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Conservation, preparation and imaging of diverse ambers and their inclusions

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ABSTRACT

Amber, a natural polymer, is fossil tree resin derived from diverse botanical sources with varying chemical compositions. As such, all amber is susceptible to the effects of light, temperature, relative humidity, and oxygen, as well as exposure to certain chemicals, and will deteriorate over time in collections if left unprotected. Here we review approaches for the conservation, preparation, and imaging of amber specimens and their inclusions, and address indications and causes of amber degradation, as well as recommendations for a suitable storage environment. We also provide updated preparation and embedding protocols, discuss several techniques for imaging inclusions, and address digitization efforts. A stable storage environment is essential to mitigate or avoid deterioration of amber, which often manifests as crazing, spalling, breaking and colour changes. Based on previous conservation studies of fossil resins, we generally recommend storage in a climate-monitored environment with a relative humidity of ca. 50%, 18 °C, and stress that light exposure must be kept to a minimum. For stabilization and anoxic sealing, amber specimens should ideally be embedded in an artificial epoxide resin (EpoTek 301-2 or similar is currently recommended). Amber should not be treated with or stored in vegetable or mineral oils (even for a short time for examination or photography), or come into contact with alcohol, disinfecting agents, hydrogen peroxide, or other destructive solvents or mixtures, since any of these materials can irreversibly damage the amber. Most photography of inclusions for research and digitization purposes can be successfully accomplished using light microscopy. Scanning electron microscopy (SEM) is sometimes used to uncover fine details, but is an invasive method. However, X-ray based methods (utilizing micro computed tomography, or micro-CT) are becoming more frequently used and increasingly indispensable in the examination of minute internal structures of inclusions, and to fully visualize important structures in opaque amber. Micro-CT makes it possible to digitize an inclusion three-dimensionally, and thus enables digital specimen 'loans'. Light microscopal images are still widely used in the digitization of amber specimens and are an essential alternative to micro-CT imaging when resources or time are limited. Overall, due to the vulnerability of all fossil resins, we recommend that conservation of amber samples and their inclusions be prioritized.

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1. Introduction

Amber is ancient polymerized tree resin that falls into several classes based on botanical origin and consequent chemical and physical properties. Ambers of various ages from many deposits across the globe preserve a great variety of organisms three-dimensionally and with remarkable fidelity, including many that are not typically found or well-preserved as compressions or impressions in rock. Even soft-bodied taxa, such as microorganisms (e.g. bacteria, fungi, and protozoans), are sometimes found within these fossil resins, as well as mosses, liverworts, seed plants, arthropods (especially insects and arachnids) and even vertebrates (e.g. Barden et al., 2020; Bauer et al., 2005; Dunlop et al., 2018; Heinrichs et al., 2018; Kettunen et al., 2019; Penney, 2010; Sadowski et al., 2017a; Schmidt et al., 2006; Stebner et al., 2017; Xing et al., 2020). Due to the exceptional preservation of inclusions in such fossil resins, even to the subcellular level, amber fossils provide new insights for various lines of research within and across such fields as zoology, botany, mycology, palaeoecology, geochemistry, systematics and evolution (e.g. Baranov et al., 2019; Grimaldi and Ross, 2017; Haug et al., 2020; Kaasalainen et al., 2017; Labandeira, 2014; Lambert et al., 2008; McCoy et al., 2017a,b; Penney, 2002; Rikkinen and Schmidt, 2018; Sadowski et al., 2017b). Therefore, the scientific value placed on amber inclusions and the collections they are housed in is indisputable.

However, despite the exceptional preservation of bioinclusions in amber from ancient deposits throughout the world, any amber that is removed from anoxic sediments will begin to deteriorate over time (Bisulca et al., 2012, p. 2), since it is now subject to the effects of visible and ultraviolet light, heat, and changes in relative humidity (RH). Therefore, the preservation and conservation of amber specimens in museum and private collections is essential. Previous studies on the conservation and preparation of fossil resins have addressed specific hazards for amber collections, storage recommendations and preparation techniques (e.g. Bisulca et al., 2012; Caldararo et al., 2013; Corral et al., 1999; Girard et al., 2009; Grimaldi, 1993; Hoffeins, 2001; Koteja, 1990; Nascimbene and Silverstein, 2000; Penney and Green, 2010; Pastorelli and Glastrup 2011; Pastorelli et al., 2011, 2013a, b; Shashoua, 2002; Shashoua et al., 2006; Sidorchuk, 2013; Thickett et al., 1995; Waddington, 2011; Waddington and Fenn, 1988). We suggest, however, that an evaluation and synopsis of these earlier approaches, coupled with a review of recent advances, are needed to encourage and promote state-of-the-art preparation and curation of amber collections, as well as optimal digitization of amber inclusions.

Here we review approaches for the conservation, preparation, and imaging of amber specimens and their inclusions, and address the indications and causes of amber degradation, as well as recommendations for a suitable storage environment. We further provide updated preparation and embedding protocols, discuss several techniques for imaging inclusions, plus current digitization efforts.

A number of natural history museums house amber collections. Some pre-eminent collections are found in such depositories as the Museum für Naturkunde Berlin (MfN: 37,000-43,000 amber specimens; N. Lentge-Maaß, pers. comm.); the American Museum of Natural History in New York (AMNH: 15,000–20,000 Mesozoic and Cenozoic amber specimens from multiple deposits; Bisulca et al., 2012); the Museum of the Earth in Warsaw (20,000 amber specimens; Kosmowska-Ceranowicz, 1990); Staatliches Museum für Naturkunde in Stuttgart (30,000 amber specimens; Naturkundemuseum BW, 2020); the Muséum National d'Histoire Naturelle in Paris (MNHN: 25,000 amber specimens; A. Nel, pers. comm.); the Smithsonian National Museum of Natural History in Washington D.C. (12,000 amber specimens; M. S. Florence and J. K. Nakano, pers. comm.); the National Museum of Denmark (about 17,000 amber objects; Jensen and Jensen 2000, cited from Shashoua, 2002); the Collections of the Geoscience Centre at the University of Göttingen (GZG: 30,000 amber specimens; Reich et al., 2018); Senckenberg Research Institute and Nature Museum (~15,000 amber, copal and resin specimens); just to name a few.

Amber collections encompass a wide range of amber objects. Besides inclusions of various organisms, such collections often house raw (unpolished) amber, copal (resins from the Pleistocene or the Holocene), and amber artifacts, such as beads, amulets, and carvings (Grimaldi, 1996), as well as historic amber specimens which are glued to object slides and embedded in glass chambers using Canada balsam or dammar resin (Klebs, 1880; Neumann, 2010). Although the focus of this paper is on amber specimens with organismal inclusions, the described agents of deterioration, as well as storage recommendations, also apply to other amber objects.

2. Botanical sources and classification of fossil resins

As fossilized tree resin, amber is composed of "volatile and non-volatile terpenoids and/or phenolic secondary compounds" (Langenheim, 2003, p. 24; Ragazzi and Schmidt, 2011; Vávra, 2009). Fossil tree resins are usually divided into five classes (see Seyfullah et al., 2018a, table 3; herein Table 1) on the basis of their chemical structure, as suggested by Anderson and Crelling (1995) using pyrolysis-gas chromatography-mass spectrometry analysis (Py-GC-MS). Most fossil resins can be attributed to Class I or II, each of which have polymeric skeletons, as does the much less common Class III (polystyrene) amber. Class IV and V resins have non-polymeric skeletons, and are thus unable to form true ambers, making them exceedingly rare in the fossil record (Seyfullah et al., 2018a).

Amber deposits exist worldwide, and various botanical sources have exuded resins as far back as the Carboniferous Period ($\geq$ 320 Ma), the earliest by an as-yet-undetermined lineage (Bray and Anderson, 2009). During the Mesozoic, gymnosperms (conifers) produced resins that share some basic chemical characteristics and have generally been designated as Class Ib, based on Py-GC-MS (Anderson, 1995, 2001, 2006). The earliest coniferous resin containing arthropod inclusions is Late Triassic in age (~230 Ma) and was recovered in northern Italy (Schmidt et al., 2012; Seyfullah et al., 2018b; Sidorchuk et al., 2015), produced by a member or members of the extinct family Cheirolepidiaceae. By the Cretaceous, resin-producing conifers were widespread, and many organismal inclusions have been recovered from various Cretaceous ambers across the Northern Hemisphere (see Seyfullah et al., 2018a, table 1 and references therein).

Meanwhile, a number of significant Cenozoic deposits have yielded large amounts of fossiliferous amber from two lineages of angiosperms with very different chemistries (see Seyfullah et al., 2018a, table 1, for a complete list of amber deposits and references): (1) In India and China (as well as in several other Asian deposits), the majority of Eocene and Miocene ambers recovered are attributed to Dipterocarpaceae, and are designated as Class II (dammar) resins (Dutta et al., 2009; Rust et al., 2010; Shi et al., 2014). The dipterocarp lineage likely goes back to the Late Cretaceous; (2) In contrast, Eocene amber from France and Miocene ambers from Mexico, the Dominican Republic, Peru and Ethiopia were produced by representatives of the angiosperm family Fabaceae (and in the case of the Miocene ambers, members of the genus *Hymenaea*), and are categorized as Class Ic (Anderson et al., 1992; Bouju and Perrichot, 2020; McCoy et al., 2017a; Nohra et al., 2015; Poinar Jr. and Brown, 2002; Solórzano Kraemer, 2010).

Another, but very different, Cenozoic amber is succinite, the most common fossil resin of the Eocene Baltic amber Lagerstätte, which likely has coniferous origins (Langenheim, 1969, 2003), along with the chemically related and more geographically restricted succinites of the Bitterfeld and Rovno amber Lagerstätten. Baltic amber succinite (in the following referred to as Baltic amber) was the first amber investigated by the use of Py-GC-MS, and thus was designated Class Ia (due to the presence of succinic acid, which all known coniferous Cretaceous resins lack (Anderson and LePage, 1995; Langenheim, 1969, 2003; Langenheim and Beck, 1965). Succinite sometimes co-occurs with diverse other amber types, such as Glessite and Gedanite. A prominent example is the Bitterfeld Lagerstätte (Kosmowska-Ceranowicz, 2015; Yamamoto et al.,

Table 1
Classification system for ambers, taken from Anderson et al. (1992; and citations therein); Anderson and Botto (1993); Anderson (1994, 2006); Anderson and Crelling (1995); Bouju and Perrichot (2020); Bray and Anderson (2009); Grimaldi et al. (2000a); Grimaldi and Nascimbene (2010); Nohra et al. (2015); Poulin and Helwig (2012); Rust et al. (2010); Seyfullah et al. (2018a); Vávra (2009; and citations therein); and Yamamoto et al. (2006).

Class	Characteristics	Examples	Inferred botanical affinity
Class I	Based on polymers of labdanoid diterpenes, including especially labdatriene carboxylic acids, alcohols and hydrocarbons		
Class Ia	Based on polymers and copolymers of labdanoid diterpens (regular configuration), including communic acid and communol; incorporation of significant amounts of succinic acid	Succinite: Baltic area (shores), Samland (Kaliningrad, Russia) Glessite: Bitterfeld (Saxony, Germany)	Pinaceae? Sciadopityaceae? Burseraceae, *Betula* (Betulaceae)
Class Ib	Based on polymers and copolymers of labdanoid diterpenes (regular configuration), including/not limited to communic acid, communol and biformene; devoid of succinic acid	Cretaceous Raritan amber (New Jersey) Mid-Cretaceous French amber (Charentes) Mid-Cretaceous Burmese amber New Zealand amber	Cupressaceae Cheirolepidiaceae? Araucariaceae? Cupressaceae/Taxodiaceae *Agathis* (Araucariaceae)
Class Ic	Based on polymers and copolymers of labdanoid diterpenes (*enantio* configuration), including/not limited to ozic acid, ozol and enantio bioformenes; devoid of succinic acid	Miocene Mexican amber Miocene Dominican amber Miocene Ethiopian amber Eocene Oise amber Carboniferous amber from Illinois	*Hymenaea mexicana* (Fabaceae) *Hymenaea protera* (Fabaceae) *Hymenaea*? (Fabaceae) Fabaceae Pre-conifer Gymnosperm
Class Id	Based on polymers and copolymers of labdanoid diterpens with *enantio* configuration; incorporation significant amounts of succinic acid	Canadian Arctic (Nunavut) and British Columbia	Unknown
Class II	Polymeric skeleton of bicyclic sesquiterpenoid hydrocarbons, especially cadinene; triterpenoid including di-sesquiterpenoid component as occluded material	Miocene Zhangpu amber Eocene Indian amber Eocene Arkansas amber	Dipterocarpaceae *Shorea* (Dipterocarpaceae) Unknown
Class III	Polymeric skeleton, basic structural feature is polystyrene	Siegburgite: Siegburg (Bonn, Germany) and Bitterfeld (in part) some rare New Jersey ambers	*Liquidambar* (Hammelidaceae)
Class IV	Non-polymeric, basic structural feature is sesquiterpenoid, based on cedrane (IX) skeleton	Ionite: Pliocene of California	Unknown
Class V	Non-polymeric diterpenoid carboxylic acid, especially based on the abietane, pimarane and isopimarane carbon skeletons	Highgate Copalite (Eocene of Highgate Hill area, London), Settlingite (Northumberland, UK)	Pinaceae

2006). However, these additional amber types occur in small quantities and contain few inclusions.

After deposition, given the right conditions, a botanical resin belonging to Classes I-III will begin to polymerize in a process called 'amberization' or 'maturation', becoming a material called copal (pre-amber resin, ca 2.58 Ma–1760 CE) and over time, as polymerization continues, amber (Anderson et al., 1992; Seyfullah et al., 2018a; Solórzano-Kraemer et al., 2020; Tonidandel et al., 2008). We can differentiate between extant resin, copal and amber by determining a specimen's age using appropriate collecting and dating methods (e.g. carbon-14 dating as per Solórzano-Kraemer et al., 2020; or other radiometric as well as biostratigraphic dating techniques), and by analysis of physicochemical characteristics (see Seyfullah et al., 2018a for summary and references). Not all drops or runnels of exuded resin survive over time to become amber, since not all resin is buried, and taphonomic conditions after burial may not be suitable for preservation. In a larger sense, this can apply to entire ancient forest ecosystems. The process and degree of amberization depends directly on a number of factors, including the level of protection from the elements, particularly resistance to oxidative degradation, as well as factors like thermal maturation, and in some cases the avoidance of microbial decomposition (Delclòs et al., 2020; Langenheim, 1969; Seyfullah et al., 2018a). Moreover, geological events (such as volcanic or tectonic activity), geographic location and palaeoenvironmental conditions can affect the microhardness of ambers and their chemical structure (Stach et al., 2019).

Amber deposits are generally preserved in lowland nearshore environments with marine influences that create or enhance anoxic conditions, such as deltas, peat bogs and estuaries (Bisulca et al., 2012; Grimaldi, 1996; Seyfullah et al., 2018a). In addition, clay layers above lignitic lenses containing amber can act as a chemical buffer as well as a physical barrier to atmospheric exposure. The ages and geological histories of individual amber deposits vary significantly, such that generally, older Cretaceous ambers tend to be more friable and subject to swifter degradation than more recent (Cenozoic) fossil resins (Bisulca et al., 2012), with the exception of mid-Cretaceous Burmese amber, which is surprisingly resilient. This is likely due to an as-yet-unidentified chemical component or components in its macromolecular structure, making Burmese amber more resistant to deterioration than other Mesozoic resins (Bisulca et al., 2012, p. 13). It is interesting to note that among Cenozoic resins, coniferous Baltic amber is more resilient than the various angiosperm resins like Dominican (Class Ic) or Indian amber (Class II).

