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# USING CONFOCAL LASER SCANNING MICROSCOPY TO IMAGE TRICHOME INCLUSIONS IN AMBER

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ABSTRACT

Confocal laser scanning microscopy (CLSM) is an analytical technique usually applied to biological and medical samples. It is used to produce high resolution in-focus three dimensional images of thick sections by targeted fluorescence. Trichomes held in amber fluoresce in the far red range whereas amber fluoresces in the ultraviolet. This allows the trichomes to be resolved easily from the amber by CLSM. Samples of amber from two regions were selected for analysis. Baltic amber (Eocene) is well known for its trichome inclusions which have, in the past, been used as a diagnostic feature of that amber. Mexican amber (Middle Miocene) from Simijovel, Chiapas, Mexico also contains abundant trichomes. Samples of amber from both these locations were successfully imaged and re-constructed in 3D using CLSM. This technique enables detailed analysis of the trichome structure without damaging the sample.

RESUMO [in Portuguese]

A microscopia confocal de scanning laser é uma técnica analítica que é geralmente aplicada em amostras biológicas e médicas. Esta técnica produz imagens tridimensionais em foco de alta resolução de secções espessas por fluorescência. Os tricomas aprisionados no âmbar fluorescem na zona mais distante do espectro visível do vermelho enquanto que o âmbar fluoresce no ultravioleta. Isto permite identificar facilmente os tricomas do âmbar por microscopia confocal de scanning laser. Amostras de âmbar nas duas regiões foram seleccionadas para análise. O âmbar do Báltico (Eocénico) é conhecido pelas suas inclusões de tricomas que foram já usadas como características diagnóstico para este tipo de âmbar. O âmbar do México (Miocénico médio) de Simijovel, Chiapas, México também contém tricomas em abundância. Amostras destas duas localizações foram reconstruídas com sucesso em 3D usando microscopia confocal de scanning laser. Esta técnica permite efectuar análises detalhadas das estruturas dos tricomas sem prejuízo da amostra.

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## INTRODUCTION

The use of confocal laser scanning microscopy (CLSM) for the study of inclusions in amber is a recent development. Böker & Brocksch (2002) produced a series of images of insects in Baltic amber using CLSM and identified the potential for 3D imaging of minute detail of taxonomically important morphological structures such as the mandibles and genital organs. Since then, however, very little has been published on CLSM analysis of amber inclusions despite the apparent benefits.

Another study using this technique, amongst other microscopic techniques, looked at some Spanish amber from Álava (Ascaso et al. 2003, 2005). The study looked at a protozoan with fungal hyphae trapped in amber and produced a 3D image based on a series of optical sections recorded by the CLSM. It is perhaps surprising, considering the detail and quality of the images that can be produced, that more studies have not incorporated this technique. More recently, Speranza et al. (in press) have used light microscopy, CLSM & widefield fluorescence microscopy to image microscopic fungi embedded in amber, thus confirming the benefits of a 'fluorescence' approach.

Trichomes are present in the vast majority of angiosperms and have been considered for some time to be of importance in comparative systematic studies (Theobald et al. 1979). They have an important role as defensive structures, especially in repelling phytophagous insects (Levin 1973). There is often more than one trichome type on any one taxon, but certain types may be more common to one taxon than another (Theobald et al. 1979). The taxonomic value of trichomes is therefore limited, but the Baltic trichomes are not inconsistent with them being from a type of oak.

In this study, certain elements of the microflora are examined. The "stellate hairs", or trichomes, are common in Baltic amber and have been considered as a characteristic of this type of amber (Weitschat & Wichard 2002). These trichomes are found associated with, and attached to, the male oak flowers and are therefore thought to belong to oak also (Weitschat & Wichard 2002) although there may be more than one type of trichome present. During this study samples from Chiapas, Mexico were also examined and were found to contain abundant trichomes. No previous record of trichomes in Mexican amber has been found in the literature.

Weitschat and Wichard (2002) describe the 'stellate hairs' found in Baltic amber as structures that develop on the flower and leaf buds of the oak that are shed in great numbers every year. They also state that no studies have been able to clarify the origin or their significance for amber.

