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Recibido: 24 de abril 2022 - **Aceptado:** 18 de septiembre 2022 - **Publicado:** 7 de diciembre 2022

Para citar este artículo: Samar Riad El-Abdallah, Ashley Kammet, Kally Matsunaga, Selena Smith, & Alexandru Tomescu (2022). Resolving the anatomy of mature sclerifed conifer seed cones: Complementarity among three methods. *Publicación Electrónica de la Asociación Paleontológica Argentina* 22 (2): 45–57.

Link a este artículo: http://dx.doi.org/10.5710/PEAPA.18.09.2022.425

ISSN 2469-0228

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Publicación Electrónica
ASOCIACIÓN PALEONTOLÓGICA ARGENTINA
Año 2022 - 22(2): 45–57
ARTÍCULO

RESOLVING THE ANATOMY OF MATURE SCLERIFIED CONIFER SEED CONES: COMPLEMENTARITY AMONG THREE METHODS

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Abstract. Phylogenetic studies of conifers that involve morphology are hindered by gaps in the anatomical characterization of seed cones, a direct result of difficulties encountered in sectioning cones in mature stages, which are often hardened due to sclerification. Here, we compare the resolving power of three methods—paraffin sectioning, petrographic thin-sectioning, and X-ray microcomputed tomography (micro-CT)—in documenting the morphology and anatomy of mature seed cones at different scales of detail. We use *Taxodium* as a case study, based on which we make recommendations on the complementarity of these methods, and we present a paraffin sectioning protocol for softening sclerified tissues. Paraffin sectioning, while providing high anatomical resolution, can only be used for small specimens, is labor-intensive, and hampered by hard tissues. Petrographic sectioning is fast and effective on larger specimens, but has low anatomical resolution and is limited to dry non-fleshy material. Micro-CT, if available, is fast, produces high resolution with no size limitations, and allows virtual sectioning and accurate 3D rendering; however, understanding of histology requires comparisons of CT images with results of the other methods. Although they overlap, each of the three methods provides unique insights on anatomy at different scales of detail. Thus, combining all three methods is ideal for producing high-quality data at all scales of anatomical and morphological detail.

Key words. Anatomy. Conifer. Microcomputed tomography. Paraffin sectioning. Seed cone.

Resumen. RESOLVIENDO LA ANATOMÍA DE CONOS MADUROS ESCLERIFICADOS EN CONÍFERAS: COMPLEMENTARIDAD ENTRE TRES MÉTODOS. Los análisis filogenéticos de coníferas que incluyen caracteres morfológicos se ven limitados por la falta de información en la caracterización anatómica de conos ovulíferos, una consecuencia de la dificultad de seccionar conos en estadio maduro, ya que se encuentran esclerificados. En este trabajo, comparamos el poder de resolución de tres métodos—cortes delgados de parafina, cortes delgados petrográficos y microtomografía computada de rayos X (micro-CT). Utilizamos a *Taxodium* como caso de estudio, y en base a los resultados obtenidos recomendamos complementar todos estos métodos, así como también presentamos un protocolo para la obtención de cortes de parafina para ejemplares con tejidos esclerificados. Las secciones delgadas de parafina, mientras que proveen una alta resolución anatómica, solo pueden ser utilizadas en ejemplares pequeños, es una técnica laboriosa y se ve dificultada por los tejidos duros. Los cortes delgados petrográficos son más rápidos y efectivos en ejemplares grandes, pero su resolución anatómica es baja y su aplicación se limita a ejemplares sin tejido fresco. El micro-CT, cuando está disponible, es rápido, produce cortes de alta resolución sin limitantes en el tamaño del ejemplar, y permite realizar cortes digitales y modelos 3D precisos; sin embargo, para poder identificar las estructuras anatómicas es preciso complementar con cortes obtenidos mediante alguno de los otros dos métodos. Estos métodos, aunque se superponen, proveen información anatómica con diferentes escalas de detalle. Por lo tanto, es ideal combinar estos tres métodos para así obtener información anatómica y morfológica de detalle de alta calidad a cualquier escala.

Palabras clave. Anatomía. Coníferas. Microtomografía computada. Cortes delgados de parafina. Cono ovulífero.

DETAILED data on seed cone anatomy are crucial for comparative studies with implications for conifer systematics and phylogeny. Seed cones are complex structures that provide a wealth of characters in terms of both morphology and anatomy (Rothwell *et al.*, 2011; Escapa & Catalano, 2013; Smith *et al.*, 2016; Herrera *et al.*, 2017). Typically, a conifer seed cone consists of a stem that forms the cone axis, with ovule-bearing structures attached to it. These structures have been interpreted as reduced branch shoots (secondary shoots) that present different degrees of fusion to the bracts that subtend them (summarized by Florin, 1954); they are referred to as ovuliferous scale, generically, although the

term bract-scale complex better encompasses their inferred homology. At first glance, thin sections illustrating the cross-sectional anatomy of an ovuliferous scale reveal a broad range of structures that can provide a multitude of characters for comparative and systematic studies. To name a few, such structures include micropapillae on epidermal cells, with their morphology and spatial distribution, cuticular thickness variation, transfusion tissue distribution, sclereid anatomy and frequency, or vascular bundle structure. These and other features detailing diverse aspects of seed cone anatomy and morphology can significantly increase the number of characters used in phylogenetic studies, with beneficial results for resolution of relationships.