The conservation and preparation of amber specimens must take into consideration the diverse chemical and physical properties of these fossil resins, which are directly related to an amber's botanical source, age, depositional environment, plus any significant taphonomic factors.

3. Amber deterioration

When amber is removed from a deposit, the material becomes susceptible over time to the effects of ultraviolet and visible light and heat, as well as to fluctuations in humidity. Diverse ambers with unique chemistries, or which may be deposited under somewhat different taphonomic conditions, can each react in specific ways, but all will deteriorate over time, and all will benefit from a collection environment that mitigates or prevents exposure to the elements (Bisulca et al., 2012). Hence, in order to conserve an amber collection, specimens should be examined for signs of deterioration.

Here, we describe various types of damage which occur in amber and likely indicate an unsuitable storage environment (summarized in the Supplementary Material, Fig. S1 for didactic purposes):

Crazing – the formation of a network of cracks over the surface of an amber piece (Fig. 1A) – is augmented by exposure to both fluctuating humidity and ultraviolet light, and if left untreated, these fine cracks can lead not only to surficial flaking, but also infiltration of the specimen over time (Fig. 1F), erupting along internal fractures, even directly compromising inclusions. In the worst cases, breakage of the amber piece occurs, along with destruction of its inclusions (Fig. 1G; Bisulca et al., 2012). Indications that a specimen has internal damage include spalling, exfoliation and powder, as well as the formation of a desiccated rind or crust on the amber surface (Thickett et al., 1995; Waddington, 2011). Networks of minute cracks may also develop inside an amber piece close to the surface of an inclusion (Fig. 1H, I), especially in cases where any portion of the inclusion was exposed at the amber surface for a long period of time (Fig. 1D, E; Kaasalainen et al., 2015, 2020; Kettunen et al., 2019).

Colour changes (i.e. 'darkening', 'yellowing', or 'reddening') of amber are especially prevalent in some older collections. For instance, Baltic amber is originally predominantly honey-orange in colour, but if left unprotected and exposed to an elevated or fluctuating temperature (and possibly enhanced when combined with low humidity and/or exposure to atmospheric oxygen), it will turn reddish and darker over time (Fig. 1B, C; Bisulca et al., 2012). Such darkening will eventually obscure any inclusions, and is only reversible in specific cases by trimming/grinding away some of the amber. In accelerated thermal aging tests of Baltic specimens, significant yellowing was observed (Pastorelli et al., 2013a). In amber pieces left untreated for long periods of time, especially in long-term historic collections, inclusions themselves may eventually darken, sometimes becoming quite dark or even black, so that cellular details are no longer discernable (Fig. 1E; Bisulca et al., 2012). When this happens, little can be done to reverse the damage.

Exposure to specific elements either singly or in combination, given enough time or intensity, will lead to various forms of deterioration in all fossil resins. The deterioration of amber is induced and increased by exposure to UV-light, the visible spectrum of daylight, high temperatures, shifts in temperature (including freezing), high or low or shifting RH, and any combination or fluctuation of the above-named factors. Further hazards include various forms of oxidation, exposure to pollutants, cleaning agents, fungi or bacteria (Beimforde and Schmidt, 2011; Bisulca et al., 2012; Girard et al. 2012; Pastorelli, 2009; Shashoua, 2002; Shashoua et al. 2006; Waddington and Fenn, 1988; Wang et al., 2014; see Sections 5.1 and 5.2).

Prolonged exposure to UV-light (100–400 nm) and visible daylight (390–750 nm), especially behind window glass without UV blocking filters (Dunnill, 2014), will cause severe damage to Baltic amber, since it induces the oxidation of the molecular structure of the amber (photodegradation; Bisulca et al., 2012; Pastorelli et al., 2011). Moreover, intense light can induce a photochemical decarboxylation reaction and the formation of dark coloured quinones which leads to the browning of amber (Heinrichs et al., 2013). However, among the five amber types tested by Bisulca et al. (2012), Eocene Baltic amber was determined to be the most stable when exposed to light, while Cretaceous New Jersey amber was found to be the most unstable.

Levels or changes in RH can cause or contribute to deterioration in fossil resins, but detrimental effects vary between different ambers. While Dominican amber degrades quickly under low relative humidity, Baltic amber will not tolerate a relative humidity that is either too high or too low (Bisulca et al., 2012; Shashoua, 2002). If the RH is too low, Baltic amber off-gasses formic acid and acetic acid, an indication that degradation is occurring (Pastorelli and Glastrup 2011). If the RH is too high, Pastorelli et al. (2013b) showed that thermally-aged Baltic amber undergoes hydrolysis, during which succinate esters are hydrolyzed into communol and succinic acid. This supports the notion that an increased RH in combination with thermal stress can accelerate amber degradation.

Elevated temperature in conjunction with changes in oxygen level has been shown to achieve specific colour changes, particularly in Baltic amber as described above. In a study by Wang et al. (2014), different colour changes in Baltic specimens were intentionally created by heat treatment in combination with controlled oxygen supply. For instance, a high temperature of 210 °C plus a high oxygen concentration produced a red colour in Baltic amber. A lower temperature of 50–60 °C coupled with slow oxidation over 60 to 100 days resulted in a beeswax-like discoloration. Deep-frying amber followed by baking (long-term heating) created tiny internal cracks giving a "sparkling effect." So-called "sun-sparks" are disk-shaped cracks created by heating in conjunction with a rapid change in pressure (Dahms, 1906; Wang et al., 2014). Autoclaving (combining heat and pressure) might not only damage amber and change its chemical properties (Wagner-Wysiecka, 2018), but also alter, shrink or darken its inclusions, so that certain characters are hardly visible after treatment (Hoffeins, 2012; Szwedo and Sontag, 2009). Like heating, a bath in boiling oil (e.g. linseed oil) clarifies amber and induces discoloration, a method that has been widely used in jewelry production (Dahms, 1906; Tornquist 1911). Hence, depending on temperature, the duration of heating, pressure and oxygen concentration, various colour changes and internal reflective cracks can be induced in Baltic amber (Wang et al., 2014). This further substantiates how a combination of different environmental factors can be especially harmful to amber.

Another combination of two or more environmental factors, in this case fluctuating RH and exposure to UV-light and/or daylight, will inevitably lead to significant crazing of amber specimens (Bisulca et al., 2012; Pastorelli et al., 2011). Fluctuations of these factors, especially over a short period of time, are particularly harmful. For instance, a series of abrupt changes in RH along with exposure to UV-light significantly increased the level of crazing in amber specimens that underwent this regimen, "since [a] polymer needs enough time to reach equilibrium with ambient conditions" (Bisulca et al., 2012, p. 8).

Fossil resins are also highly susceptible to pollutants and cleaning products. It has been shown that substances like ammonia, formic or acetic acid and hydrogen sulphide can significantly damage Dominican amber specimens, causing darkening, 'crizzling' and exfoliation (Waddington and Fenn, 1988). Moreover, biocide vapours of naphthalene, paradichlorobenzene and camphor can lead to a partial dissolution of the amber. This sensitive reaction to substances which may occur in museum collections needs to be considered during both storage and exhibition (Waddington and Fenn, 1988). Pastorelli (2009) and Pastorelli et al. (2013b) showed that acidic and alkaline environments cause chemical changes in Baltic amber specimens, specifically alkaline hydrolysis (saponification) or acidic hydrolysis of the succinate ester, resulting in the formation of communol and communic acids. Since this process involves oxygen, it can be prevented by storing amber in an anoxic environment (Pastorelli, 2009; Pastorelli et al., 2013b).

Oxidation is the most problematic hazard for amber, since it is intrinsically linked to other environmental factors, particularly temperature, light and airborne pollutants, all of which contribute to the oxidation process (Pastorelli et al., 2013a). Oxidative radical reactions break down the polylabdanoid chains of the amber, causing depolymerisation, which begins on the amber surface. Once the surface is physically damaged, oxygen can diffuse into the amber, inducing more depolymerisation (Pastorelli et al., 2013a). This process can also lead to colour change (yellowing/darkening) and the eventual fragmentation of the amber specimen (Pastorelli et al., 2013a). However, the pace and degree of deterioration primarily depends on the type of amber. Since ambers from different deposits differ in age, botanical source and resulting chemistry, they each possess distinct functional groups that react differently to oxidation (Bisulca et al., 2012).

So-called 'pyrite disease' has long been recognized as a form of deterioration that can cause severe damage to fossils in palaeontological collections (Becherini et al., 2018; Cavallari et al., 2014; Larkin, 2011).

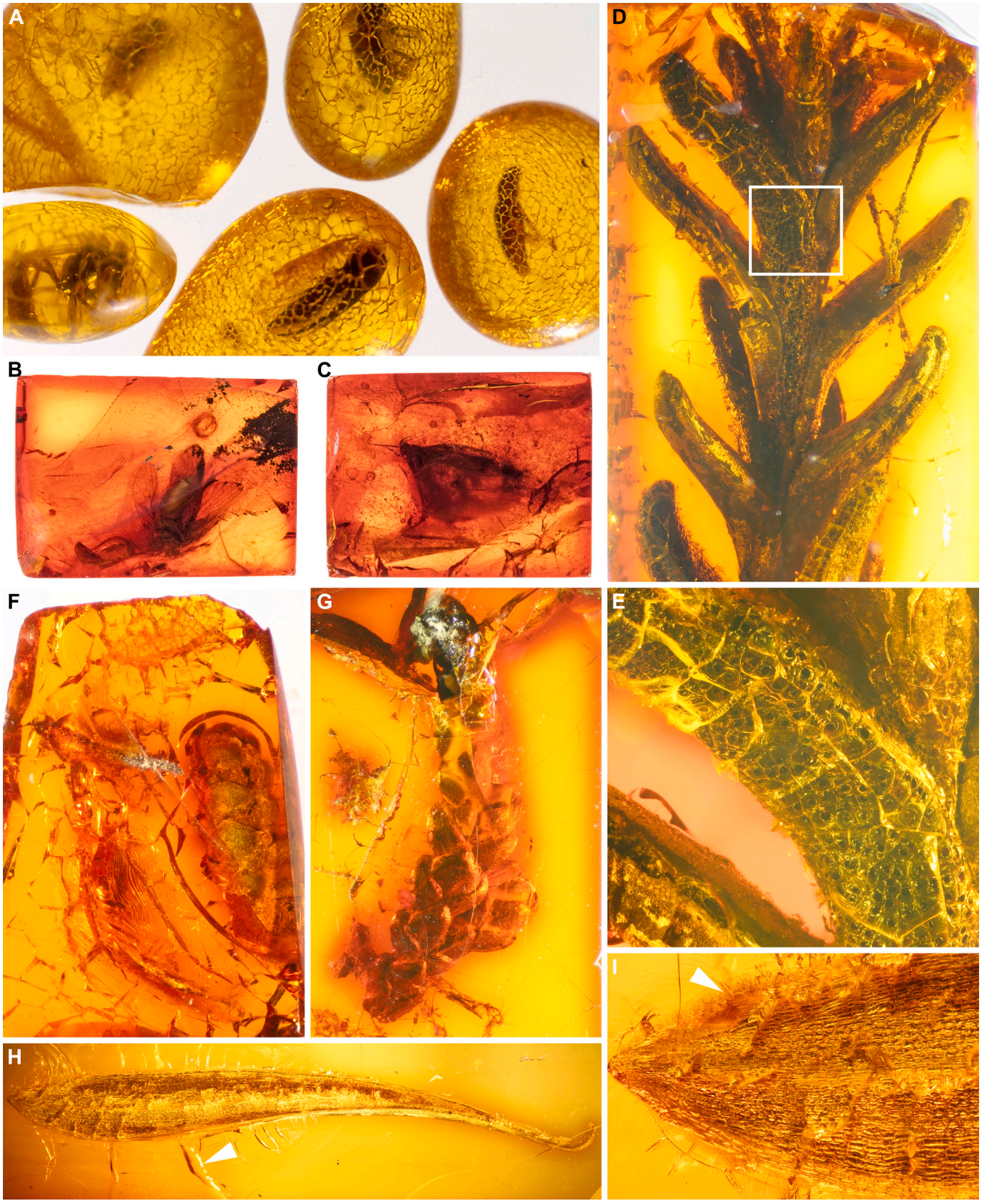


Fig. 1. Amber deterioration. A: Dominican amber specimens with insect inclusions exhibiting crazing (American Natural History Museum, New York); note the network of fissures covering the entire surface. B, C: Baltic amber with insect inclusions (Simon Amber Collection; Museum für Naturkunde Berlin); originally a honey-orange colour that turned reddish as the inclusions darkened. D, E: Inclusion of a partial conifer shoot from Baltic amber (Königsberg Amber Collection, University of Göttingen); white-line inset is magnified in 'E', showing that the surface of the inclusion darkened and became riddled with fissures, so that epidermal features are indiscernible. F, G: Cupressaceous inclusions from Baltic amber (Künow Amber Collection, Museum für Naturkunde Berlin) with deep cracks exposing the inclusion at the surface (G). H, I: Conifer needle from Baltic amber (Königsberg Amber Collection, University of Göttingen) with fine fissures (arrowheads) that protrude from the inclusion.

This process occurs when iron sulfide, in the form of pyrite or marcasite, oxidizes, leading to the formation of sulphuric acid and hydrated iron sulphates. The transformation of sulphide to sulphate is accompanied by an increase in molar volume, and thus can lead to breakage (Becherini et al., 2018). Interestingly, pyrite disease has never previously been discussed as a potential hazard for amber collections. During the current study, we observed greyish powder with yellow crystals commonly associated with pyrite disease. These were seen in and around some amber specimens, along with the formation of some dark-to-grey crystals in contact with amber inclusions (Fig. 2A–E). The formation of these crystals appears to have created stress by increasing volume, causing internal fractures within the amber that extend up to the amber's surface (Fig. 2F). Pyrite can also occur inside inclusions (Fig. 2F; Garty et al., 1982; Hartl et al., 2015) or replace inclusions entirely (Knight et al., 2010; Seyfullah and Schmidt, 2015, fig. 6c; here in Fig. 2G). However, there are currently no published studies that address pyrite disease in