The interpretation of what the inclusions in Baltic amber represent in terms of their ecology has been the subject of much debate as many of the insect inclusions suggest a wide range of biotopes (Weitschat and Wichard 2002). Even individual pieces of amber seem to contain species from what would currently be both temperate and tropical regions (Weitschat 1997). This may suggest that species represent within the amber could have had a wider range then than their extant relatives (Weitschat and Wichard 2002).

In the present study we present a technique which uses CLSM and 3D imaging to analyse the structure of trichomes.

## MATERIALS

Amber from the Baltic Region is well known for its trichome inclusions. One amber nugget was sliced and polished to allow examination of the trichomes (GLAHM 114416/3).

A piece of Mexican amber from the La Quinta Formation (Middle Miocene) from the mines in the municipality of Simojovel of the Chiapas region in southern Mexico containing numerous trichomes was sliced and polished (GLAHM 131494/3). The vast majority of inclusions in the Mexican amber are small flying insects belonging to the dipteran order (Solorzano Kraemer 2007). Solorzano Kraemer suggested that the fauna showed a close similarity with the amber inclusions of the Middle Miocene deposits of the Dominican Republic and Hispanola as a whole. It was concluded that the ecology was typically that of a lowland tropical dry forest with elements suggesting more open forest with a mangrove region. The plant that is thought to have produced the resin that became the amber in Hispanola is the angiosperm *Hymenaea protera* (Iturralde-Vinent and MacPhee 1996, Poinar et al. 1996).

## METHODS

Samples of amber in the collections of the Hunterian Museum, University of Glasgow (GLAHM) were examined using a Nikon SZM-2T trinocular stereoscopic zoom microscope to

determine the abundance of trichome inclusions. The samples with the most trichomes were then carefully sliced using a fine diamond saw at slow speed close to the visible trichomes. This was done to maximise the number of trichomes close to the cut surface. The surface was then finely polished. We imaged trichomes within 200µm of the surface to accommodate the working distance of high power objectives and reduce the optical aberrations caused by the mismatched refractive index of amber (~1.5) and air (1). Penetration of the amber by the laser beam was dependant on the colour and clarity of the amber specimen.

Confocal analysis was performed using a BioRad Radiance 2100 fitted to a Nikon Eclipse TE300 inverted microscope. Optimum excitation and emission parameters were sought to maximise the signal to background ratio. Since amber is known to exhibit UV auto-fluorescence we confined our investigations to the green-red end of the spectrum. Optimum imaging was achieved using the red-diode 637nm line with a 660LP emission filter. Pinhole radius was set to 1.1mm. A single trichome was chosen from the Baltic amber and was scanned using 2.6 zoom, 10X objective and 1024 x 768 pixel resolution. A group of several trichomes from Mexican amber were also scanned using 1.2zoom, 10X objective (NA 0.5) and 521 x 512 pixel resolution. Lambda scanning was used to produce images which were the average of 3 scans (i.e. Lambda=3) The CLSM produced 2D image slices by varying the plane of focus in the z-axis at 1µm increments. The images collected were 8-bit greyscale. Optical sections (2D) were pre-processed in MetaMorph prior to being reconstructed in 3D using AMIRA. The software produced a surface-rendered image of the trichomes. The images of the Mexican trichomes were then compared to light microscope images taken of the same trichomes immersed in Johnson's Baby Oil. The oil immersion helps to reduce reflections caused by internal fractures and because of the similar refractive indices of the amber and oil (Crighton & Carrió 2007).

## RESULTS

Trichomes from both Baltic and Mexican amber exhibited the optimum signal to background noise ratio when excited with a wavelength of 632nm and collected 660nm. This produced excellent image data which facilitated sharp thresholding and segmentation and in turn enabled surface rendering in AMIRA (figure 1).