Phylogenetic relationships provide some of the best hypotheses and perspectives on biological evolution, and inclusion of fossils in studies of phylogeny is key to improved understanding of relationships, while also revealing interesting aspects of diversification or of the tempo, mode, and details of morphological evolution. However, effective inclusion of fossils in phylogenetic analyses can only be achieved by using anatomical and morphological characters due to lack of DNA preserved in fossils. In the specific case of conifers, seed cones are among the most important plant parts for studies of phylogeny, because of the large number of informative characters that they provide (*e.g.*, Matsunaga *et al.*, 2021). Conifers have a rich fossil record, including numerous occurrences of seed cones, many of which exhibit anatomical preservation (*e.g.*, Stockey *et al.*, 2005; Rothwell *et al.*, 2011; Smith *et al.*, 2016; Atkinson *et al.*, 2021). Thus, in-depth understanding of comparative seed cone anatomy is crucial for integrating anatomically-preserved fossils in phylogenetic studies.

A survey of the paleobotanical literature shows that fossil conifer seed cones are overwhelmingly represented by mature specimens (*e.g.*, Stockey *et al.*, 2005; Atkinson *et al.*, 2014; Smith *et al.*, 2016). This is due, in part, to the fact that many species abscise their cones or cone parts (which then enter taphonomic pathways) at maturity, and in part to the higher preservation potential of mature, sclerified plant organs, as compared to the developing, fleshy juvenile stages. The latter are also less likely to enter taphonomic pathways, except for fortuitous traumatic events (*e.g.*, breaking of plant parts by wind, precipitation). Thus, inclusion of fossil seed cones in comparative and phylogenetic studies requires a solid comparative base in the anatomy of mature cones. Unfortunately, current available comparative data on extant seed cone anatomy present gaps, especially in the coverage of mature, sclerified and hardened stages of the cones. This is, at least in part, due to serious difficulties in obtaining good, anatomically-informative sections of such cones using traditional methods. In turn, this is due to the hardness of the cones, resulting from the high proportion of sclerified tissues and the often thick cuticle layers, which render the cones mechanically hard to section and preclude complete penetration of tissues with solutions during specimen processing prior to sectioning.

Here, we employ three different methods to document seed cone anatomy in *Taxodium distichum*: thin-sectioning with petrographic techniques, traditional paraffin sectioning, and microcomputed tomography scanning (micro-CT scanning). We compare the results obtained with each method in terms of types of data recovered, level of anatomical detail obtained, and efficiency. The three methods produce results that are only partially overlapping and each provide unique insights. Detailed documentation of mature seed cone anatomy requires integration of the perspectives offered by all three methods presented here.

MATERIALS AND METHODS

Petrographic thin sections

Cones are embedded in BioPlastic synthetic resin (Ward's Science, Rochester, New York, USA). Infiltration is performed in a vacuum chamber for up to 10 minutes. Upon curing, the resulting plastic block that encloses the cone is treated like a rock sample and sectioned following the procedures for petrographic thin sections, with some modifications. Every time a new section is cut, the cone tissues exposed on both sides of the plane of section (at the top of the wafer that is attached to the microscope slide, and on the side of the larger block where the specimen is exposed) are infiltrated with fresh epoxy resin under vacuum (we used Devcon (Solon, Ohio, USA) 2 Ton Epoxy). This strengthens the exposed tissues, allowing them to withstand polishing or grinding to the desired section thickness. The side of the remaining plastic block with the cone section exposed is polished and glued to a new microscope slide. Part of the block

is then cut off on the sectioning machine, leaving a thin wafer attached to the slide. After the top side of the wafer exposed by the cut is sealed with epoxy resin, the wafer is ground to the desired thickness on the sectioning machine, polished, and covered with a coverslip. Repeating these steps produces serial thin sections spaced *ca.* 2 mm apart.