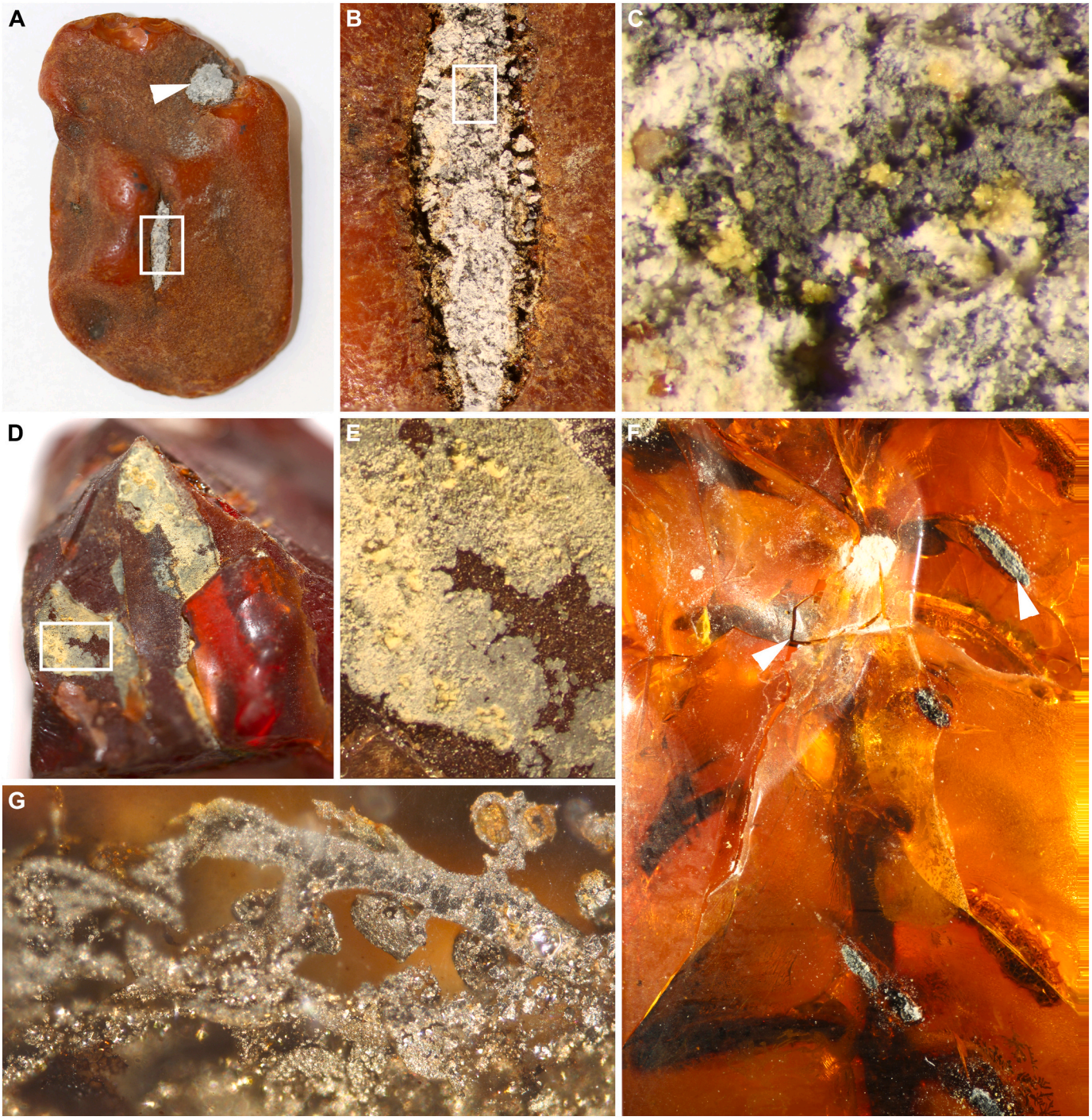


Fig. 2. Pyrite disease in amber. A–E: Two Baltic amber pieces (MB.I.8640, A–C; MB.I.8641, D–E; Simon Amber Collection, Museum für Naturkunde Berlin) with grey-yellow crystal growth in fissures at the surface. White-line insets in A and D are magnified in B–C and E. Samples were taken from the indicated areas (insets in A, D and arrowhead in A) and studied using XRD, Raman and SEM/EDS; C is magnified in Fig. 3A. F: Plant inclusion from Baltic amber (GZG.BST.24637, Königsberg Amber Collection, University of Göttingen), showing crystal growth on the inclusion (right arrowhead); the left arrowhead indicates fractures, likely caused by crystals that expanded in the amber. G: Inclusion of a bryophyte (*Frullania cretacea*, AMNH Bu-FB 1) from Burmese amber that is entirely replaced by pyrite.

amber collections, and the processes of pyrite formation in amber are not yet fully understood.

We suggest that pyrite-induced bursting during oxidation may be based on the fact that some amber inclusions themselves contain pyrite. To test whether pyrite disease occurs in amber, we studied two Baltic amber specimens from the Simon Amber Collection (MB.I.8640, MB.I.8641, Museum für Naturkunde, Berlin), each of which displayed grey and yellow crystal growth on their surfaces and in fissures infiltrating the amber (Fig. 2A–E). We examined the crystals by scanning electron microscopy/energy-dispersive X-ray spectrometry (SEM/EDS), Raman spectroscopy and X-ray powder diffraction (XRD) using analytical conditions as detailed in the Supplementary Material (S2). Raman spectroscopy (Fig. 3B), SEM/EDS (Fig. 3A; Fig. S2), and XRD (Fig. 3C) indicated the presence of pyrite, szomolnokite ($FeSO_4 \cdot H_2O$) as well as minor amounts of quartz and phyllosilicates in the amber specimens. Besides pyrite and szomolnokite, no other iron sulfides or sulfates were detected by XRD. This suggests that pyrite was the sole precursor for the hydration–oxidation reaction to szomolnokite, which proceeds according to the reactions:

$$4FeS_2 + 13O_2 + 2H_2O \rightarrow 4FeSO_4 + 2H_2SO_4 + 2SO_2$$

$$FeSO_4 + H_2O \rightarrow FeSO_4 \cdot H_2O$$

Furthermore, given that several iron sulfates of the general formula $Fe_x(SO_4)_y \cdot nH_2O$ with different crystal water contents exist (e.g., $FeSO_4 \cdot 4H_2O$, $FeSO_4 \cdot 5H_2O$, $Fe[SO_4]_3 \cdot 9H_2O$) and are typically found among iron sulfide alteration products (e.g., Dimitrova et al., 2020; Majzlan et al., 2011), it is possible that the sole presence of szomolnokite in the studied samples is due to rather constant humid storage conditions ($\geq$ 60%) in the Simon Amber Collection that favored formation of szomolnokite over more hydrated forms. Quartz and clay minerals are probably surficial contaminations or were entrapped in the resin before it cured. Pyrite in amber hints at a sapropelic environment, into which the resin dripped before being embedded, and most likely occurs in places where iron sulfide has permeated the amber bearing sediments, as is the case for most lignitic sediments (Sidorchuk, 2013). Over time, the pyrite was probably formed during the diagenesis of the amber specimens (Garty et al., 1982; Hartl et al., 2015; Kowalewska and Szwedo, 2009).

Our study shows for the first time that pyrite disease should be recognized as a possible threat to any amber specimens that are infused with pyrite or marcasite. As pyrite disease occurs under humid conditions (RH $\geq$ 60%), we suggest a constant storage environment with an RH of 50%, as well as epoxy embedding to prevent oxidation (see Sections 4.1 to 4.3). In addition, Hartl et al. (2015) observed halite crystals within a lichen inclusion in Baltic amber. The halite probably formed during or after transport of the amber in sea water (Hartl et al., 2015). Whether or not the formation of halite crystals is a potential danger to (Baltic or other) amber inclusions is unknown; however, hypothetically, crystal growth might induce enough pressure in an inclusion to cause cracking.

In summary, there are a number of factors that can contribute to the deterioration of fossil resins, and others that are implicated and require further study. Exposure to and fluctuation of light, temperature and RH, singly or in combination, as well as various other forms of oxidation, are the most severe threats to amber. Most of the previous conservation studies cited here have focused on one or very few amber types. Future studies that specifically address various deterioration agents and how they harm different amber types are needed to adjust specific conservation and storage protocols for each type of amber. There is a need for further research to address the deterioration of Class II ambers, like those recently recovered from the Eocene of India, Miocene of China and several other Cenozoic Southeast Asian deposits (Rust et al., 2010; Shi et al., 2014).

4. Amber conservation

Most harmful agents that affect amber collections can be controlled by maintaining an optimal storage environment: providing a stable RH and a controlled temperature, as well as limited light exposure (see storage recommendation below). Essentially, amber and its inclusions can best be preserved by recreating as closely as possible the stable anoxic conditions (Bisulca et al., 2012; Pastorelli, 2009; Pastorelli et al., 2013b) that preserved the ancient deposits that contained these resins in the first place. Therefore, we recommend a stable storage environment with an RH maintained at 50%, as well as epoxy embedding to prevent oxidation. Conditions and procedures are explained in the following sections.

4.1. Approaches to the preservation of fossil resins

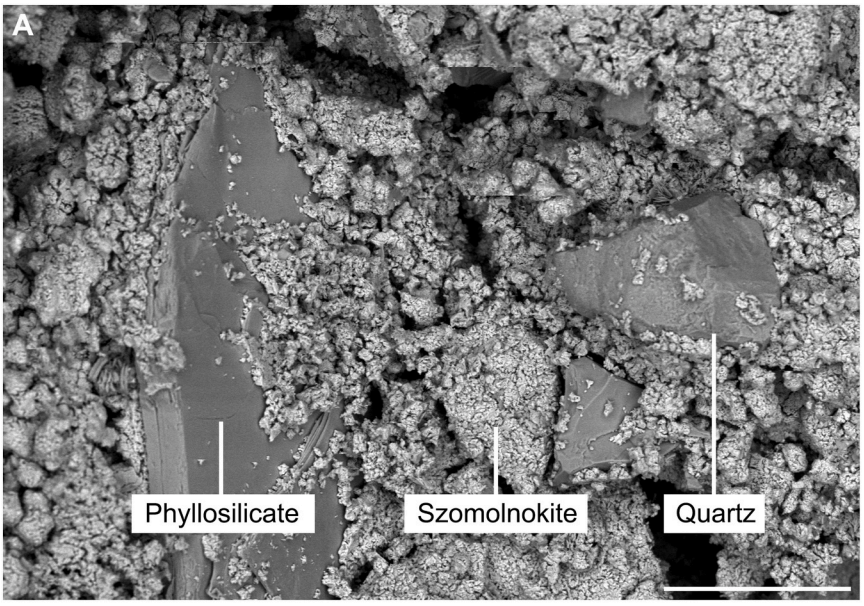
There are several methods used to preserve amber specimens and prevent exposure to harmful environmental conditions and agents.

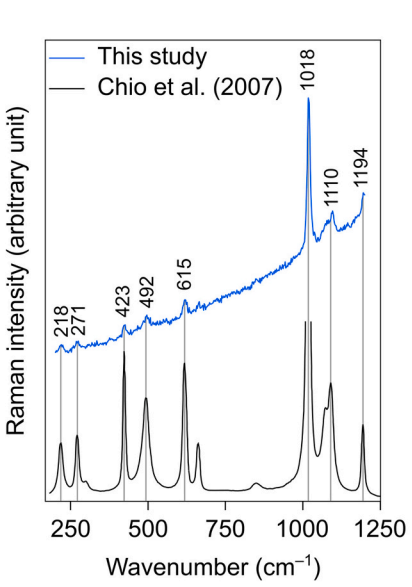
4.1.1. Immersion in a dammar-like resin

One longstanding practice is immersion in a modern liquid resin like Canada balsam (coniferous, *Abies*: Pinaceae) or dammar resin (angiospermous, *Shorea*: Dipterocarpaceae), or in an artificial liquid resin based on the naturally-produced botanical ones, any of which require permanent storage of the specimen within a glass chamber (Fig. 4A). The method was established in Königsberg (today Kaliningrad, Russia) by the amber preparator and entomologist Georg Künow (Hinrichs, 2007) and later pursued by the amber collector and scientist Richard Klebs (Tornquist, 1911). For this preparation method, Klebs (1880) used a solution of dammar resin and venetian turpentine, which was solved in turpentine oil, filtered and carefully inspissated. The amber specimen was closely trimmed and polished, then placed in a glass chamber, which was glued onto an object slide and filled with the resin mixture (Fig. 4A–H; Azar, 1997; Dahms, 1914; Klebs, 1880; Perrichot et al., 2004). Historic amber collections, such as the Künow Amber Collection (Museum für Naturkunde, Berlin) and the Königsberg Amber Collection (Collections of the Geoscience Centre at the University of Göttingen, GZG) contain many of these object slides, which have preserved amber specimens for over a hundred years. Unfortunately, in some cases the technique severely limits viewing or any further preparation for study by modern systematists due to light scattering within the glass and resins, and also because the distance of the amber inclusion from the glass surface prevents use of high-magnification microscope objectives for study. This, however, can be minimized by placing the specimen between glass coverslips for at least its two largest surfaces and by adjusting the thickness of the glass chamber as close as possible to the specimen.

In addition, dammar-like resins covering amber specimens in these glass chambers sometimes have been known to gradually deteriorate and form fissures (Fig. 4D, F, H, I), providing oxygen access and increasing light scattering. Any attempt to remove an amber specimen from one of the glass chambers requires a certain degree of skill and may risk destroying the resinous medium surrounding the amber, and thus the amber itself, along with its inclusion. However, important specimens in historical collections that initially were preserved in this way can be successfully separated from the medium, but it is a very careful meticulous process. Figs. 4H and I show one example – a lichen (*Calicium succini*) successfully removed from dammar resin-filled glass chambers of the Künow Amber Collection (Fig. 4H, I) for reinvestigation and photography (Fig. 4J, K; published in Kettunen et al., 2019). The glass cover slip was already lacking, so the dammar resin was carefully cut out from the glass chamber using a scalpel. Since dammar resin is softer than the embedded cuboid amber, this process did not damage the historic amber specimen.

In certain instances when this method of preservation has been used, damage and deterioration of the amber specimens themselves have occurred over time, due to colour changes and crazing of the immersive medium (Fig. 4D–F, H, I; Zatorska et al., 2013), or to discoloration and darkening of the amber and its inclusions (Fig. 4F, G). The latter can occur if the balsam penetrates the amber via fissures that extend inward from the amber's surface, reaching the inclusion and impregnating its





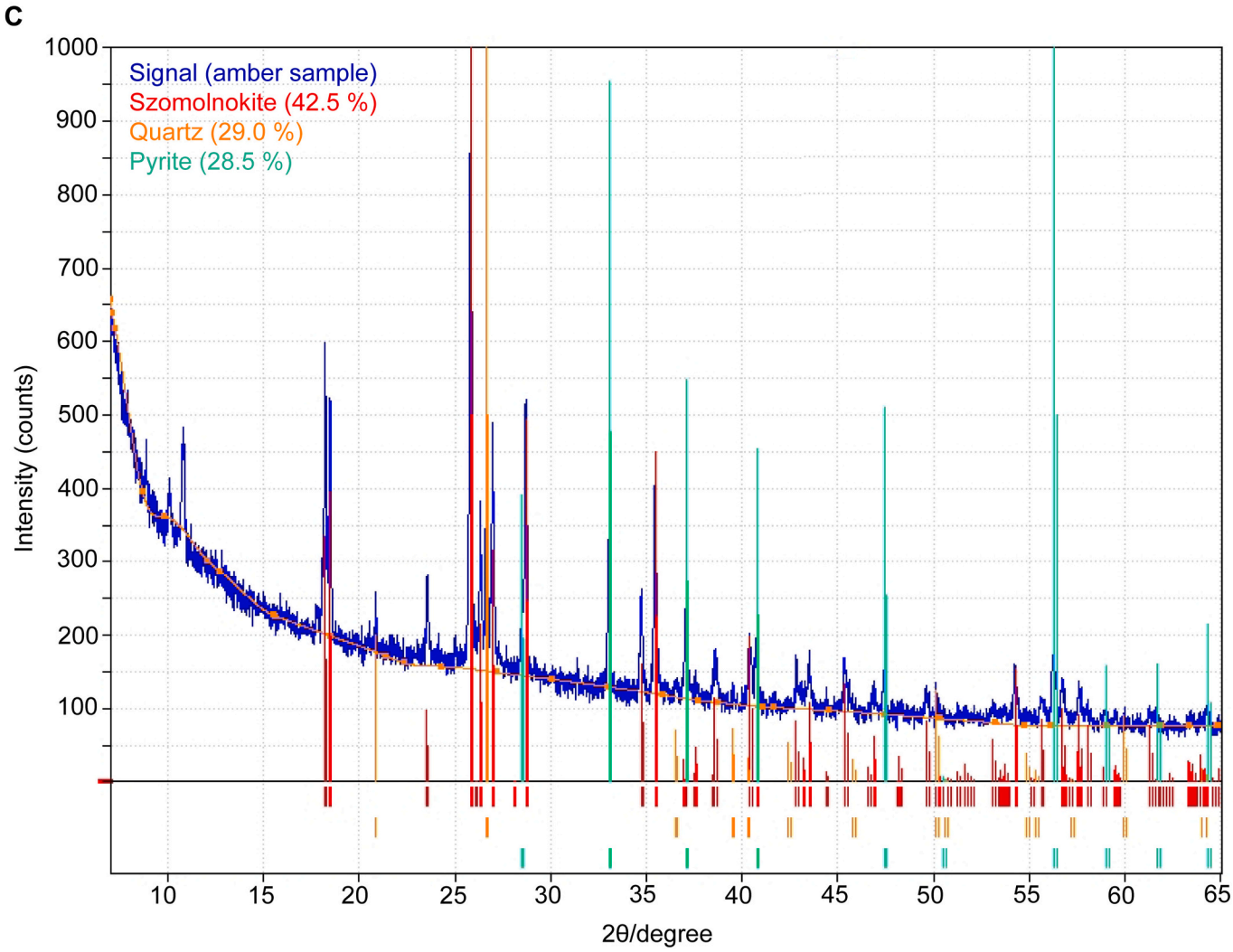


Fig. 3. Analyses of crystal growth in Baltic amber – samples taken from specimens depicted in Fig. 2A–E. A: Back-scattered electron SEM image of crystal growth (magnified from Fig. 2C), showing phyllosilicate, szomolnokite and quartz. B: Representative Raman spectrum of szomolnokite ($FeSO_4 \cdot H_2O$) in the amber inclusions compared to a reference spectrum from Chio et al. (2007). Numbers above the measured spectrum indicate Raman band positions. C: Powder X-ray diffractogram spectrum indicating the presence of pyrite, szomolnokite ($FeSO_4 \cdot H_2O$) as well as minor amounts of quartz and phyllosilicate in the amber specimens (colours as indicated in C). Scale bar: A = 100 μm.