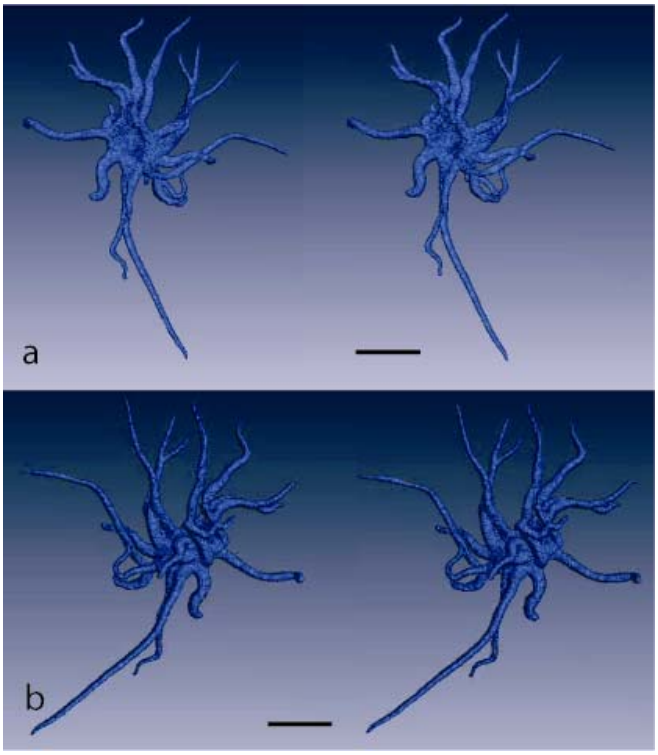



**Figure** 1. Stereo pairs of Baltic trichome (GLAHM 114416/3) showing (a) visible side, and (b) opposite