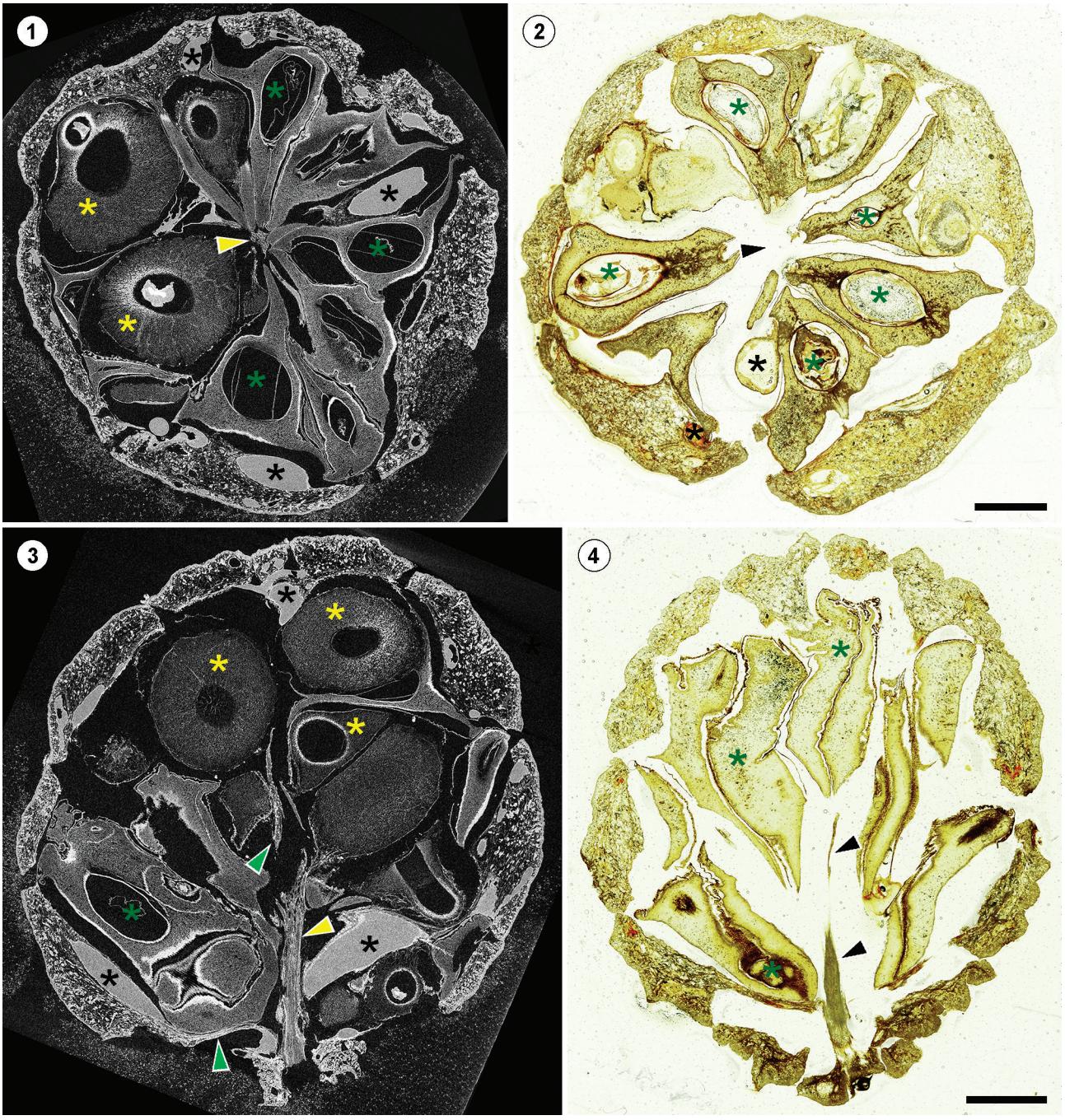


Figure 1. Whole-cone median sections of ***Taxodium distichum***. **1–2,** transverse sections; **3–4,** longitudinal sections. **1, 3,** micro-CT. The thin cone axes visible in the scans (yellow arrowheads) and the slender stalks of the cone scales (green arrowheads) may be missed between successive petrographic sections. The central volume of the cone is occupied by seeds (seed lumens indicated by green asterisks) with thick coats and with irregularly triangular outlines; the umbos of the cone scales surround the central volume occupied by the seeds and are distinguished from the latter by their less homogeneous texture. Resin canals (black asterisks), even very small ones, are resolved based on their homogenous gray coloring. Some of the seeds (yellow asterisks) are galled by parasites; their seed coat is thicker but less dense and the outermost layer is detached from the rest of the seed coat. **2, 4,** petrographic thin sections. The cone axis is present in Fig. 1.4 (arrowhead) but is missing in Fig. 1.2 (arrowhead indicates where it's expected). Not all resin canals (black asterisks) are as easily distinguished as in the CT-scans. Seeds (green asterisks) are identified by their seed coats and lumens. Scale bars= 3 mm.

(Benedict (2015) also provided a protocol for embedding and sectioning hard plant specimens using petrography techniques). For sectioning we used a Hillquist (Arvada, Colorado, USA) Thin Section Machine.

Histological paraffin sectioning

Individual cone scales (*i.e.*, bract-scale complexes) are fixed in formalin-propionic acid-alcohol (FPA) and then embedded in paraffin following dehydration in an ethanol-isopropanol solution series. Small paraffin blocks containing individual specimens are mounted on wood blocks and sectioned with a rotary microtome, mounted on slides, and stained as needed (*e.g.,* Walker's Sam quadruple stain protocol (Appendix 2) in this study). Use of a softening agent (in this study, 5% Downy (Proctor & Gamble, Cincinnati, Ohio, USA) in water) is crucial when applying this method to woody cones. In addition to a softening agent, frequent cooling of the specimen in an ice bath significantly improves the quality of sections. We found both treatments to be necessary even for small woody specimens (*e.g.*, *Microbiota*, *Sequoia*, and *Fitzroya* cones). See Appendix 1 for the detailed protocol.

Micro-computed tomography (micro-CT, µCT, X-ray microtomography)

Cones (fresh, dried, or preserved in alcohol) are imaged in a micro-CT scanner that uses X-rays to produce a series of 2D X-ray images (projections); these projections are then reconstructed to produce a 3D volume. The resulting volume can be observed from any desired angle or sectioned (virtually) in any desired plane. For this study, the micro-CT scan was performed at the at the University of Michigan Computed Tomography in Earth and Environmental Sciences (CTEES) facility of the Department of Earth and Environmental Sciences on a Nikon XTH 225 ST micro-CT system. The system is equipped with a Perkin-Elmer 2000 X-ray detector panel and tungsten reflection target. Scans were reconstructed using CT Pro 3D (Nikon Metrology, USA), which uses a FDK (Feldkamp-Davis-Kress) type algorithm. A *Taxodium* cone was scanned at 94 kV, with a current of 124 µA, with an exposure of 354 ms and 11.75 µm pixel size resolution. Digital sections were processed and analyzed in Avizo 9 Lite (Thermo Fisher, Waltham, Massachusetts, USA) for 3D rendering and virtual sectioning.