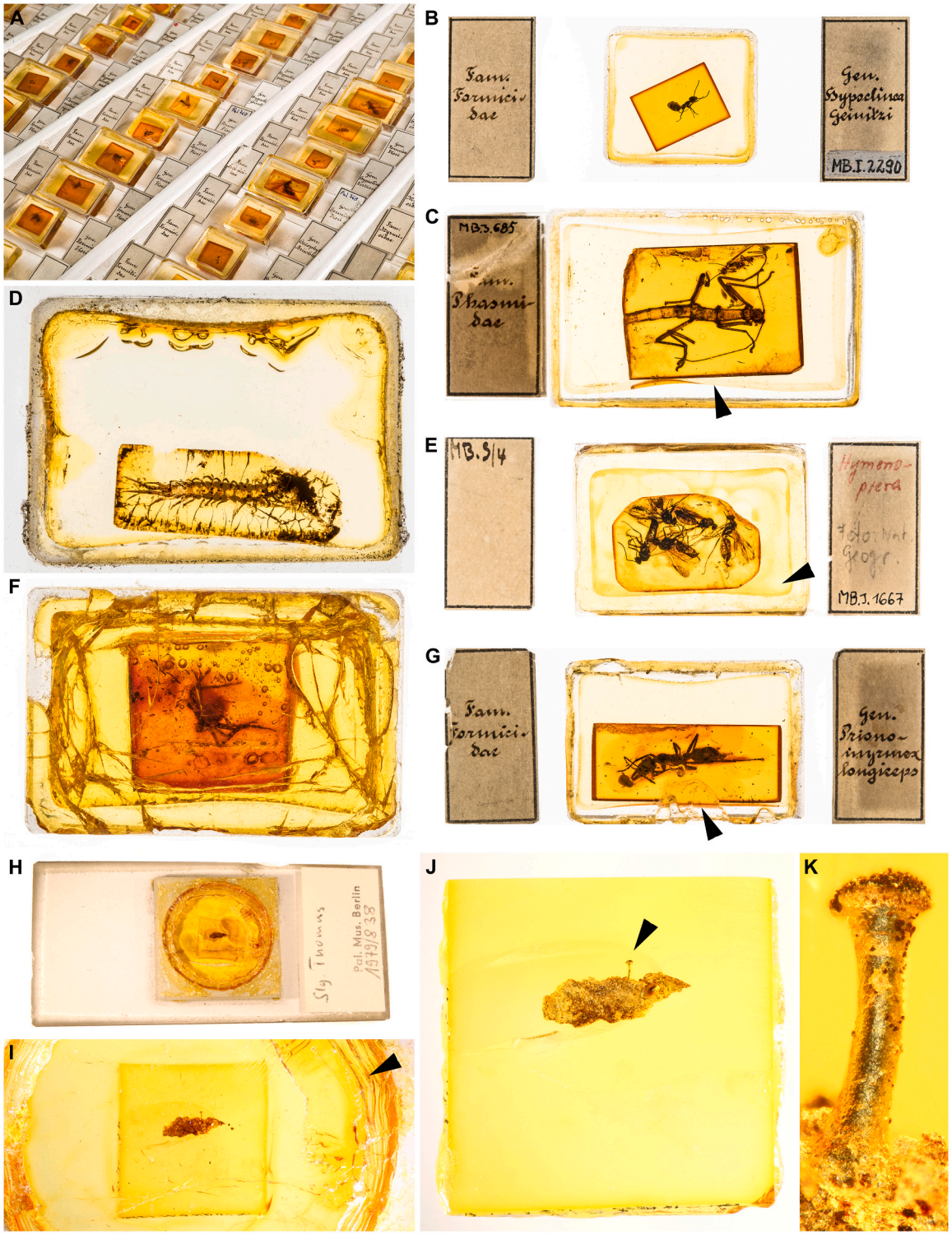


Fig. 4. Baltic amber specimens of the historic Simon Amber Collection (A–G) and the Thomas Amber Collection (H–K; Museum für Naturkunde Berlin) that were embedded in glass chambers using dammar resin and glued onto object slides over a hundred years ago. A: Overview of the object slides. B: Object slide with an ant inclusion (MB.I.2290) showing no signs of deterioration. C: Phasmidae inclusion (MB.I.0685); note the shrunken dammar resin (arrowhead) in the glass chamber, almost reaching the amber specimen. D: Glass chamber with a myriapod inclusion (MB.A.1739); besides the colour change of the dammar resin, the amber piece is covered with deep fissures. E: Hymenoptera inclusions (MB.I.1667); note the yellow colour change of the dammar resin (arrowhead). F: Glass chamber with an arachnid inclusion (MB.A.0100), enclosed in fissured, discoloured dammar resin. G: Object slide with an ant inclusion (MB.I.5827); note the fractured glass chamber (arrowhead) that exposes the specimen to external degradation factors. H, I: Object slide with a lichen inclusion (*Calicium succini*, MB.Pb.1979/0838) without coverslip; the dammar resin shows signs of deterioration, including discolouration and crazing (I, arrowhead). J, K: The amber specimen (shown in H and I) was cut out from the dammar resin, removed from the glass chamber (J) and polished (K); note the micromorphological details, such as the spores, that are now clearly visible. Images in 'A–G' by Carola Radke (Museum für Naturkunde Berlin).

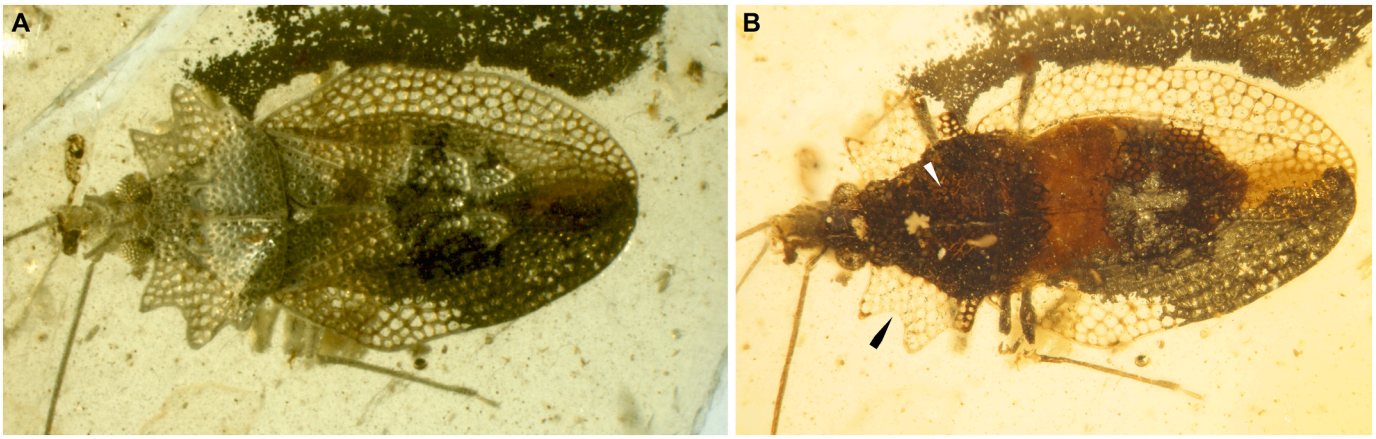


Fig. 5. Effect of Canada balsam on a Cretaceous French amber specimen affected by a fissure reaching the insect inclusion (*Ambarcader eugenei*, MNHN.F.A30053). A: Aspect of the inclusion in 2005, immediately after embedding in Canada balsam. B: Current aspect showing the discoloration (black arrowhead) and darkening (white arrowhead) resulting from the impregnation of the insect cuticle by the Canada balsam. Note the pyritized portion of the left wing that seems less affected.

integument, or in some cases even diffusing within the amber matrix without apparent fissures (Nel et al., 2021: fig. 1). When this happens, the insect cuticle or plant epidermis is actually still preserved but more difficult to visualize because these have become too dark or nearly invisible (Fig. 5A, B). Amber specimens from historic object slides have sometimes been so severely damaged that details of their inclusions are no longer discernable (Fig. 4F). This typically happens when the glass chamber is damaged (Fig. 4G), does not seal properly (or if a coverslip becomes detached, Fig. 4H, I), exposing its interior, and over time allowing oxygen to enter the dammar resin. Therefore, it is strongly recommended that historic specimens be regularly checked for signs of degradation. In severe cases of damage, restoration of the particular specimen should be considered. This includes careful removal of the amber piece from the glass chamber and surrounding medium (e.g. using a scalpel, as described above), grinding and polishing of the amber specimen and then embedding the specimen in an epoxy resin (see Section 4.2 below for protocols).

4.1.2. Varnish 'bath'

Another conservation method used in some private collections is the coating of amber specimens in a 'varnish' (mostly one-component polyurethane resin, e.g. Acrüdur R 40 with thinner, Rüegg company, Germany). A cotton fiber is glued to the surface of a piece of amber (Fig. 6A), which is then lowered and immersed in a mixture of the varnish and a diluting agent. Following this procedure, the amber specimen is hung up by the fiber to dry in a dust-free chamber until the coating (which now covers the entire specimen) has cured (Gröhn, 2015; Gröhn and Kobbert, 2017). This method is preferred by some private amber collectors who wish to simplify the conservation of their specimens, so that access to a professional lab is not necessary (and is occasionally used in exhibitions to preserve the natural look of individual amber pieces). This coating appears to protect amber pieces superficially from degradation. However, there is no published research on this form of conservation, whether it prevents deterioration over time, and there are no studies on the effects of light, RH, temperature and oxidation on these varnished amber pieces. It is important to note that such coats of varnish are disadvantageous if amber specimens need further preparation, particularly grinding and polishing, during which the varnish coating exfoliates (Fig. 6B), and powder from grinding accumulates between the varnish and the amber (Fig. 6C). Furthermore, surface damage can occur between the coating and the specimen, in the form of spalling of the amber, such that the coating needs to be entirely ground away to obtain an even, smooth surface. In addition, any handling of the specimens may cause scratches in the varnish layer, since the polyurethane resin is even softer than the amber. It is not known how varnish might interfere with physical- and chemical analyses of the amber, such as infrared spectroscopy, or how it might change the composition and properties of the amber. In archaeological and art collections, paraloids are sometimes used to coat and protect amber carvings and beads (e.g. Paraloid B-67 and B-72, in combination with mineral spirits or xylene). Another coating substance used for this purpose is Regalrez ® 1126, a hydrogentated hydrocarbon resin (Ham et al., 2009; Lin and Rizzo, 2014; Teodor and Macovei, 2008; Zatorska et al., 2013). However, it is unknown how any of the various coatings may affect inclusions, or what other long-term effects might occur. Moreover, any use of xylene "might inadvertently extract soluble molecular fragments from [some] non-Baltic ambers and therefore compromise future provenance analysis using Py-GC-MS" (Lin and Rizzo, 2014, p. S102). Because of the above-described disadvantages in handling specimens, and also since long-term effects are unstudied, we recommend that none of these coating methods should be used for largescale collections.

4.1.3. Embedding in a high-grade 'glass conservation' epoxy

The preservation method currently favored by researchers is to embed amber in a high-grade 'glass conservation' epoxy (e.g. EpoTek 301-2), which can in turn be trimmed and polished to conform to the shape of each amber piece, while hermetically sealing it to create an anoxic environment (Corral et al., 1999; Nascimbene and Silverstein, 2000). Embedding amber pieces in an artificial resin was first tried by Schlee and Glöckner (1978), who used a polyester resin (e.g., GTS manufactured by Vosschemie company, Uetersen, Germany). The method was applied to amber housed at the Staatliches Museum für Naturkunde in Stuttgart. The use of polyester as a medium has since continued in some major private amber collections (such as the Hoffeins Amber Collection) and has been further developed since then (Hoffeins, 2001).