side not seen from top surface of polished amber (scale = 0.2mm).

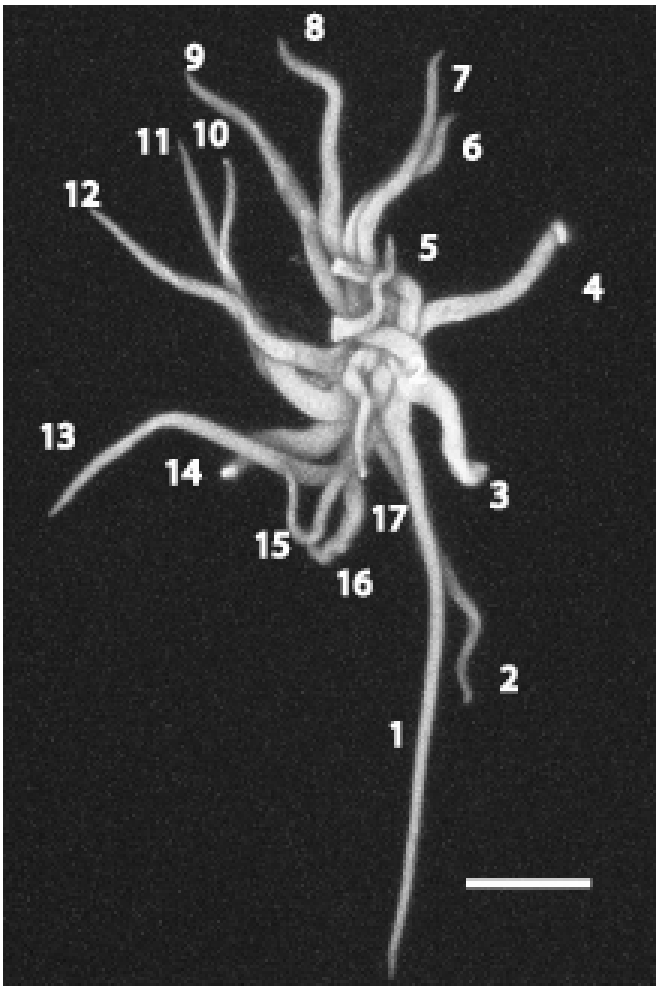
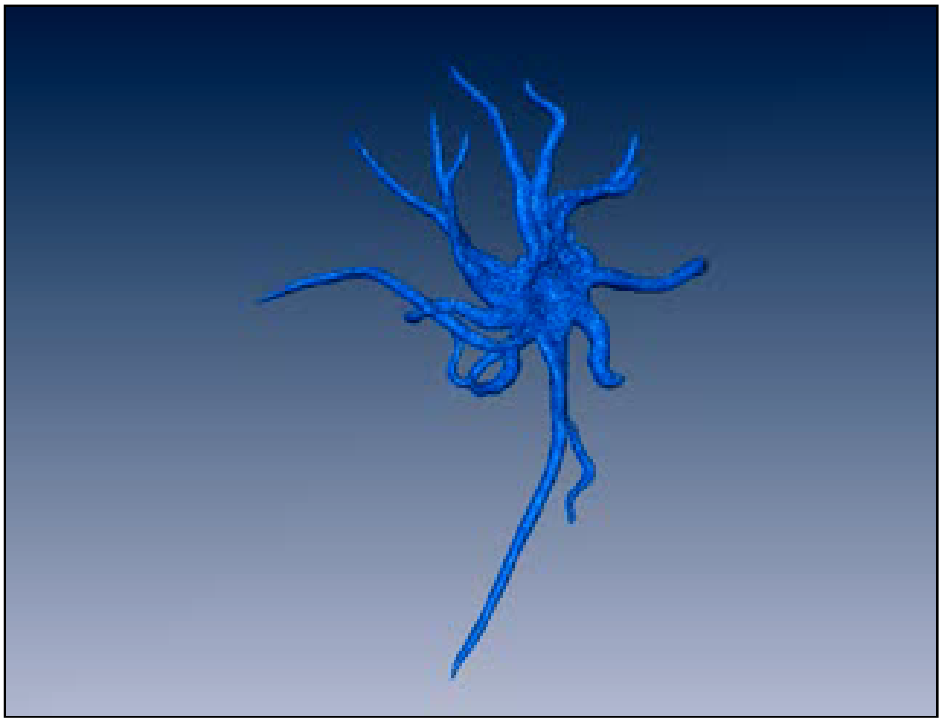