RESULTS AND DISCUSSIONS

Taxodium case study

Overall morphology. Characterizing the position and size of the cone axis as well as the pattern of attachment of the cone scales to the axis, is needed for understanding the overall architecture of the cone. Because of the relative irregularity of shape and orientation of cone scales and seeds in *Taxodium*, no individual plane of section through the cone makes it easy to understand their geometry. Additionally, locating the cone axis and choosing a suitable longitudinal plane of section is difficult because of the lack of external indicators for the exact position of the cone axis. *Taxodium* has a thin and relatively short cone axis (Fig. 1.1–1.4), and comparatively large cone scales, which are densely packed. As a result, the cone axis is not visible from the outside and its orientation is not easy to predict when choosing planes of sectioning for producing serial sections, especially those with longitudinal orientation. Furthermore, the stalks of the cone scales are very thin and short, therefore easy to miss in widely spaced serial sections (like the petrographic thin sections that are spaced 2 mm apart). However, in the case of *Taxodium*, the size of cones makes it impossible to cut paraffin sections of whole cones (although this can be done for species with smaller cones). Micro-CT, which allows virtual sectioning in any desired plane and 3-D reconstructions with ease and at high resolution (Smith *et al.*, 2009), overcomes these issues (compare Fig. 1.1, 1.3 with Fig. 1.2, 1.4; see also videos of micro-CT scan stacks stored on the Open Science Framework, at https://osf.io/s64hp).

Tissue differentiation. Differentiating tissues in whole-cone sections (both longitudinal and cross sections) is possible in both petrographic thin sections and micro-CT, because of the conspicuous differences in tissue density and texture between cone scales, cone axis, and seeds. Specifically, seed coats are more homogeneous texturally and denser, with smaller cells, compared to the cone scale tissues which consist of larger cells; these differences are more conspicuous, even at low magnifications, in micro-CT material than in thin sections (Fig. 2.1–2.3).

Whole-cone sections of both petrographic sections and micro-CT material reveal the seeds, seed coats, and resin ducts within the cone scales (Fig. 1.1–1.4). Resin canals are

more conspicuous at lower magnifications in micro-CT material, wherein they are easily recognized by the homogeneous gray color of the resin when present (Fig. 1.1, 1.3), whereas in petrographic thin sections they are less conspicuous (Fig. 1.2, 1.4). Some of the cones observed here contained seeds parasitized by galling insects (Fig. 1.1, 1.3). Micro-CT captured very well the differences in seed coat structure between the healthy seeds (with thinner, denser seed coat) and the parasitized seeds (with thicker, less dense seed coats).

Zooming in on individual cone scales as seen in cross section (Fig. 2.1–2.3), sclerenchyma cells, more abundant on the abaxial side of scales, are easily seen in both micro-CT, in which their thicker cell walls make them appear brighter (Fig. 2.1), and in paraffin sections, where staining of the secondary walls renders them very conspicuous (Fig. 2.2). The sclerenchyma cells are considerably less obvious in petrographic thin sections, in which they can be recognized only at higher magnifications (Fig. 2.3, see also Fig. 4.4–4.5). The thin but dense outermost abaxial layer of the scales, which consists almost exclusively of sclerenchyma, is conspicuous in both micro-CT and paraffin sections, but can hardly be even gleaned in the petrographic thin sections (compare Fig. 2.1, 2.2, with Fig. 2.3).

Resin canals are conspicuous in all three methods at higher magnifications. However, small resin canals are harder to resolve in micro-CT, because they can be confused with large sclerenchyma that have a very similar tone of gray, and they are also inconspicuous in petrographic sections. These smaller canals can only be confirmed by observation of their epithelial cells, which are visible only in histological paraffin sections at high magnifications (Fig. 3.1–3.3)—such observations are not possible in micro-CT (unless resolution is highly increased) or in petrographic thin sections (due to the thickness of the sections, which precludes observation of small individual cells)—.

Most of the cone scale vascular bundles are very small in *Taxodium*. As a result, important vascular features, such as the collateral organization of vascular bundles and the radial alignment (or otherwise) of cells within the xylem and phloem can only be documented in detail in histological paraffin sections (Fig. 3.4) and are not distinguishable in micro-CT and petrographic thin sections because of the small cell sizes and absence of histological staining. In contrast, even the smallest vascular bundles are easy to observe in histological paraffin sections because of the capacity for high resolution and amenability to staining (Fig. 3.3–3.4). Additionally, continuous phloem and xylem rays of the sec-