Embedding in an artificial resin enhances viewing on as many as six (typically flat) surfaces (in which opposite sides are parallel) and, particularly with the use of an epoxy, strengthens the amber by filling any cracks, surficial pores or fissures (Fig. 7A–H), enabling the close preparation required to view details of inclusions, while protecting each piece for long-term study and survival in museum collections (Fig. 7I–K). Furthermore, epoxy can clarify semi-translucent amber and increase the visibility of inclusions, as we observed in Miocene amber from New Zealand (Schmidt et al., 2018a). EpoTek 301-2 replaces the use of earlier less advanced epoxies (such as Buehler Epoxicure mentioned by Nascimbene and Silverstein, 2000), and significantly, unlike earlier epoxies, did not exhibit yellowing in accelerated aging tests (Bisulca et al., 2012). There is some evidence to suggest that at least certain polyester products may not be ideal for embedding amber (in one case, cured polyester was

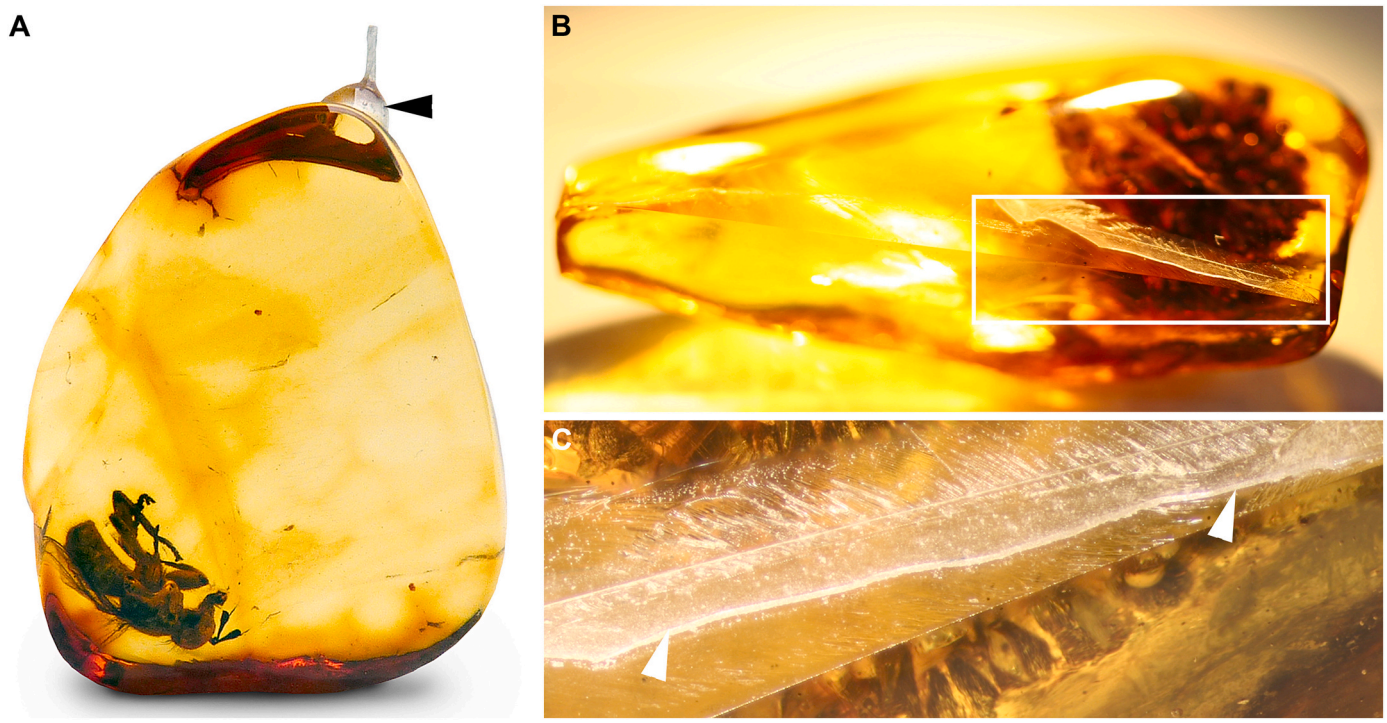


Fig. 6. Baltic amber specimens coated with varnish as a conservation method. A: To coat the amber, a cotton fiber is glued to the surface (arrowhead), by which one can immerse the amber into the varnish. B: Amber specimen with a varnish coating that exfoliated after cutting into the amber. C: Exfoliated varnish coat, magnified from B (white-line inset); arrowheads indicate powder from grinding that accumulated between the coating and the amber. Image in 'A' by Carsten Gröhn (Glinde).

observed to peel off polished surfaces of North Carolina amber specimens), and it should also be noted that some manufactured polyester products have been observed to quickly turn yellow after embedding (Schlee and Glöckner, 1978), while no such peeling or colour changes have been reported in the use of modern 'glass-conservation' epoxies.

While fossil resins are chemically diverse, those studied thus far all appear to benefit from the epoxy embedding technique. Most recently, initial trimming and polishing of Class II ambers has revealed fresh surfaces that are typically 'gummy' and can be smeared by contact. Embedding these pieces in a high-grade epoxy produces all the benefits seen for other ambers, but also eliminates cumbersome handling of specimens, as well as the virtual impossibility of studying inclusions in detail. In addition, coating surfaces of larger Class II pieces (those with multiple inclusions, or any-sized piece infected by internal marcasite seams) using a small paint brush dipped into the liquid epoxy-hardener mixture, has been shown to clarify and protect surfaces after curing while the piece is cut into smaller sections, each of which can then be separately processed.

As with the use of balsam or dammar resins, we have sometimes observed darkening of insects, plant remains and fungi preserved in amber when epoxy penetrated these inclusions via fissures that extended inward from the amber's surface (this darkening was also noted and described for insect inclusions with the use of polyester by Hoffeins, 2001). In such cases, an inclusion's integument is still preserved but more difficult to illuminate because it has become too dark. One possible explanation is that the resin fills the light-refractive minute gap between the inclusion and the amber matrix, which renders imaging of surface structures difficult. Careful screening of such amber specimens for fissures that act as a conduit between the amber surface and any inclusion or part of an inclusion, and sealing these at the surface with semi-hardened epoxy resin, is therefore proposed. Also, we recommend taking images before and after embedding to monitor possible (reflective or refractive) changes of the inclusion.

It is possible that some copals, as well as recent 'Defaunation resins' (Solórzano-Kraemer et al., 2020), may have an adverse reaction to the embedding process, since they have not completely lost their volatile compounds. Therefore, the method is not yet recommended for these resins and requires further study.

It should be noted that, since amber types differ in their physico-chemical properties based on botanical source and taphonomic history, each amber type might behave somewhat differently during certain aspects of preparation. If preparation-conservation procedures are to be undertaken for amber with unknown or untested properties, test trials with less valuable specimens should be implemented beforehand.

4.2. Preparation techniques and protocols for embedding various ambers in an epoxy

Equipment for the preparation and embedding of amber includes a stereoscopic microscope (with adjustable fiber-optic or LED lighting), a flat lap with the appropriate grinding/polishing discs (wet silicon carbide), a small rock-cutting saw (with water reservoir), a high-grade conservation epoxy (like EpoTek 301-2 or Araldite 2020®: parts A and B; or similar), small reusable or disposable embedding cups (or self-made silicon moulds, or, dependent on size of the amber specimens, ice cube trays made of silicone), 5-min epoxy, glass rods, small paint brushes, and a vacuum pump apparatus (specifications as described in Nascimbene and Silverstein, 2000) or vacuum oven.

A. Screening

1. If preparing crude amber pieces from a deposit, initially wash these in water.
2. Select samples and screen these for inclusions.

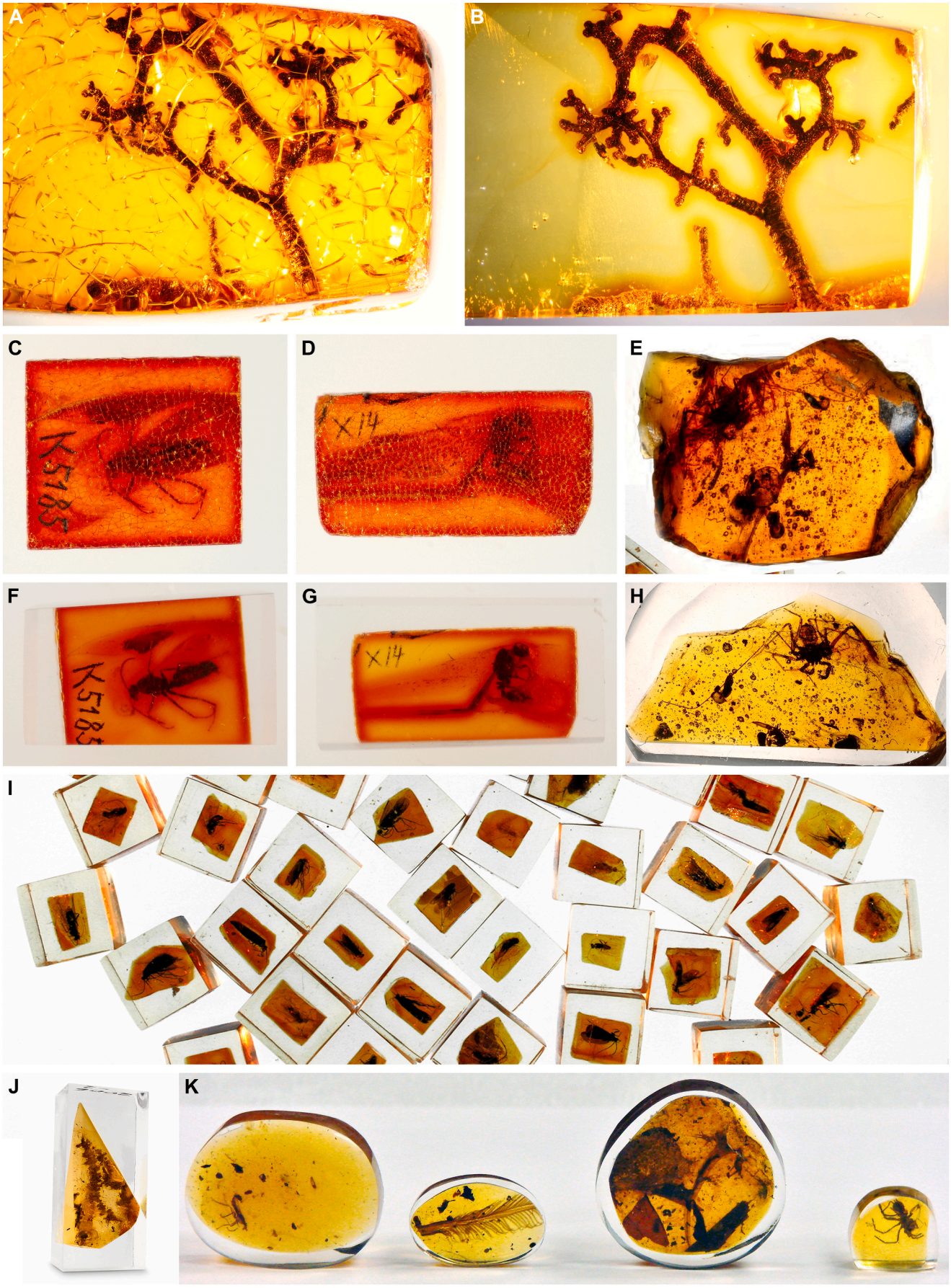


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Fig. 7. Preparation of amber specimens, and embedding in artificial resin (epoxy). A, B: Lichen inclusion in Baltic amber (Königsberg Amber Collection, University of Göttingen) before (A) and after (B) preparation and embedding in epoxy. The amber piece cleared and fissures were removed, enhancing the visibility of the inclusion (B). C–H: Arthropod inclusions (from amber collections of the American Natural History Museum, New York): Baltic insect inclusions (C, D, F, G) and an amblypygid (E, H) inclusion from Dominican amber before (C–E) and after embedding (F–H); note crazing of the amber surface in C and D, which disappeared after embedding. I–K: Baltic (I, J) and Burmese amber pieces (K; James Zigras Collection) embedded in epoxy. Images in 'I' by Carola Radke (Museum für Naturkunde Berlin) and in 'J' by Carsten Gröhn (Glinde).

a. Grind and polish 1 or 2 flat surfaces to create windows to clarify inclusions, or to view any potentially hidden or partially hidden inclusions (the latter applies to less than perfectly clear amber) and to let in light. (This can be done manually or by using a flat lap, in either case applying a stream of water).
b. Amber that is full of organic material or contains regions that are hidden from view will need to have some surfaces more thoroughly and systematically ground/polished, admitting more light to reveal any further inclusions.
c. Large dark or organically rich pieces can sometimes be carefully 'slabbed' using a small rock-cutting saw or downsized using a scalpel. The slabs can then be individually polished and screened (this applies particularly to Class II fossil resins, e. g. Eocene Indian amber).
d. Place pieces with inclusions in individual plastic containers (or temporarily in plastic ziplocks) and label each.

Note that when a single piece of amber is divided into two or more pieces, it is important to label the divided pieces accordingly, so the information is not lost (e.g. Piece Number + inclusion: a,b,c…n).

B. Initial preparation: grinding and polishing

For pieces with inclusions, it is important to achieve the best views possible prior to embedding, and in many cases, prior to photography, which is performed first for some specimens. It is also necessary to take into consideration the fragility of the amber, so as not to compromise or destroy inclusions that are particularly vulnerable. Since surficial scratches and fissures cause light diffraction and can disturb the optimal visualization of an amber inclusion, these should in most cases be removed as much as possible initially, through grinding and polishing. We use 20.32 cm (8-in.) diameter Carbimet and Microcut wet/dry polishing discs (Buehler) mounted on a variable-speed flat lap that produces a steady stream of water. Successively finer grit sizes are used for each surface: 320; 600; 800; 1200; 2400. In order to closely grind/polish amber inclusions that are especially small (e.g. mites), amber samples can be attached to a specialized holder and ground using a small polishing machine (e.g. OpenScience PollyOne; see Sidorchuk, 2013 for protocols).

When polishing amber surfaces, it is sometimes possible to skip a grit size (different amber deposits yield ambers with varying physicochemical characteristics, so that obtaining a final polish should take into consideration individual amber types). Between each step, the amber specimen should be cleaned with water to remove any grinding residue, in order to prevent the transfer of particles to the next carbide disc with finer grit size. As a last step, amber can be further hand or machine polished using a 4000 (or even smaller) grit polishing paper, and/or a leather polishing cloth. Avoid polishing with a polycrystalline diamond suspension, since the small crystal particles of 1–3 μm in size may enter fine fissures, creating an obstructive sparkling film inside of the amber.

Note: In order to conduct tests on any specific amber piece, a section (or sections) of the piece that has no inclusions can be removed, labeled, and then set aside for possible future analyses, for instance of the chemical properties of an amber specimen (such as infrared spectroscopy; Beck, 1982). Such tests should be conducted (or made possible) before embedding, since epoxy may negatively affect the properties of any amber piece that it comes into contact with and confound the results of the tests.

1. Carefully grind and polish as many of the amber surfaces as possible to optimize viewing (create up to 6 flat polished surfaces). Ideally, each pair of opposite surfaces created are parallel, or closely aligned with appropriate features of the inclusion(s). Trim/polish reasonably and safely close to the inclusion or inclusions.
2. Remove excess or obstructing amber material, insofar as practical (especially dark or carbonized outer rinds).
3. Produce as many unobstructed, flat (appropriately close) views of inclusions as warranted/optimal (e.g. dorsal, frontal, lateral, etc.), dependent on the type of inclusion and on the scientific approach. Note that characteristics of individual inclusions can vary significantly, and that older historical specimens may in some cases be especially fragile and thus require extra care in handling.
4. Amber pieces should be carefully trimmed and polished in order to fit comfortably in embedding cups or silicon forms (manufactured cups tend to range from 1.25–4.0 cm [approximately 0.5–1.5in.] in diameter, such that smaller or larger pieces will instead require either smaller hand-made silicon forms or larger hand-made or manufactured silicon forms).
5. If possible, create one flat surface on the side opposite or furthest away from the inclusion(s). This is done to make it possible to temporarily bind the amber to the bottom of the form or cup using a very light drop or 'smear' of quick-setting epoxy (see C2 below), which also orients the inclusion or inclusions. However, for the tiniest specimens (e.g. miniscule amber droplets or minute fragments), one can align pieces that even have curved or uneven surfaces by merely waiting for the quick-setting epoxy to start curing, then carefully orient and place the piece in the cup while the quick-setting epoxy is in its most viscous stage (see C2c).

When manual grinding/polishing is indicated: To gently remove very small amounts of amber, or when handling significant but very tiny pieces of amber, it is often advantageous to grind and polish them manually using a series of wet silicon carbide papers (we recommend grit sizes between FEPA P 600 [25.8 μm particle size] and 4000 [5 μm particle size], Struers company) to safely produce smooth surfaces for investigation and to better control the amount of abrasion.

Important note: We do not recommend initially cutting into or grinding/polishing amber that is especially friable or internally weakened due to intrusive seams of minerals like pyrite or calcite, or is otherwise compromised. Instead, such pieces should be handled with care, and initially lightly 'painted' with the high-grade epoxy, or in cases of significant degradation or fragility, coated in this way, then placed under vacuum (see C and D below) before further work is performed.

