alpha and beta pinene, which are alkenes containing a reactive four-membered bicyclic ring.
- Toluene and xylene are both aromatic solvents. They belong to the monocyclic aromatic hydrocarbons. With few exceptions, the basic structure of these aromatic compounds is a benzene ring. Toluene, also known as toluol (and sometimes referred to as methylbenzene or phenylmethane), is a mono-substituted benzene derivative. The name toluol refers to tolu balsam, which is an extract from the tropical tree *Myroxylon balsamum* (Fabri et al. 1996; Soffritti et al. 2004). Even though toluene is much less toxic than benzene, careful handling is absolutely essential. Xylene, also called xylol, is a mixture of three isomers of double methyl-substituted benzene derivatives. The name refers to its initial discovery in crude wood spirits (Fabri et al. 1996).

## Results

Not all of the tested solvents were capable of dissolving fully Cambay amber. Acetone only affected the outer surface of the amber after several days, and essentially made it brittle and friable. Petroleum ether, with its multiple flash points, made the amber elastic and opaque. Furthermore, it macerated the samples. After only about 45 minutes, the amber already began to form linear streaks. Several hours later, amber immersed in petroleum ether became a loamy, relatively glutinous mixture (flash point of 40°C). Pure petrol ether produced the same effects.

As already mentioned by Rust et al. (2010), Cambay amber dissolves well in toluene. The solvent gave good results after one hour, and only scarcely affected the inclusions. However, due to the major deleterious health effects of toluene, it is not the method of choice. As described above, we also tested xylene as an aromatic solvent. Like toluene, it was able to dissolve the amber within one hour. It should be preferred instead of toluene, since it is less harmful to humans. Rust et al. (2010) further discussed the use of chloroform. As expected, chloroform

Table 1. Chemical reaction of the amber to different solvents, their chemical formula, and the duration of the reaction.

Solvent	Chemical formula	Time	Comments
Acetone	$C_3H_6O$	$\infty$	Only affected the surface of the amber after several days. The stone became brittle and friable.
Dichloromethane	$CH_2Cl_2$	$\sim 1$ h	No lightening could be observed. Strong effect on the amber. Effects on inclusions were not tested yet.
Trichloromethane	$CHCl_3$	$\sim 0.5$ h	Lightening of the amber with a strong and fast result. Dehydration of the inclusions when left in the solvent.
Dimethylformamide	$HCO-N(CH_3)_2$	$\infty$	Amber floated on the solvent. Did not show an effect after a week.
Formaldehyde	$CH_2O$	$\infty$	Amber floated on the solvent. Did not show an effect after a week.
Petroleum ether	$C_nH_{2n+2}$ ( $n=5\sim 8$ )	$\infty$	Petroleum ether, with its multiple flash points, made the amber elastic and opaque. Furthermore, it macerated the samples. The amber began to form linear streaks. Several hours later, amber immersed in petroleum ether became a loamy, relatively glutinous mixture (flash point of $40^\circ C$ ).
Turpentine oil	predominantly $C_{10}H_{16}$ ( $\alpha$ -Pinen)	$\sim 2$ d	Took longest to dissolve the amber, but finally with a good result. Effects on inclusions were not tested yet.
Orange oil	predominantly $C_{10}H_{16}$ (d-limonene)	$\sim 3-4$ h	Strong effect on the amber. Until now no observed damage to inclusions.
Toluene	$C_6H_5CH_3$	$\sim 1$ h	Strong effect on the amber. Best results when mixed with a small amount of ethanol. Dehydration of inclusions when left in the solvent.
Xylene	$C_6H_4(CH_3)_2$	$\sim 1$ h	Stronger effect than toluene. Effects on inclusions were not tested yet.

worked well as a solvent. When the amber was placed in the clear liquid, it began to decolorize and dissolve. The amber dissolved within half an hour. We also tested dichloromethane because of its similar chemistry. When immersed in dichloromethane, lightening of the amber was not observed. Whereas chloroform was able to completely dissolve the material within half an hour, dichloromethane took about an hour to do so.