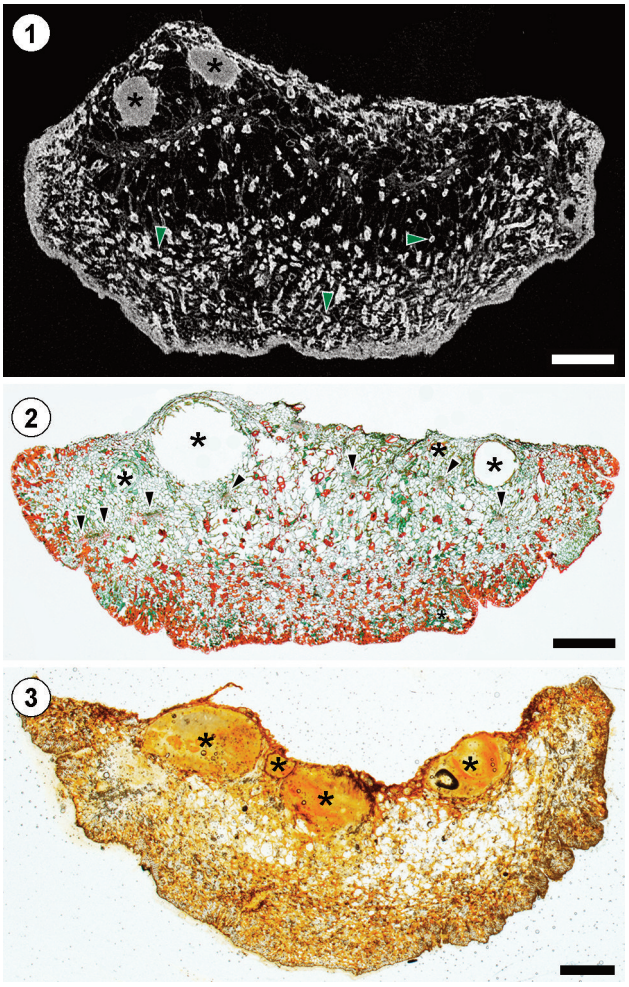
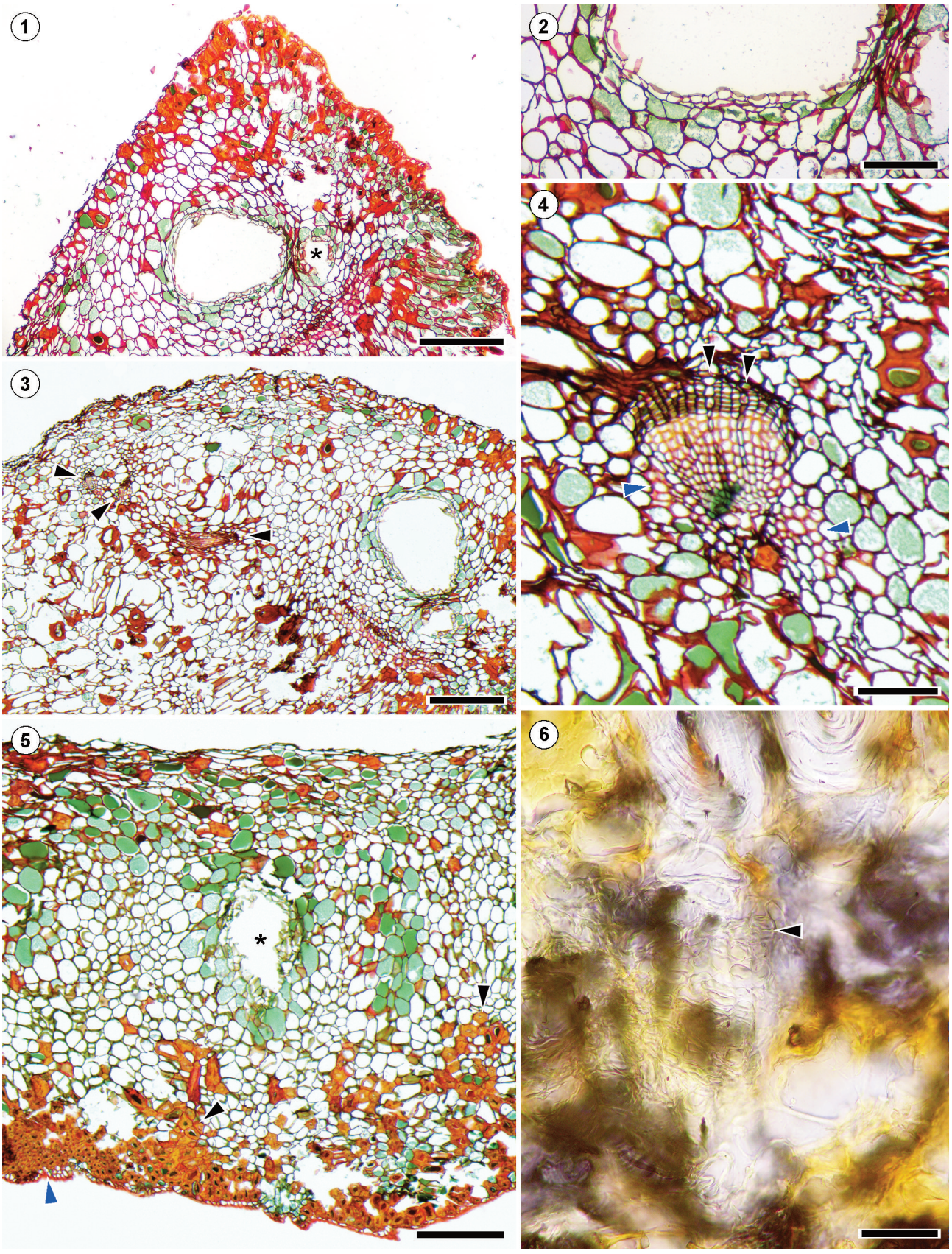


Figure 2. Cone scale transverse sections of ***Taxodium distichum***. Note differences in tissue density apparent in all three sections between abaxial side (cells smaller and more tightly packed) and adaxial side (larger cells). **1,** micro-CT; sclereids (examples at green arrowheads) are abundant in the abaxial, denser tissues of the cone scale. Sclereids and resin canals (asterisks) are conspicuous in micro-CT scans due to their homogenous, gray coloring; however, smaller resin canals can be confused with sclereids and other sclerenchymatous cells that have similar gray tones. **2,** histological paraffin section; sclereids and other sclerenchyma are conspicuous due to their red staining; note the abundance of sclereids on abaxial side of scale. Vascular bundles (black arrowheads) are also conspicuous, especially at higher magnifications. Resin canals (asterisks) lose their content during fixation and paraffin embedding and appear empty; the smallest ones could be confused with large parenchyma cells unless epithelial cells are observed (at higher magnifications; see Fig. 3.2). **3,** petrographic thin section; the fine histology cannot be resolved at low magnifications, and only large resin canals (asterisks) are conspicuous, as are the differences in tissue density. Scale bars= 1 mm.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ondary tissues are distinguishable from other cells of the vascular bundle (Fig. 3.4) and transfusion tracheids are found on the periphery of the primary xylem (Fig. 3.4). Vascular bundles are transparent, therefore inconspicuous, in petrographic sections (Fig. 3.6). Additionally, the thickness of petrographic sections blurs the outlines of small cells, which are also hard to resolve in the absence of diagnostic staining. Vascular bundles are hard to resolve in micro-CT as well, and the smallest of them will be easily missed because of the lower resolution provided by this method.