**Figure** 2. Outline of Baltic trichome (GLAHM 114416/3) with indication of number of radii (scale = 0.2mm).

The trichomes in Baltic amber also fluoresced at lower wavelengths, but not as pronounced. The amber immediately surrounding the Baltic amber trichomes also fluoresced particularly in the green wavelengths. The Baltic trichome chosen had a single long radius and 16 shorter radii (figures 1, 2; animation 1).

The trichomes chosen in this study from the Mexican amber form a group of linked stellate trichomes (figures 3, and 4; animation 2) of eight to ten radii in one plane (rotate – terminology used is according to Theobald et al. 1979) with a central disk (slightly lepidote?) from which the radii eminate. Although some of the trichomes studied had less than ten radii, the form of these trichomes is similar to that found in the Euphorbiacean angiosperm *Croton* (Webster et al. 1996).



**Animation** 1. Rotating image of a Baltic amber trichome (GLAHM 114416/3) using CLSM rendered in 3D using AMIRA software.

**Animation** 2. Rotating image of a group of Mexican amber trichomes (GLAHM 131494/3) using CLSM rendered in 3D using AMIRA software.

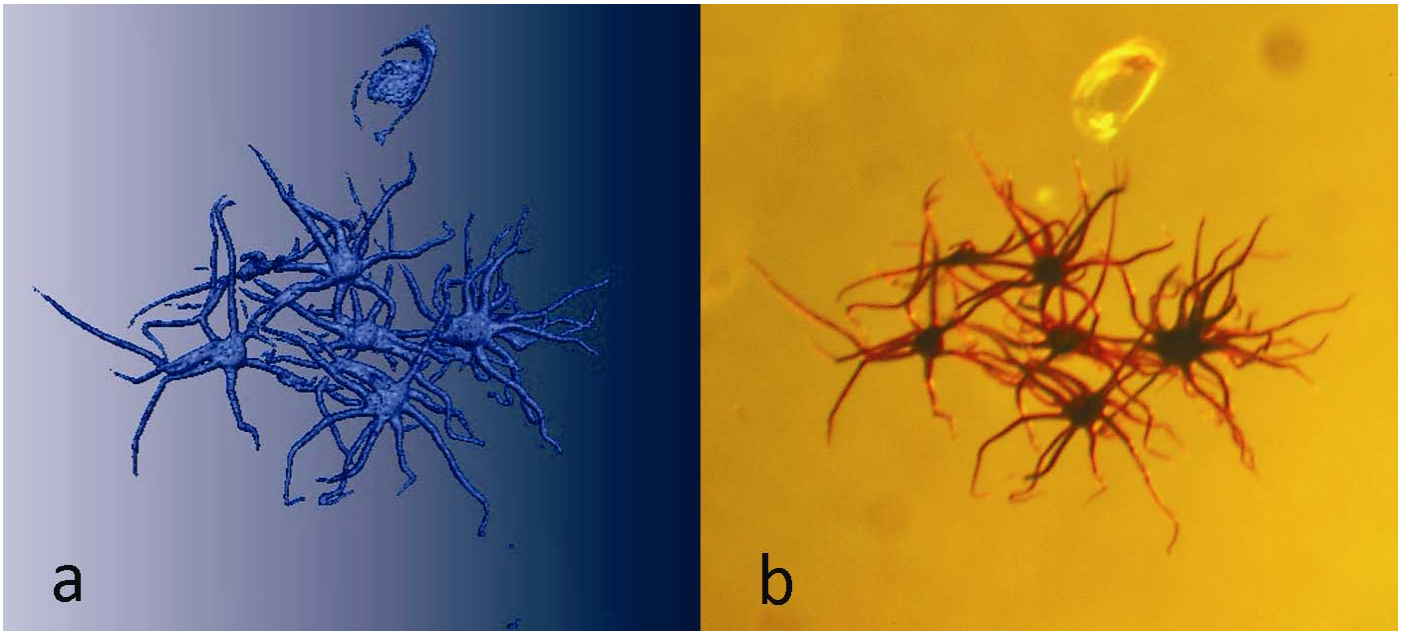



**Figure** 3. Trichome group in Mexican amber showing (a) visible side using CLSM, and (b) the same group using a transmitted light microscope (scale = 0.2mm).