C. Pre-embedding steps

Prior to embedding, we generally recommend taking images of prepared amber inclusions for research and digitization of the specimens, since any polymer coating naturally increases light scattering when using high-magnification lenses/microscope objectives. Exceptions to this protocol are most Class II amber specimens, which should be minimally handled before embedding. Freshly trimmed and polished surfaces of Class II amber pieces are typically somewhat 'sticky' and can easily smear (these are dipterocarpaceous resins, like Eocene Indian Cambay amber and Miocene Chinese Zhangpu amber, which contain cadinane and cadalene-based sesquiterpenoids). For the majority of

ambers (Class I fossil resins), photography can be used to document the condition of an amber specimen before and after embedding (see section 5.1), particularly for older damaged specimens.

1. Prepare and label cups or silicon forms/moulds

a. Depending on the size of the amber pieces, to conserve epoxy, use either self-made silicon moulds (e.g., made of sanitary silicone as described by Sidorchuk, 2013), silicon ice cube trays, or cups (reusable or "single-use") provided by manufacturers of epoxy resins (e.g. Buhler). If using single-use cups, cut material off the upper rim of the cup to lower its height keeping in mind the size of the amber piece. A slightly lower cup rim can often make placing/orienting specimens easier. Note that cup/mould size and rim should be high enough to adequately submerge the amber so that bubbles will not collect on the uppermost surface.
b. For exceptionally large amber specimens, correspondingly larger manufactured reusable silicon forms are available (from Buehler and others). Label all cups/forms with collection numbers (or similar) to prevent dissociation of the specimen and the labels.

2. Mix a small amount of quick-setting epoxy (QSE; widely available commercially, e.g. from Bob Smith Industries ["Quick-Cure Epoxy"] as well as the Weicon Company ["Epoxy Minute Adhesive"]). Such inexpensive epoxies serve as a 'glue' that will prohibit the 'up-floating' of the amber piece when embedded (and will initially set in 3 to 6 min).

a. Using a glass stirring rod or similar, apply a thin film or drop to a small area of the bottom of each form or cup (either toward the center, or in such a way that a pre-selected portion of the amber's bottom surface will come into contact with the QSE).
b. Orient each piece, pick it up (using forceps or a jewelry prong holder, depending on the shape of the piece), then affix the appropriate amber surface to the bottom of the form, in contact with the QSE (Fig. 8, step 1). Be certain that the quick-setting epoxy does not permeate or cover the inclusion, and that it is minimally applied (as a thin restricted coating) to an amber surface on which it will later be ground away.
c. Make sure the inclusion(s) in each piece is/are oriented optimally away from the 'glued' surface. The advantages of using this quick-setting epoxy are (1) one can place and orient the specimen in any direction right before the QSE begins to set; and (2) if applied properly, the amber will not float during the actual embedding, nor during the subsequent long-term curing process (2–3 days).

3. Mix appropriate amounts of high grade epoxy resin and hardener (by weight ratio as indicated). Epo-Tek 301–2 (or similar) is recommended, for which the ratio of part A (resin) to part B (hardener) is approximately 2.5 (see Table 2). Mix thoroughly until the liquid becomes entirely clear. This step may require as much as 6 to 10 min (or until the 'striations' in the mixture completely disappear).

a. Stir gently to minimize bubbles in mix.
b. Place mixture under vacuum to eliminate most remaining air bubbles.
c. Let the mixture stand for up to an hour to fully clarify liquid.

D. Embedding

Important note: work in a well ventilated area (or under a fume hood) when handling epoxy, since both epoxy components (parts A and B) do off-gas vapours, which may be harmful. Use of nitrile gloves to inhibit dermal exposure is recommended. Following the initial embedding of a specimen, there are several options on how to complete the overall embedding process, which are discussed in 'H' below.

1. Pour the mixed epoxy solution into the cup or form, so that the amber piece is fully immersed, with the liquid epoxy surface above the height of the amber piece (Fig. 8, step 2).

a. Add just enough epoxy to inhibit bubbles from collecting on the uppermost amber surface.
b. With an insect pin or sewing needle, move all bubbles in the liquid mixture well away from the amber, upward and outward toward the rim of the cup.
c. Make sure no bubbles lie directly on any amber surface – (immediately and gently) move away with a pin.

2. If you do not have access to a vacuum pump or vacuum oven, set the forms or cups aside for curing (over 2–3 days). Amber pieces that exhibit no significantly compromised surfaces (like some newly excavated, prepared specimens) may not necessarily require embedding under vacuum. However, we strongly recommend applying a vacuum, since this insures removal of all air bubbles, fills hard-to-see fissures and thus optimizes the efficacy of the embedding process.

E. Vacuum pump

1. Place the moulds or cups on the vacuum platform (Fig. 8, step 3). (Note: when using a 'vacuum oven' such as a VO200 or VO29 Memmert at ambient temperature, steps E2, E3 and E4c are unnecessary.)
2. Apply a thin film of petroleum jelly to the rim of bell-jar (this will create a seal when a vacuum is applied).
3. Place the bell-jar onto the stage to enclose the moulds – make sure that all air valves of the vacuum assembly/stage are closed.
4. Engage vacuum pump or oven (we recommend a vacuum pressure of 50 mbar).

a. Leave specimens under vacuum for approximately five to ten minutes.
b. Let vacuum subside gradually.
c. Carefully remove bell-jar (wipe off rim).

F. Post vacuum

1. With an insect pin or needle, move any remaining bubbles away from amber surfaces, and upward toward liquid's surface. Check this again within an hour.
2. Set specimens aside in a safe out-of-the-way and dust-free place to cure (for instance in a fume hood or other appropriate closed space) for approximately three days.

G. Preparing specimens after curing of epoxy

1. Note location of inclusions in each embedded amber piece.
2. Trim specimens carefully – you can cut and polish the epoxy surface the same way you would the amber itself.
3. Grind and polish as indicated for each specimen to optimize viewing and important features of inclusions (Fig. 8, step 5.1).

H. Options to complete the embedding process: *Re*-embedding or applying a coating to one surface

After initially embedding, trimming and polishing an amber specimen, there are several ways to complete the embedding process and fully conserve the piece and its inclusion(s) for long-term preservation and storage, dependent on the particular specimen, its condition, the orientation (and number) of inclusion(s), and the type of amber.

Applying epoxy to a single exposed surface: At the American Museum of Natural History (AMNH), this completion process (which typically takes place after inclusions are studied) often involves coating the one

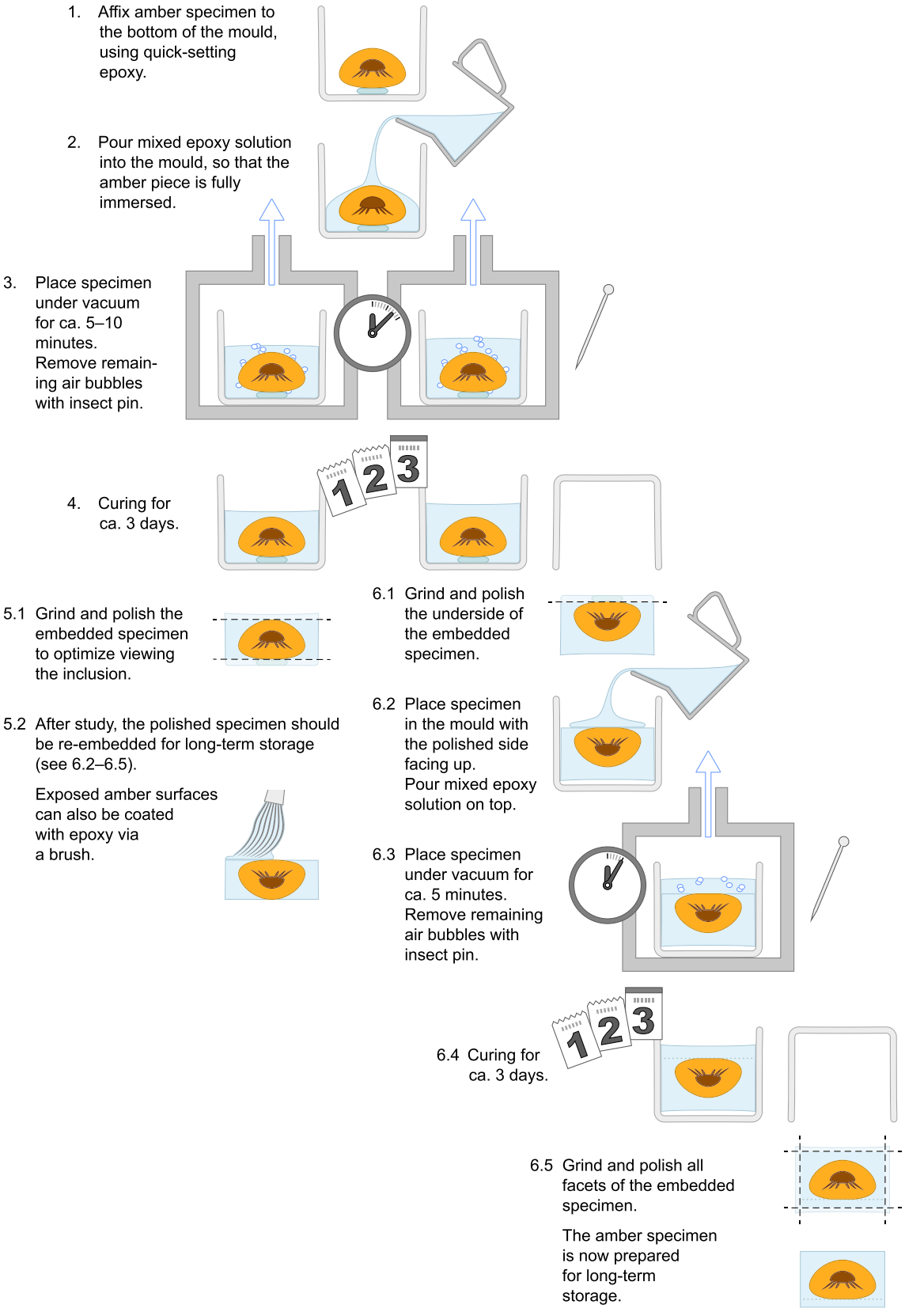

Fig. 8. Simplified scheme guiding through the process of epoxy preparation. For details, refer to Section 4.2.

Table 2
Epo-TEK 301-2, part A (resin) and Epo-TEK 301-2, part B (hardener), weight by grams for embedding amber specimens.

Epo-TEK 301-2, part A	Epo-TEK 301-2, part B	Ratio
Resin	Hardener	
150	60	2.5
100	40	2.5
50	20	2.5
25	10	2.5
17.5	7	2.5

remaining exposed (bottom) surface with (fully mixed) EpoTek 301–2, using a small brush (Fig. 8, step 5.2; the surface in question must be oriented horizontally, so the epoxy doesn't run off the amber). Because the other amber surfaces typically remain epoxy-coated following embedding (each with a thin fully-polished layer of epoxy), this process hermetically seals the piece, and in most cases after curing (2.5–3 days), the applied epoxy clarifies the view for that surface. If needed, the newly-coated surface can also be finely ground and polished. This method has the added advantages of conserving epoxy and decreasing the amount of time it will take to otherwise re-embed the specimen.

Re-embedding/two-layered embedding: If one needs to cut into the amber after the initial embedding, possibly to separate two inclusions, or further trim away material / remove occluding amber from multiple sides to obtain optimal views of an inclusion, the piece will need to be re-embedded after initial examination, and either before or after research is completed, dependent on the circumstances and requirements for study. Re-embedding can also generally be applied to amber specimens that are being conserved for long-term storage and possible future research, such that, after the process is completed, epoxy (usually several mm) will cover the piece on all sides. Re-embedding is done utilizing the same protocols outlined above for the initial embedding (Fig. 8, step 6.1–6.5).

4.3. Storage environment

Considering amber's susceptibility to deterioration when exposed to a variety of environmental factors, a stable storage environment is essential for any collection of these fossil resins, including amber specimens that are embedded in epoxy. To create and maintain a suitable indoor environment for most types of fossil collections, including amber collections, the overall range for RH should be between 37 ±2% and 53 ±2% (Mecklenburg et al., 2004; Pastorelli et al., 2013b). Even so, the ideal RH range for each specific type or deposit of amber appears to differ to some extent. For range of temperature, studies indicate that it should not be higher than 22 °C, and also recommend that it be no lower than 17 °C (Pastorelli, 2009; Thickett et al., 1995). However, there are no studies yet that actually examine the influence of even lower temperatures (<17 °C) on amber. Nevertheless, it has definitively been shown that temperatures higher than 22 °C, as well as freezing temperatures, are harmful to amber (Pastorelli, 2009; Wang et al., 2014). Light exposure should always be limited (Bisulca et al., 2012; Girard et al., 2012; Pastorelli, 2009; Pastorelli et al., 2011; Waddington 2011). However, if amber specimens are on display, UV blocking glass with specific filters should be used to prevent harmful radiation (Dunnill, 2014). It should be noted that artificial resins also require stable storage environments and protection. Although Epo-Tek 301-2 is among the most resilient of artificial resins, it still needs to be shielded from light exposure to inhibit yellowing (Down, 2001).

Extensive amber collections housed in the American Museum of Natural History (AMNH) in New York, and in the Senckenberg Institute Frankfurt (including Baltic, Dominican, Burmese, Lebanese and other ambers, as well as copals and more recent resins) are stored in special cabinets within which RH is monitored to remain 45–50%, and temperature is kept between 18 and 21 °C. To prevent hydrolysis from taking place, Pastorelli et al. (2013b) suggests using pollution scavengers to control the pH value within the storage environment, since hydrolysis in Baltic amber leads to the formation of acids. Pastorelli et al. (2013b) also discusses the possible use of humidity scavengers to help prevent hydrolysis; however, there is concern that this might lead to a decrease in RH on a micro-environmental scale, harming the amber if the RH becomes too low (Pastorelli et al., 2013b). Based on the long-term success of storage protocols and housing of amber collections at AMNH and at the University of Göttingen, we recommend a stable RH of approximately 50% and an ideal temperature of 18 °C to maintain an optimal storage environment for amber collections.

In some cases, storage conditions can be adequately controlled by a collection room's interior climate (e.g. air conditioning), or on a microclimatic scale through the use of climate chambers (e.g. Memmert HPP 750). The latter are particularly advantageous, since the storage environment can then be specifically adjusted, and an alarm will indicate any excessive fluctuation of environmental parameters. In contrast, the climate inside a typical storage room is likely to experience significant heat fluctuations through the opening and closing of windows or doors. It is strongly recommended that amber collections should be housed in closed steel cabinets, while wooden drawers and storage furniture should be avoided, since wood can off-gas acidic vapours (Schieweck, 2020, table 1), which may harm the amber. In addition, cabinets should not be placed near heaters or windows, as both contribute to environmental fluctuations. Since amber specimens are scientifically valuable, rooms and cabinets should be properly secured.

Moreover, each amber specimen should be housed in an essentially anoxic environment (e.g. anoxic sealing through embedding with epoxy resin) and under neutral pH conditions (Pastorelli, 2009; Pastorelli et al., 2013b). To maintain a neutral pH, storage material needs to be appropriate for amber specimens. These materials should include a form-fitted plastic container, and ideally, an acid/alkaline-free soft paper to envelope the amber specimen. Another possible option is the use of Plastazote foam, which is chemically inert. By using a scalpel, an appropriately-shaped depression can be carved out of the foam, and the specimen placed within (cf. Thickett et al., 1995). The carved space should not be either too tight or too loose, in order to avoid mechanical stress. Moreover, the lid of the plastic container should not touch the amber specimen, since this can induce pressure or scratch an amber surface. For any long-term storage, plastic bags or cardboard boxes do not adequately protect a specimen from mechanical damage (though plastic bags are occasionally used because they conserve space). Furthermore, there may be some risk that plasticizers in plastic bags could harm amber over time, possibly affecting its chemical composition (sensitive analytical methods such as mass spectrometry might reveal such an interaction, and future study is recommended). Some collections use acid-free cotton or wool to envelope amber specimens within each plastic container. However, the delicate fibers can sometimes become attached to sticky amber facets (such as freshly-polished surfaces in Class II ambers), fine surface irregularities or fissures in the amber, and are difficult to completely remove.