The cuticle, which can be thick and whose features can provide important characters, is hard to resolve in micro-CT but is readily observed in histological paraffin sections, where it can be revealed by differential staining (Fig. 4.1–4.3). Interestingly, because of their thickness, the slightly thicker petrographic sections allow a better look at the three-dimensional microstructures of the cuticle, such as papillae or simply the microrelief of the surface of cone scales (Fig. 4.4 and Fig. 5).

General considerations

None of the three methods presented here produces results superior to the others in all respects. Each has advantages and disadvantages when compared from different points of view (processing time, labor and equipment requirements, resolution, types of data acquired) (Tab. 1). Whereas all three methods require specialized lab equipment, micro-CT is far more expensive than the other two in this respect. Nevertheless, micro-CT is the least time-consuming (requiring up to a few hours per sample) and least demanding in terms of labor for raw data acquisition, while also being non-destructive (a possible advantage for some samples in natural history collections, as is the case for many reproductive fossil structures). However, processing digital data for complex structures and specialized volume renderings can become more time-consuming (see Tab. 1). For comparable sample sizes, the other two methods take one week or more for raw data acquisition, although this also includes long waiting times (Tab. 1) for sample infiltration with solutions, resin curing, or softening and icing. Additionally, producing 3D renderings from paraffin sections is labor-intensive and time-consuming; it requires photographing each section and digital processing of each image.

Micro-CT produces the highest Z-stack resolution (distance between successive serial sections), which, depending on the equipment and its settings and the sample size, can be <1 µm. Paraffin sectioning produces Z-stack resolution comparable to the lower end of the resolution spectrum of micro-CT, *ca.* 10 µm, which makes its products amenable to 3D renderings. The Z-stack resolution of petrographic thin sections is far lower (≥2 mm), because of the thickness of material lost in the saw kerf when each section is cut and ground. This makes petrographic sectioning unsuitable for small samples (*ca.* 1 cm or smaller, such as the seed cones of *Chamaecyparis*, *Fitzroya*, or *Microbiota*) or for 3D renderings.

Because it does not involve physical sectioning of specimens ("sectioning" is digital), micro-CT eliminates all difficulties that are typically encountered when sectioning hard woody cones often using the other two methods. Micro-CT produces virtual 3D objects that can be further virtually sectioned in any orientation, allowing for easy observation of details, as well as understanding of the three-dimensional

Figure 3. Anatomical details of ***Taxodium distichum***. **1–5,** histological paraffin sections; **6,** petrographic thin section. **1,** cone scale margin. Note histological differences between adaxial tissues (left) and abaxial tissues; sclerenchyma are more abundant abaxially and at the margin of the scale; the cuticle is much thicker abaxially; one large and one small (asterisk) resin canals are present; the space above the small resin canal is a sectioning artefact (tear). Scale bar= 500 µm. **2,** resin canal. Paraffin sectioning is the only method that allows for unequivocal recognition of resin canals as epithelial cells lining the canal can be identified; resin canals may be confused with sectioning artifacts (such as tears; see Fig. 3.1 and Fig. 3.5). Scale bar= 150 µm. **3,** adaxial and central tissues of the cone scale (partial overlap with Fig. 3.1). Note difference in histology: smaller and more regular-shaped cells on adaxial side vs. irregularly sized and shaped cells in central area of the scale (below the adaxial tissues); three vascular bundles (arrowheads) and a line of smaller cells (bottom right) occur along the boundary between the two histologic regions. Scale bar= 400 µm. **4,** detail of collateral vascular bundle (phloem at top) with conspicuous radial files of secondary xylem tracheids and secondary phloem cells, as well as two rays (black arrowheads). Small groups of transfusion tracheids are present laterally to the primary xylem of the bundle (blue arrowheads). Scale bar= 400 µm. **5,** central portion of the scale. Abaxial tissues are denser with more sclereids (tan-staining), the abaxial cuticle (blue arrowhead) is thicker than the adaxial and apparent at this magnification (see also Fig. 4.2–4.3). Note a tear (sectioning artifact; asterisk) that may be confused with a resin canal but lacks epithelial cells. Scale bar= 100 µm. **6,** vascular bundle (arrowhead) in petrographic thin section. Cell walls appear transparent (*e.g.*, two large sclereids with concentric layering of secondary walls at top of image) and may only be resolved at very high magnifications; the vascular bundle and cell types, in general, are difficult to resolve in petrographic sections. Scale bar= 400 µm.

external and internal organization of the specimens (in the case of cones: arrangement of the scales, seed positioning, coarse architecture of the vasculature, and resin canal system). Additionally, because virtual sectioning can be performed in any plane, a single cone can provide information on all relevant planes of section for different cone parts. For example, a single specimen was used to produce results of those in Figure 1.1 and 1.3. In contrast to this, the methods that involve physical sectioning require multiple specimens (cones or cone scales) for observing multiple planes of section. However, the raw data obtained with micro-CT can be ambiguous in the absence of the types of information provided exclusively by the other two methods. For example, although cells (especially the larger ones) are easily observed in micro-CT slices, they can be differentiated based exclusively on their shapes and sizes, whereas cell wall composition or other cellular and subcellular features that are emphasized by staining cannot be documented. As a result, tissues may also be difficult to distinguish, unless they are characterized by marked differences in cell size and shape or cell wall thickness. Consequently, some features on micro-CT visuals require validation by other methods (ideally stained paraffin sections) for comprehensive interpretation.