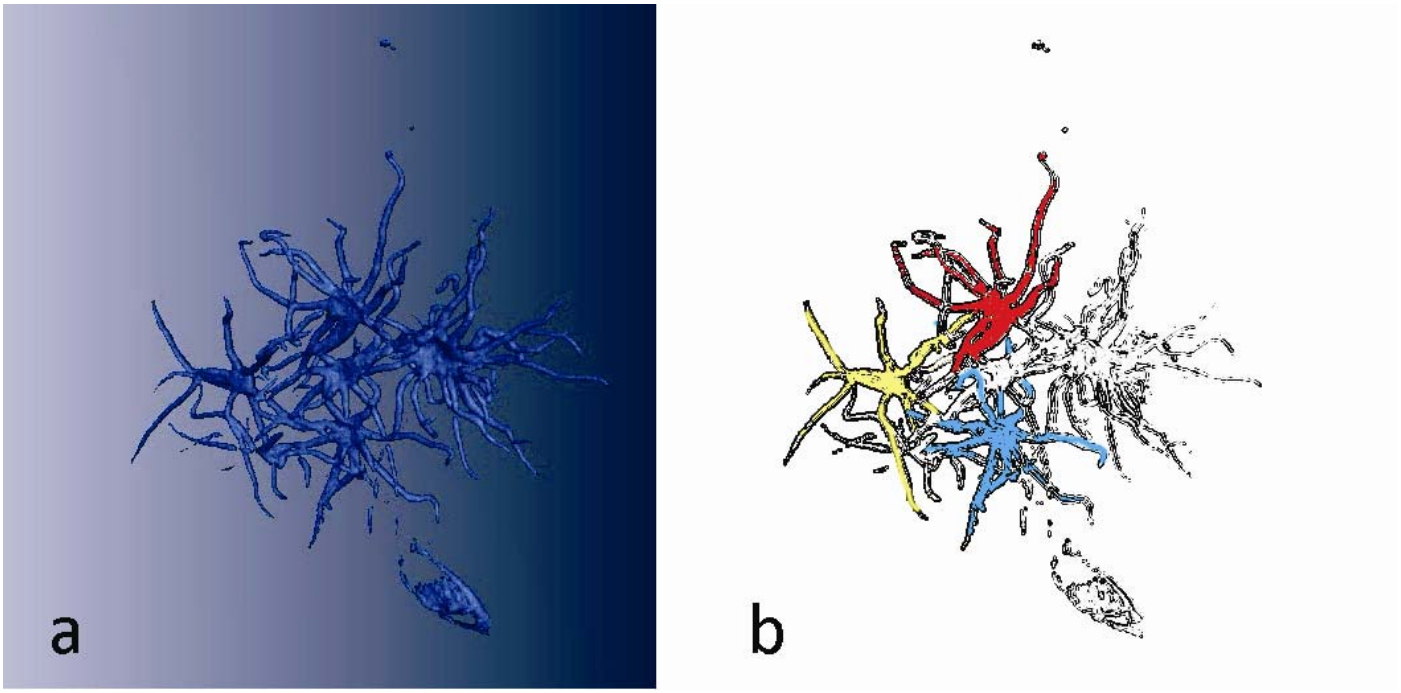



**Figure** 4. Trichome group in Mexican amber (GLAHM 131494/3) showing (a) opposite side using CLSM, and (b) the same group in outline with three trichomes with their radii coloured (scale = 0.2mm).

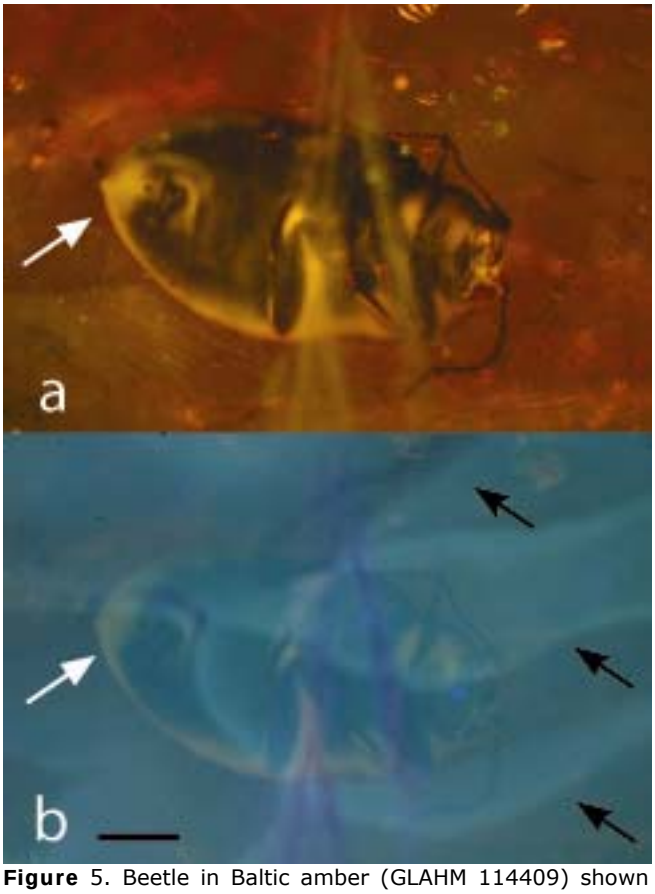



**Figure** 5. Beetle in Baltic amber (GLAHM 114409) shown using (a) natural transmitted light and (b) ultraviolet fluorescence. The white fungus (white arrow) on the cuticle of the beetle (a) fluoresces slightly more in ultraviolet than the amber (b). Flow structures (black arrows) of the original resin can also be seen in the amber using ultraviolet (scale = 1mm).