Coating, photographing or storing amber in mineral oil, white oil of cedarwood, alcohol, a solution of thymol in water, glycerin, paraffin or beeswax (Penney and Green, 2010; Sidorchuk, 2013; Thickett et al., 1995) is not recommended, as the named substances can infiltrate the amber, obscuring or even in some cases irretrievably damaging or dissolving the amber and/or its inclusions (Schlee and Glöckner, 1978; Wunderlich, 1983). Copal and Class II ambers are particularly susceptible. In addition, some of the aforementioned substances (specifically glycerin, paraffin and beeswax) can be difficult or impossible to remove and will likely interfere with authentication or provenance analyses, such as IR (Beck, 1982) or FTIR (L.J. Seyfullah pers. obs.). The degradation of amber stored in liquids has been witnessed in historic specimens of the Künow Amber Collection (Museum für Naturkunde Berlin). In this collection, we found a jar containing ant inclusions in Baltic amber stored in alcohol by Richard Klebs (1850–1911), probably since the late 19th to early 20th Century (Fig. 9A; Hinrichs, 2007). Amber specimens within the jar were densely covered with cracks, and some

had a whitish or yellow colour (Fig. 9B, C). Moreover, the jar had been exposed to sunlight until 1984 (Hinrichs, 2007), which likely also contributed to the degradation.

A further issue of importance is fire safety and security. Amber burns at ca. 300 °C and thus should be stored in a fire-safe location.

4.4. Modern microorganisms settling on amber specimens

4.4.1. Microorganisms on amber collected in lacustrine and seashore environments

Girard et al. (2009) pointed to the possible presence of living diatoms, fungi and other microorganisms at the surface and inside fissures

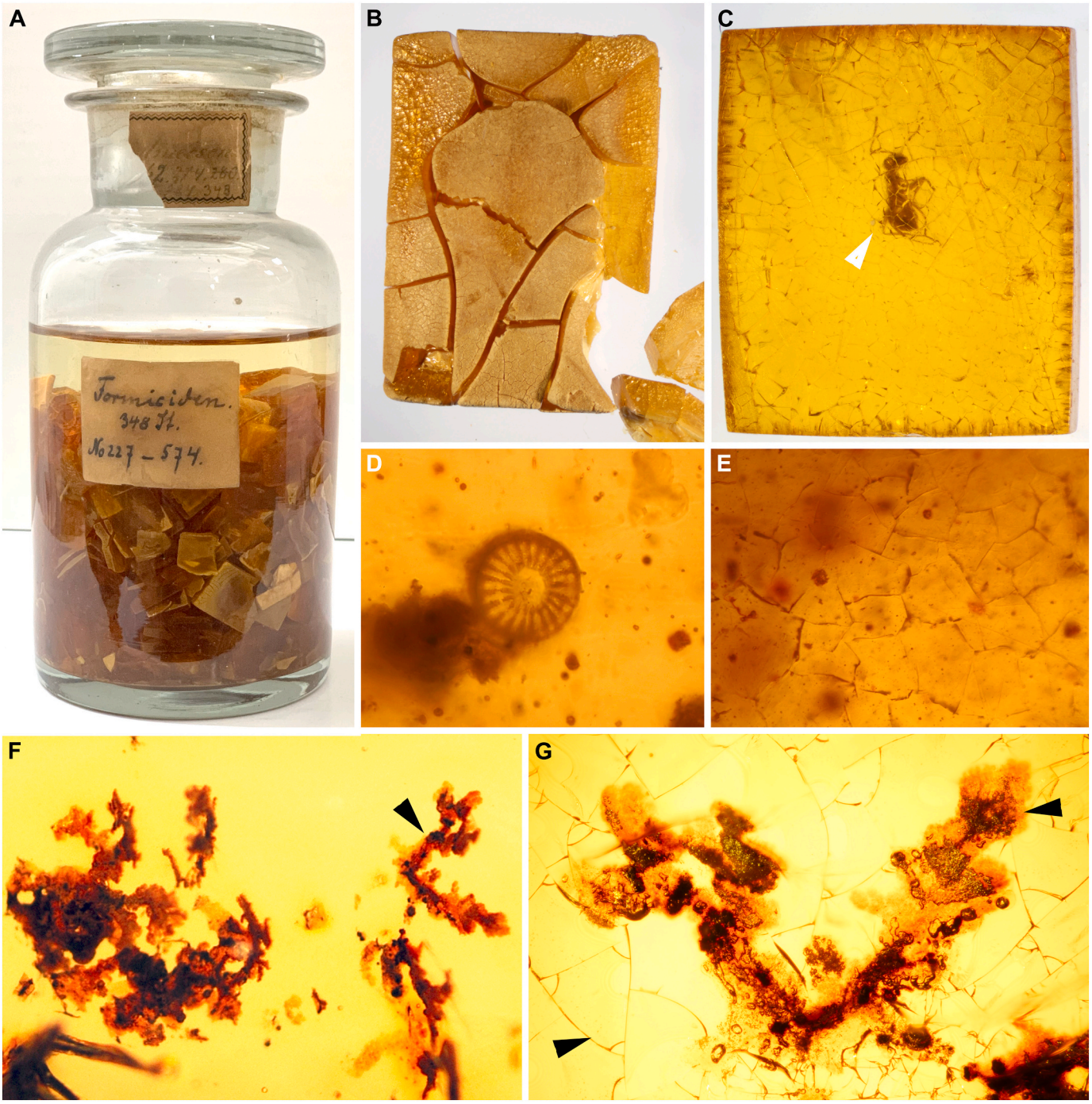

Fig. 9. Degraded amber specimens after being stored in or treated with various liquids. A: Baltic amber specimens (Museum für Naturkunde Berlin) with inclusions of ants, stored in alcohol for over a hundred years. B, C: Two amber specimens that were removed from the jar, showing a whitish-yellow colour, deep fissures and cracks; the arrowhead (C) indicates an inclusion. D, E: A diatom inclusion from French Cretaceous amber before (D) and after (E) the treatment with 35% hydrogen peroxide and 5% hydrofluoric acid. The inclusion is completely destroyed (E) and the amber is infiltrated by cracks. F–G: Inclusions of lichens (*Phyllopsora dominicanus*) in a piece of Dominican amber before (F) and after being treated with vegetable oil (G); lichen in F (arrowhead) is magnified in G. The amber surface exhibits multiple fissures (left arrowhead, G) and the inclusions are degraded (right arrowhead, G). Image in 'D' from Schmidt et al., 2018b, in 'F' by Jouko Rikkinen (Helsinki).

of amber samples collected in littoral and lakeshore environments, and suggested that these microorganisms could possibly be confused with actual inclusions of fossil microbes in amber. The authors of the study suggested (1) ultrasonic cleaning of the samples, (2) submersion in 35% hydrogen peroxide for five hours, and (3) submersion in 5% hydrofluoric acid for five minutes.

We strongly recommend against applying this decontamination protocol, since amber and its inclusions may be severely damaged by hydrogen peroxide and hydrofluoric acid. Fig. 9D shows a unique fossil diatom enclosed in French Cretaceous amber that was destroyed by use of this method before it could be studied in detail. Fig. 9E depicts the presence of numerous fissures in the amber matrix that only appeared after this treatment.

In fact, microbes present inside fissures and cavities in amber are even light-microscopically distinguishable from inclusions that are surrounded by solid amber matrix, as shown by Beimforde and Schmidt (2011).

4.4.2. *Bacteria and fungi on collection-stored amber pieces*

Storage of amber samples in rooms with a high RH (e.g. humid basements) may support growth of fungal mycelia and bacteria on amber surfaces, and such growth may even extend into fissures and cavities within the amber (Beimforde and Schmidt, 2011). It has been suggested that inorganic or organic matter in fissures can act as a substrate and create suitable conditions for microorganismal growth. According to our observations, such microbes are not able to penetrate into the solid resin. Thus there is no immediate risk to the amber specimens. In any case, amber specimens should not be treated with disinfecting agents to stop or prevent microbial growth, since these agents will likely penetrate the amber and its inclusions and affect their physical and optical properties. For optimal conservation, however, the collections should be stored in controlled climate conditions as described in Section 4.3 to prevent growth of mould or bacteria on the amber, and in particular on the collection labels.

5. Imaging of amber inclusions

5.1. *Light microscopy*

Images of amber inclusions are necessary for research purposes. In addition, such images are important to consider and include in the development of digital databases. Moreover, they can be a helpful baseline for detecting and documenting signs or any progression of deterioration, and can be applied to an entire collection as warranted.

We generally recommend taking relevant images of amber inclusions, especially microscopic ones or specimens exhibiting very fine details, before embedding the amber in epoxy for permanent storage, specifically before applying protocols discussed in embedding step H (see above). If an amber specimen is covered by epoxy on all sides, the level of light scattering within the specimen may become more pronounced, and the use of high-magnification lenses is then made more difficult because of the smaller free working distance. Exceptions to pre-embedding photography apply to the handling of very fragile amber specimens (e.g. with significant cracks, pyrite disease, or similar issues) that would likely break when initially preparing them (grinding and polishing), as well as to Class II amber specimens, as discussed in the introduction to Section 4.2 C. In fact, the embedding process can actually clarify viewing for some inclusions, and in these cases, post-embedding images should be considered. It should also be noted that the epoxy layer covering one or more surfaces can be partially or even fully ground away as needed to reduce light scattering and improve access with high-magnification lenses if dictated for subsequent research.

Even prior to embedding, amber specimens may initially exhibit a degree of internal light scattering. This most often occurs when imaging inclusions in amber blocks of irregular shape or with curved surfaces. Immersion of the amber specimens in glycerol, mineral oil, 'immersion oil' or vegetable oil for photography has sometimes been recommended by some researchers to neutralize these optical distortions, and it appears to be a widely accepted practice for imaging amber inclusions (Grimaldi, 1993; Penney and Green, 2010; Sidorchuk, 2013). However, although photography of inclusions inside amber specimens with irregular surfaces is indeed challenging, any kind of mineral oil or vegetable oil applied to the amber may irreversibly penetrate the fossil resin, permanently alter its optical features, and compromise its conservation (this is especially true for Class II ambers, as well as for copal, which such treatment will likely destroy). It is important to stress that every amber is different, and that each may react in unexpected ways to various treatments. While arthropods, for instance, may at least in some cases be less affected by oil treatment because of their strong cuticle, other types of inclusions may suffer more immediately and severely.

Fig. 9F shows a fossil lichen from Dominican amber that was immersed in vegetable oil for imaging ca. 12 years ago (Rikkinen and Poinar Jr., 2008). The amber surface is still oily, and the enclosed lichen, plus a portion of an enclosed moss, now both appear very translucent/hyaline (Fig. 9G). To avoid such irreversible (and damaging) change to inclusions, we recommend placing amber specimens in water for imaging instead of using any oil.

A further method to reduce optical artifacts is the use of sugar solutions (e.g. made of corn syrup or agave syrup), which reduce refraction and reflections of the amber and increase visibility of inclusions (Antropov, 2011; Grimaldi, 1993; Rasnitsyn, 2002; V. Perrichot pers. obs.). However, the sugar might remain in micro-fissures or small cavities within the amber and crystallize. Moreover, such sugar remnants may act as a carbon source supporting microbial growth. Therefore, we recommend that this method should not be applied to amber pieces with obvious cavities, exposed inclusions or fissures.

In most instances, the best photographic results will be obtained if the amber surface above the region of interest is ground and polished as close as possible to the inclusion. This surface should then be horizontally oriented under the microscope. We adjust prepared amber specimens on a glass microscope slide using small pieces of modelling tack in a way that insures that the polished surface is horizontal, then apply a drop of water to that surface, and cover this gently with a glass coverslip. The procedure reduces any light scattering from fine surface scratches and improves optical resolution. If oil immersion objectives are used, optical immersion oil should only be applied sparingly to the upper surface of the cover slip, while ensuring that oil will not float over the edge of the glass to come into contact the amber specimen (Schmidt et al., 2012).

Depending on the required magnification, either a stereo or a compound microscope will be sufficient for study (Penney and Green, 2010). The simultaneous application of incident and transmitted light is necessary to appropriately illuminate an inclusion from all available angles (Penney and Green, 2010; Schmidt et al., 2018a). External 'cold' lights with long goosenecks are very useful, as they allow flexible adjustment of the illumination. To decrease or eliminate temperature stress on the amber, fiber optic or LED lights are essential, since they do not overly heat the amber specimen. Penney and Green (2010) also stressed the advantageous use of different tonal backgrounds, such as white or black, to create various contrasts when viewing an inclusion. For the study of fungal spores and pollen, as well as for use on very thin amber slides, the application of differential interference contrast microscopy (DIC) should be considered, since it enhances contrast in microstructures.

Photographic imaging of an inclusion is done with cameras that are installed on the microscope. To more fully accommodate the three-dimensionality of an amber inclusion, image stacks of each focal plane can be taken, which are then merged using stacking software (Penney and Green, 2010; Schmidt et al., 2012). Some cameras (e.g. Leica DFC 490, or AxioCam MRc5), which are intrinsically linked to an imaging analysis software system, produce automatic image stacks and corresponding digital measurements (with automated insertion of scale bars in each

image). Although this is very convenient, the image quality of the cameras offered by the microscopy companies is sometimes unsatisfactory, since they produce photos of only 5 to 8 megapixels. A better alternative is the use of digital cameras like those manufactured by Canon (e.g. EOS 5D or 80D) with a 24 to 50 megapixel range, that are installed on the microscope using an adapter, and which work independently of a computer system. Scrolling stepwise through the amber specimen with the microscope's fine drive will produce images that are taken from each focal plane by remote control. The individual focal planes are then digitally stacked to produce a single photomicrographic composite, for example using the software package HeliconFocus Pro. At the end of each image stack, a photo of an object slide with a calibrated scale bar can be taken to record the magnification. Using imaging software, the scale bar for each image can then be produced and inserted manually.

5.2. SEM, TEM and X-ray computed tomography

To examine internal structures or minute features of an inclusion (e.g. the internal organs of an insect, pollen, reproductive features of a closed flower, etc.), scanning electron microscopy (SEM), transmission electron microscopy (TEM), or X-ray computed tomography (a micro-CT-scan) can sometimes be used. Koller et al. (2005) thin-sectioned a cupressoid twig in Baltic amber. Under TEM, microcellular details of the tissue were revealed, allowing an assignment of the fossil to a conifer genus. To utilize SEM in amber studies, the inclusion typically needs to be surficial – that is to lie on the amber's surface – or be exposed by cutting into the amber.