Like micro-CT scans, petrographic thin sections of bioplastic-embedded specimens are informative about the three-dimensional organization of entire cones; even larger

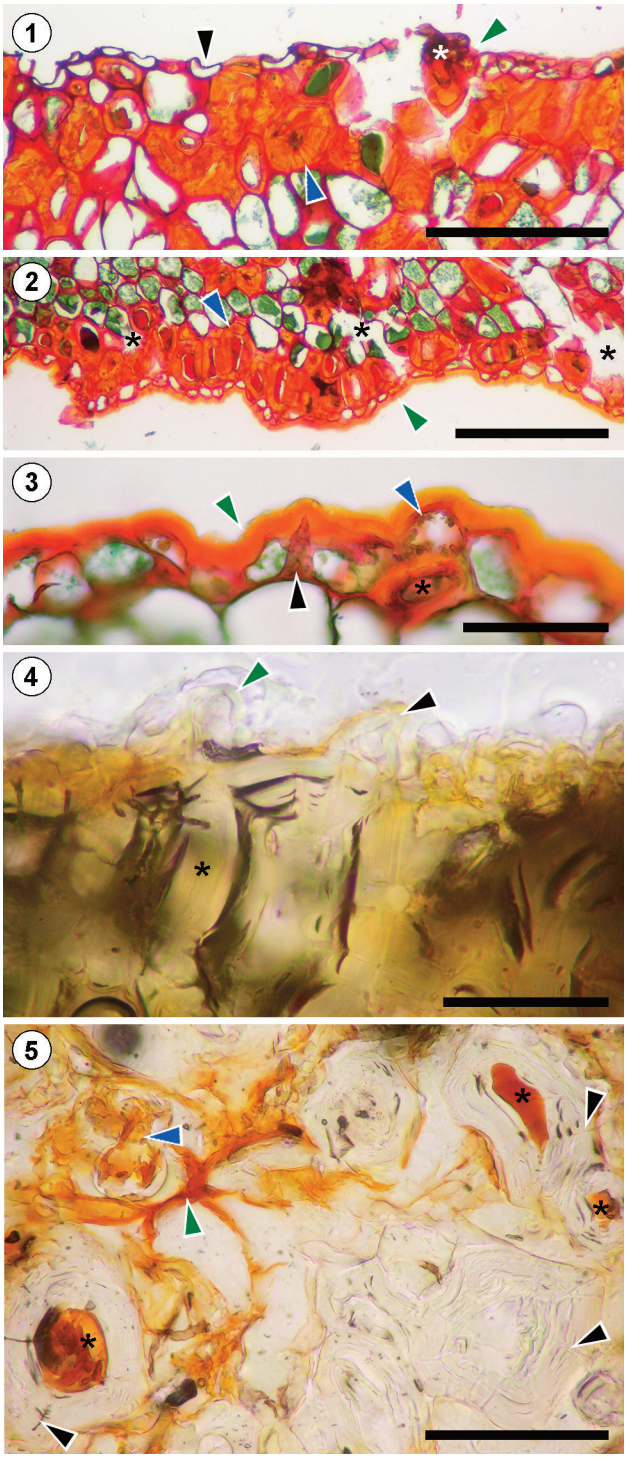

Figure 4. Fine anatomical details of ***Taxodium distichum***. **1–3,** histological paraffin sectioning; **4–5,** petrographic thin sections. **1,** details of adaxial epidermis. Epidermal cells (black arrowhead) are easily distinguished from other tissues. Paraffin sectioning may render sections with missing parts; here, only a remnant of the adaxial cuticle (green arrowhead) is left; section tearing may result in cells overlapping (asterisk). Details of sclereid cell walls (*e.g.*, ramiform pits, blue arrowhead) can be resolved. Scale bar= 200 µm. **2,** detail of abaxial epidermis in histological paraffin section. The thick cuticle (green arrowhead) is conspicuous; small epidermal cells can be distinguished from the large hypodermal sclereids (blue arrowhead), which are abundantly present. Tears (asterisks) can easily develop in the thin paraffin sections. Scale bar= 200 µm. **3,** detail of adaxial epidermis in histological paraffin section. Some detail of the cuticle topography (green arrowhead) is visible at higher magnification. Details of epidermal cells, such as a putative idioblast (black arrowhead) or internal structures (blue arrowhead) may be observed. Note the isolated sclereid (asterisk) between epidermis and subjacent layer. Scale bar= 200 µm. **4,** detail of the adaxial epidermis in a petrographic thin section; the cuticle (green arrowhead), highly translucent, is inconspicuous; papillae of epidermal cells (black arrowhead) can be resolved, but cells may be difficult to distinguish from each other in many areas. The prominent, albeit transparent, cell walls (asterisk) allow for identification of sclereids. Scale bar= 200 µm. **5,** cellular details of scale tissues. Distinguishing cells from one another is difficult, as cell walls are transparent in petrographic thin sections. Some primary cell walls (*e.g.*, parenchyma walls, green arrowhead) may be dark and conspicuous, but the thickness of petrographic sections renders many cells indistinguishable from each other and many structures (blue arrowhead) unidentifiable. Darker material or structures can be observed in the lumen of some sclereids (asterisks). Although the cell walls of sclereids are transparent, their stratified structure and ramiform pits (black arrowheads) are apparent at high magnifications. Scale bar= 100 µm.