Orange Oil dissolved the Cambay amber quite well, within an average time of 3–4 hours. Turpentine Oil was also able to dissolve this amber, but took much longer than any other solvent, about two days. Dimethylformamide was not able to dissolve the amber after 1 week, nor was formaldehyde. Cambay amber floated on these solvents. The dissolution reaction of the amber to different solvents is summarized in Table 1.

## Discussion

The Cambay amber dissolved fully in several of the tested solvents, and the dissolution characteristics of the reactions are similar to those of recent dammar resins and copal. As early as 1896, Glimmann and Tschirch reported that chloroform has a strong solving effect on recent dammar resin. They further found that toluene did not completely dissolve recent resin. In contrast, the Cambay amber in our experiments did completely dissolve, with the exception of a small residue of impurities. Furthermore, Tschirch and Glimmann (1896) noted that petroleum ether could solve recent dammar resin partially, whereas in our study, the Indian amber did not actually dissolve, but became soft and macerated. The discrepancy between the two results is likely due to subtle chemical differences between the Cambay amber and recent dammar resin. Even though it is known that Cambay amber derives from Dipterocarpaceae, which presently comprise 470 Asian species (Maury-Lechon and Curtet 1998), the precise dipterocarp tree that produced the resin has not yet been identified.

We were able to find two new effective solvents for dissolving Indian amber that had not been described before in this context. Furthermore, we determined that xylene is a better and less harmful solvent for Indian amber than toluene. The stronger effect of xylene compared to toluene can be attributed to the two electron-releasing functional groups in xylene which enable a stronger positive mesomeric effect. Additionally, we used a mixture of the three possible isomers for the best dissolution results. As expected, chloroform completely dissolved the amber. Using dichloromethane, it takes longer to completely dissolve pieces of amber than it does in chloroform. This is not surprising, due to dichloromethane two functional groups compared with chloroform's three functional groups.

Fortunately, we were able to show that at least Orange Oil and turpentine oil, both virtually nontoxic solvents were able to dissolve Cambay amber in a reasonable time. The nonpolar components of both oils, the terpenes, may be able to react with the nonpolar parts of the amber. The total compositions of these terpenes are not yet completely specified. They are a group containing various miscellaneous hydrocarbons that operate more strongly (due to their functional groups) with the components of the amber than they do with each other.

A common test to differentiate amber from copal is to apply acetone to the surface of fossil resin pieces. In general it is reported that amber is unaffected, whereas copal reacts to the acetone. However, Indian Cambay amber also reacts to acetone. Tested pieces became brittle and chapped. Ragazzi et al. (2003) reported that copal would dissolve in ethanol. Although ethanol was not explicitly tested here, other experiments have shown that Cambay amber is not affected for at least three days. It is noted that only nonpolar solvents were able to solute the amber.

As mentioned previously, the Cambay amber is usually dark, very brittle, and it can break easily during preparation. Thus, in some cases, it may be impossible to examine arthropod

inclusions adequately for systematic purposes. In such cases, dissolving the amber matrix is a capable alternative investigation method. It allows the extraction of entire inclusions for various analyses, although the material is usually extremely fragile after extraction. The isolated arthropods, as well as other organic inclusions, can be examined with scanning electron microscopy to observe fine ultrastructural preservation (Figs. 1–3), enabling submicron resolution of morphological details. To prepare samples for scanning electron microscopy they are rinsed with alcohol and placed on stubs where they could dry for a short time. After drying the mounted samples were sputter-coated with gold. Additionally, the remarkable preservation of arthropod cuticles allows histological analyses of major parts or even whole organisms and plant material, enabling detailed examinations with transmission electron microscopy as well as histochemical and other molecular methods (JR, NM, and Anke Schmitz unpublished material). Finally, it must be emphasized that rare or scientifically important inclusions should not be candidates for extraction, provided they can be examined properly within the amber, and this protects unique specimens from any dangers of disintegration, however minimal.

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