The exceptions are Class II ambers (Anderson et al. 1992), e.g. Cambay (India), Zhangpu (China), or Arkansas (USA) amber. These recently excavated and studied Cenozoic ambers are fully dissolvable. For instance, samples of Class II Eocene amber from India were fully dissolved with toluene, as well as with orange oil (Rust et al., 2010; Beimforde et al., 2011). This allowed inclusions to be completely extracted (although this was an exceedingly delicate process, since the fully exposed inclusions were quite fragile). This method was also successful for Holocene copal from Colombia (Penney et al., 2013). Interestingly, 70% or greater dissolution with chloroform was reported for Cretaceous Class Ib amber from Lebanon (Azar, 1997; Azar et al., 2010, p. 286). This allowed for the extraction of fully intact insect and plant parts (but not the extraction of complete insect or plant specimens). Also of note: Class Ic amber from Oise, France, was successfully softened in a mixture of acetone and turpentine oil (80/20), allowing the complete extraction of pollen grains (De Franceschi et al., 2000). But it should be noted that Class I ambers (Ia, Ib, Ic and Id) are not fully soluble, and will at best only produce such fragments or parts of inclusions, or tiny resilient structures like pollen grains, when immersed in a solvent.

For most ambers, inclusions can be exposed using razor blades or a scalpel to remove overlying amber. Then, the fragments or parts of the inclusion can be removed and placed on carbon-covered SEM mounts, for example using a wet hair from a superfine brush. After sputtering the stub with gold/palladium (10–12 nm thickness), samples can be examined under SEM. This technique is particularly useful for pollen studies, since it enables examination of the layers and ornamentation of the pollen on a micro-to-nanometer scale (Fig. 10A–D; e.g. Sadowski et al., 2020, fig. 22). The method can also be applied to other botanical inclusions (Fig. 10E–G), as well as to expendable partial or complete insect inclusions, like a stingless bee specimen in Dominican amber (Grimaldi, 1996, p. 119), or to lichen inclusions in Baltic amber (Hartl et al., 2015, fig. 2). However, TEM and SEM are both destructive methods and may irreversibly damage an amber specimen, which means that possible gain of knowledge must be balanced against conservation of specimens.

An alternative for assessing internal structures on even the subcellular level – a method that is generally considered non (or significantly less) destructive – is high-resolution X-ray computed tomography (micro or μCT). Previous studies have shown that X-ray based methods can accurately dissect an amber inclusion digitally to reveal extremely fine details, whether of an animal (Fig. 10I–N) or a plant. Furthermore, micro-CT enables the study of opaque or translucent ambers, in which inclusions are invisible using standard light microscopy (Lak et al., 2008). Thus micro-CT scanning is becoming a standard method in amber research (e.g. Cnudde and Boone, 2013; Crepet et al., 2016; Gandolfo et al., 2018; Moreau et al., 2017; Oliveira et al., 2016; Penney and Green, 2010; Sadowski et al., 2018; Xing et al., 2017). When scanning an amber inclusion using micro-CT, the amber sample first needs to be mounted on a specimen holder. Then the amber specimen is rotated in front of the X-ray source. X-rays penetrate the amber, dependent on the density of the sample, and hit a detector. An image series is created from every angle, for which each image pixel is measured in micrometers (Cnudde and Boone, 2013; Penney and Green, 2010). The images are digitally stacked using either specific commercial software (e.g. Amira-Avizo [ThermoFisher] or Volume Graphics [VG Studio Max]), or non-commercial software (e.g. Dristhi, Dragonfly, ImageJ etc.). To achieve the highest possible resolution, images of minute structures in an inclusion are achieved using ultra-high resolution X-ray computed tomography (UHR CT), propagation phase-contrast X-ray synchrotron microtomography (PPC-SRμCT), or synchrotron-radiation-based X-ray micro-computed tomography (SRμCT), providing exquisite images of animal and plant inclusions and their internal features (e.g. Grimaldi et al., 2000a; Grimaldi et al., 2000b; Moreau et al., 2017; Penney et al., 2007; Perreau and Tafforeau, 2011; Sadowski et al., 2018; Solórzano Kraemer et al., 2011, 2014; Soriano et al., 2010).

It should be noted regarding micro-CT scans that, although they often produce exquisitely detailed images, for some types of amber (e.g. Burmese amber, Grimaldi and Ross, 2017) and some specific inclusions, density differences between the amber and the inclusion are not always sufficient to produce clear or complete images, and resolution of fine structures may be below light-microscopical resolution. The actual diagenesis or preservation of certain inclusions in specific ambers may contribute to such density and resolution issues. As examples, leaves of liverworts or mosses, which are composed of a single cell layer, as well as minute compressed inclusions, often exhibit poor contrast and do not always reveal fine structures when scanned. The quality of micro-CT scans also depends on the taphonomic preservation of the inclusion: some inclusions are hollow and only leave an imprint of the outer surface within the amber.

In addition, access to facilities with synchrotron radiation-based micro-CTs is limited, can be expensive, and the radiation produced by a synchrotron typically causes a brownish discoloration/darkening of the amber as well as of the epoxy coating if used at too high an energy level (Fig. 10H), the degree being dependent on the particular amber. Such brown discoloration can generally be removed by placing the amber under a short-wave (UV) black light over the course of a few minutes to a few days (again, depending on the amber) or in some cases by exposure to daylight for 2–3 days. But the browning may be irreversible if the synchrotron radiation level is too high, so it is recommended that protocols for the use of lower radiation levels be adopted to avoid burning the amber (Lak et al., 2008; Tafforeau et al., 2006). Also, the temperature of the amber specimen should be monitored when using a black light, since, as mentioned earlier, fossil resins are susceptible to damage by heating. Van de Kamp et al. (2013) suggest testing the effect of synchrotron radiation with a "[barren] piece of the same [amber] type before scanning a valuable sample" (p. 154), which, however, only applies to scans that take 20 to 30 min. Depending on the sample, some scans can take up to 10 h, which makes test scans inefficient, as access to a synchrotron is often limited. Whether amber is permanently or significantly damaged by synchrotron exposure, or by exposure to a black light afterwards, and to what degree, is not fully understood at this time.

Significantly, to our knowledge, the browning effect observed with the use of a synchrotron does not occur when using a lab-based micro-CT, making the use of the latter particularly advantageous. Furthermore, the X-ray optics of lab-based micro-CTs have evolved rapidly over the past several years to reach a comparable resolution to that achieved

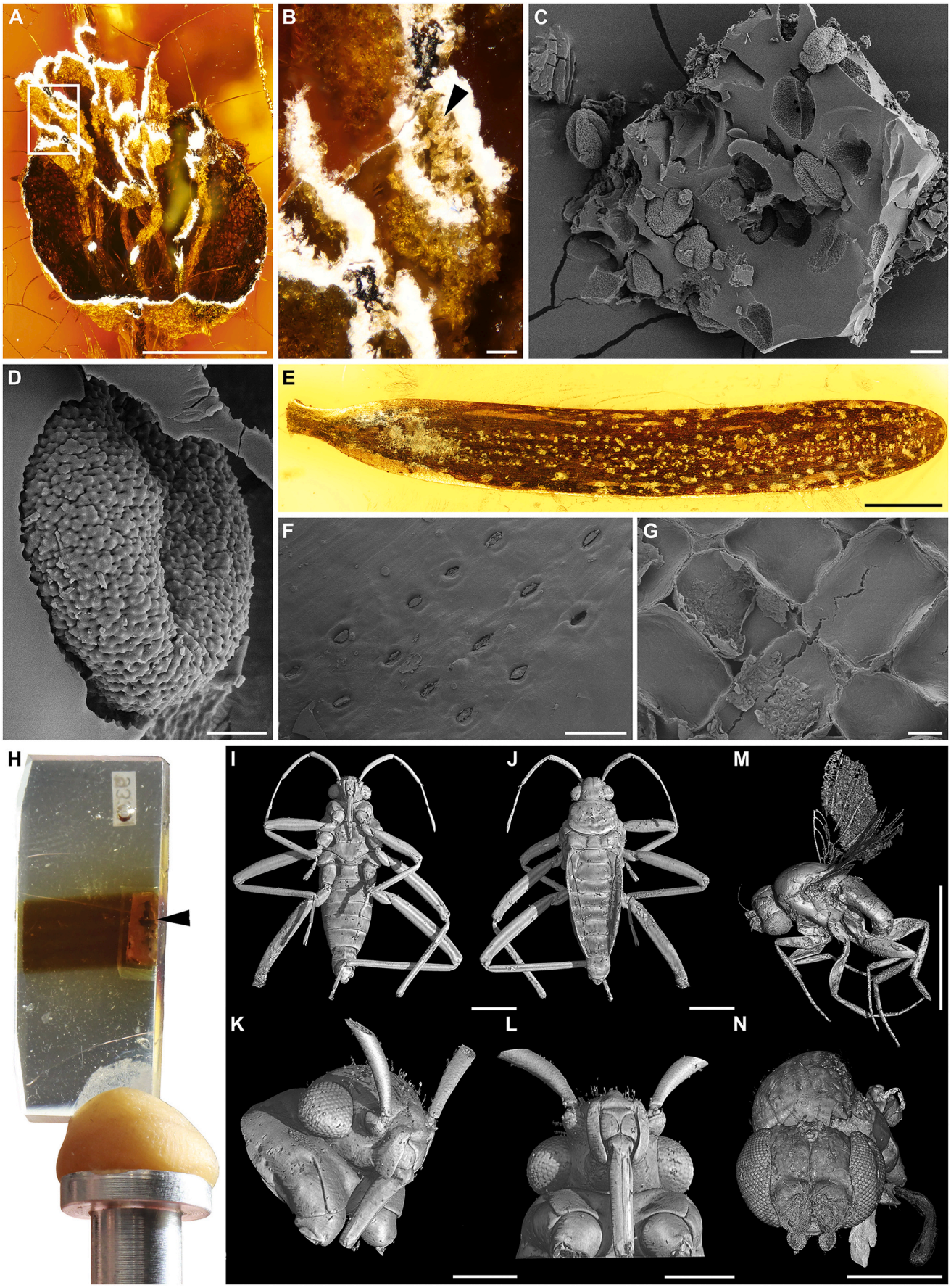

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Fig. 10. SEM and Synchrotron imaging of amber inclusions. A, B: Inclusion of a staminate flower of *Quercus* (Fagaceae) from Baltic amber (GZG.BST.24535, Königsberg Amber Collection, University of Göttingen); the anthers are exposed at the amber surface (white-line inset, A) and exhibit numerous pollen grains (black arrowhead, B). C, D: Amber sample with pollen, extracted from B with a scalpel, sputtered with gold/palladium and examined with a field emission scanning electron microscope (see Sadowski et al., 2020 for further explanation). E: Inclusion of a conifer needle from Baltic amber (*Nothotsuga protogaea*, Pinaceae, GZG. BST.23535, Königsberg Amber Collection, University of Göttingen). F, G: The amber specimen shown in E broke during preparation and exposed epidermal and cellular features (F, G) of the needle that were studied using field emission SEM. H: Characteristic brownish darkening of a Cretaceous Spanish amber specimen (arrowhead) and its epoxy coating after irradiation by synchrotron X-rays; image by Ismael Montero (Barcelona). I–L: 3D virtual extractions of a water strider (I–L: *Arcantivelia petraudi*, IGR.ARC-271.1) and a phorid fly (M, N: *Prioriphora schroederhohenwarthi*, IGR.ARC-382.1b) preserved in a fully opaque piece of Cretaceous French amber, using synchrotron imaging (PPC-SRμCT). Modified from Solórzano Kraemer et al., (2011, 2014). Scale bars: A, E, M = 1 mm; B = 50 μm; C, G = 10 μm; D = 3 μm; F = 100 μm; I, J = 2.5 mm; K, L = 0.25 mm; N = 200 μm.

using a synchrotron (pers. comm. Jörg U. Hammel, DESY, Hamburg). Based on our own experience, we have not yet observed any long-term damage to amber specimens by use of either a lab-based micro-CT or a synchrotron. In addition, Bertini et al. (2014) reported that neither micro-CT nor confocal microscopy appeared to alter the amber matrix chemically or visually, but it was noted that hard synchrotron X-rays caused a visible discoloration in irradiated amber and copal samples.

6. Digitization of amber collections

To digitize amber collections and provide finely detailed images of bioinclusions, in order to make them accessible worldwide, both light microscopy and X-ray based methods (e.g. micro-CT) may be applied. Each "digital specimen" should be accompanied by associated data related to the corresponding amber piece, including images of historic labels or research data (such as IR or chemical analyses). Each bioinclusion in amber treated in this way is considered an "extended specimen" (Lendemer et al., 2020; Webster, 2017). If using X-ray based methods, a three-dimensional digital model (or even video) of a specimen can be generated (preserved indefinitely) and shared online (such that sending or loaning valuable specimens would essentially become unnecessary). Moreover, these three-dimensional models are a great tool for teaching, as well as for visualizing minute amber inclusions during museum exhibitions. However, not every institution or museum (or individual department) has regular or dedicated access to a micro-CT. Furthermore, data processing (including segmentation and interpretation of the image sequences) requires high-performance computers, expertise, personnel and time. Thus, if digitization resources are limited, only the most important specimens should be candidates for a micro-CT scan. The alternative is imaging of amber inclusions with standard light microscopy, which is both easier and faster, but which creates an essentially 2-dimensional image. However, to achieve any reasonable image – whether through light microscopy or micro-CT (and to adequately preserve each specimen for long-term study) – preparation and conservation steps as described earlier should be performed first, especially for those specimens that are too fragile or degraded to handle safely.

7. Future research

More comprehensive studies are needed on how different types of amber react to various deterioration agents, in order to further optimize protocols for conservation (including preservation treatments and long-term storage). With the discovery and excavation of new ambers from a number of deposits worldwide, conservation studies need to address these more diverse fossil resins, and should include amber deposits like those from Australia, China, Ethiopia, India, New Zealand, Peru, and the United States, among others.

It is recommended that long-term studies comparing different embedding materials be conducted, particularly focusing on these materials' reactions to various deterioration agents.

We also need to learn more about resin and amber chemistry: how molecular-chemical properties (and physicochemical properties) change over time, and under what specific conditions. This includes targeted studies on the amberization process (diagenesis) – how resin becomes amber – which will shed light on the key-processes occurring in the formation and deterioration of amber.

8. Conclusions

In summary, amber is highly susceptible to the effects of light, temperature, relative humidity, and oxygen, and is particularly vulnerable to fluctuations in these elements, whether singly or in combination, as well as to chemical hazards. A less-than-suitable storage environment will lead to deterioration of amber specimens, discernible as crazing, spalling, breaking and colour changes, as well as the occurrence of pyrite disease. Thus, stable storage conditions are essential for any collection of amber or copal. For those fossil resins that have been included in conservation studies thus far, we recommend a relative humidity of 50%, temperature at or just above 18 °C, and limited light exposure, only occurring when specimens are temporarily removed from cabinets for study. In addition, we recommend that most amber specimens be embedded in an artificial resin for stabilization and anoxic sealing, which can prevent pyrite disease. The currently recommended embedding medium for use with fossil resins is EpoTek 301-2 or similar. Amber specimens should be placed in sealed plastic containers and stored in steel-cabinets (in a climate-monitored environment) or in climate chambers.

Amber should not be treated or stored in vegetable or mineral oils, alcohol, disinfecting agents, H_2O_2, or other destructive solvents or mixtures, since these materials irreversibly damage the amber.

Most photography of inclusions can be successfully accomplished using light microscopy, and this especially applies to digitization images. SEM, or TEM can sometimes be used to achieve detailed images of inclusions; however, both are considered invasive methods. Important specimens may qualify for micro-CT scanning, in order to examine internal structures or minute features of an inclusion. Light microscopal images or micro-CT based three-dimensional models are both useful for digitization purposes; however, micro-CT scanning is very time-consuming, expensive and produces data that require significant memory capacity. We thus emphasize that the conservation of fossil specimens should be prioritized, as manpower and time are limited.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.earscirev.2021.103653.

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