An attempt was also made to look at insect inclusions using the same method following the success of Böker & Brocksch (2002). The insects did not fluoresce in the far red as the trichomes did, but in the ultraviolet part of the spectrum. As the insect inclusions were generally larger than the trichomes, it was more difficult to produce a 3D image as the inclusions extended deeper than the imaging capabilities of our system and were larger than the field of view of the objective. Using a lower power objective may help by increasing the field of view, but there may still be a problem with the penetration. The amber also fluoresced in the ultraviolet, making it difficult to distinguish the insect from the amber. The white fungus commonly found in Baltic amber fluoresced differently from the surrounding amber and was clearly visible (figure 5).

## DISCUSSION

Baltic amber has been well known for its inclusions for centuries, and speculation on its origins as a tree resin dates certainly as far back as Pliny the Elder in 49 AD and perhaps earlier (Healy 2004, Clark 2010). Many have suggested that the origin of the resin may have been a variety of pine tree (Healy 2004, Clark 2010) and some have suggested the name *Pinites succinifer* (Göppert 1846). Despite the similarity of the resin to the conifer families Araucariaceae and Pinaceae, it may be that the tree that produced the resin was more closely related to the Sciadopityaceae which is now represented by a single species *Sciadopitys verticillata* (Japanese Umbrella-pine) (Wolfe et al. 2009).

This study was unable to obtain the depth penetration, nor the differential fluorescence between the insect and the amber resin, necessary to obtain insect images similar in quality to those obtained by Böker & Brocksch (2002). Both the insects and the amber fluoresce in the ultra-violet part of the spectrum making it difficult to distinguish between them.

Trichomes are known to contain (blue-green) autofluorescent flavanoids. The samples of amber have strong autofluorescence in the UV range which enables discrimination of amber from the embedded trichome. Our initial observations of a strong autofluorescence in the far red spectrum suggest the presence of an alternative compound or a modified form of flavonoid present within amber-included trichomes. Alternatively, the trichomes may have been transformed and/or chemically altered over time. Whilst this certainly warrants further study, the main aim of this project was to examine the structure of the trichomes and assess the usefulness of CLSM.

The Mexican trichomes examined in this study differ from the trichomes from Baltic amber and may belong to the Family Euphorbiaceae.

The individual Baltic trichome that was chosen for the study has a stellate form with seventeen radii (figures 1, 2; animation 1). Overlapping radii give the appearance of bifurcation, but when viewed in 3D, it is evident that they are separate radii. The trichome appears to have two stellate clusters superimposed on each other (geminate). One of the radii is longer than the others perhaps representing a stalk. The trichome here is consistent with the structure of trichomes of the Fagaceae, or oak family (Hong-Ping et al. 1990, Nixon 2002, González-Villarreal 2003a, 2003b).

CLSM may be further developed to help with producing 3D images of inclusions in amber. Although the technique seems to be limited by the size of the inclusions and the depth at which the inclusion appears, Böker & Brocksch (2002) have shown that it is possible to produce high definition 3D images of larger insect inclusions. The equipment at the University of Glasgow is not optimised for such analyses, but has been useful in producing 3D images of very small trichome inclusions. Further refinement of this technique and equipment may allow useful systematic study of inclusions in amber by providing very high resolution 3D imagery of the anatomy of inclusions.

Scanning of amber using computed tomography and phase contrast X-ray synchrotron microradiography has provided the opportunity for 3D analysis of larger fossil inclusions (Polcyn et al. 2002, Lak et al. 2008, 2009). This technique provides very high resolution 3D images of the fossil contents of the amber and has been particularly useful in examining opaque amber (Lak et al. 2008). The long term effects of exposure to a high energy monochromatic beam on amber are unknown, but the benefit of being able to visualise the contents of opaque amber is great.

CLSM has limited usefulness in the analysis of amber inclusions due to the lack of penetration and the reliance on the differential fluorescence between the inclusions and the amber. Nevertheless, CLSM has potential value in recognising different fluorescing properties perhaps providing an indication of different compositional or structural architecture.

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