cones can be sectioned, although the size of the slides that hold the sections is a limiting factor for cones larger than 5 cm (which consequently have to be sectioned in multiple segments). Petrographic sectioning is straightforward and does not require additional frequent softening treatments, like those required for histological paraffin sectioning of hard woody parts. In other words, the same protocol is used without modifications, irrespective of the amount of hard tissues (*i.e.*, cone type). Good quality sections with no tearing or missing parts are easily produced. However, petrographic thin sectioning is limited to dried specimens and cannot be used on cones that still have fleshy parts or tissues. The dried specimens are usually also heavily lignified, which renders staining for tissue differentiation uninformative. As a result, tissues can be difficult to distinguish, although differences in coloration can still allow for better cell and tissue type differentiation than in micro-CT scans (Fig. 4.5). Nevertheless, because of the relatively rough treatment of the plant material involved in the production of petrographic thin sections (rotary saw cutting, thinning by abrasion), as well as the relatively thick sections produced, cell-level resolution is lower compared to the other two methods, and even small vascular bundles or resin canals may not always be resolved. As in the case of micro-CT, full histological resolution requires validation by comparison with stained histological paraffin sections.

Traditional paraffin sectioning of hard, woody cones requires the most significant amount of case-by-case experimentation and modifications, among the three methods compared here. Such experimentation involves primarily tissue-softening protocols. Additionally, the size of specimens is limited, so for larger cones (as is the case for *Taxodium*) the scales have to be sectioned individually. Nevertheless, histological paraffin sectioning remains the best method for observing fine anatomical features. Because histological staining is possible, they allow for differentiation of tissues and cell types, cell wall chemistry, as well as observation of high-resolution cellular details. As a result, even fine vascular strands and vascular architecture can be observed in detail. Detailed study of vascular anatomy (*e.g.*, organization of cells, identification of secondary xylem and phloem rays, transfusion tissue, etc.) is only possible with staining and the high resolution available with histological paraffin sec-

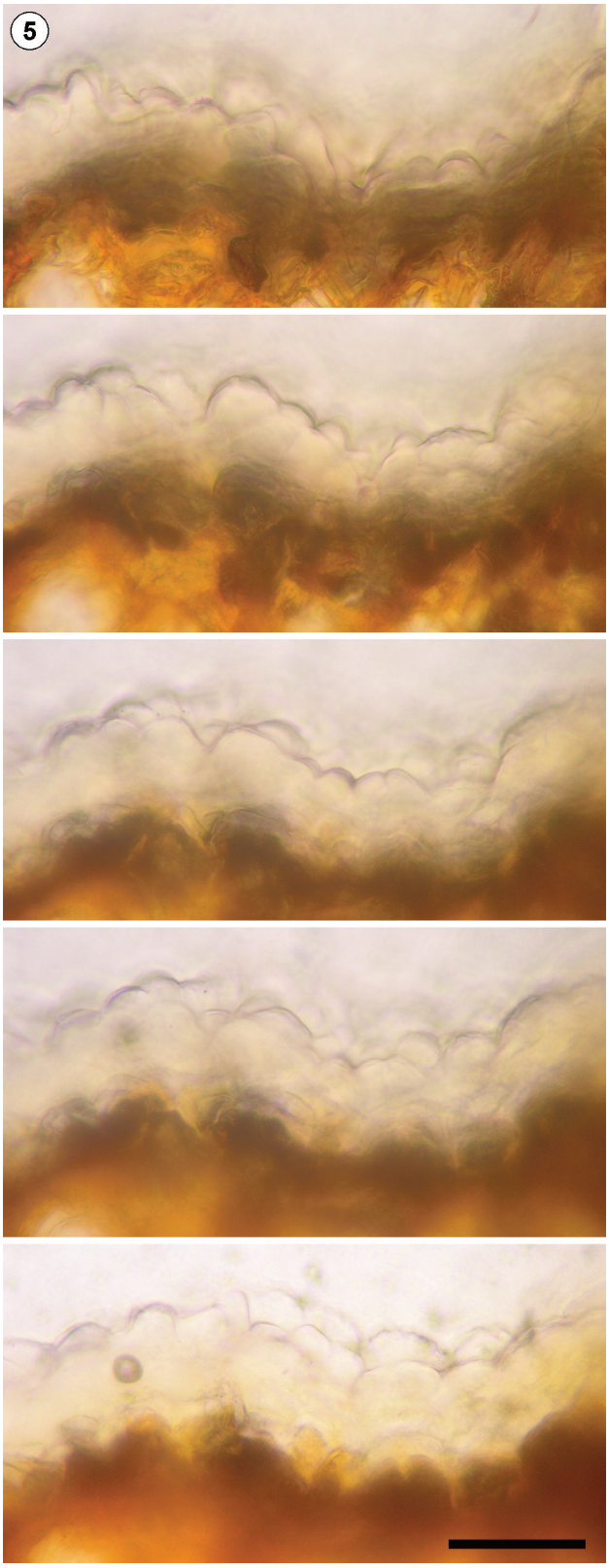

Figure 5. Fine anatomical details of the ***Taxodium distichum*** cuticle. Multiple planes of focus reveal cuticle microtopography in petrographic thin sections, due to the thickness of the sections. Scale bar= 200 µm.

TABLE 1 – Comparison of three methods of documenting conifer seed cone morphology and anatomy

Method	Processing time (for specimens of equivalent size)	Equipment	Products	Z-stack resolution	Best for observing	Advantages	Disadvantages
Thin-sectioning with petrographic techniques	45–50 hrs	Petrographic thin-section machine Vacuum chamber Bioplastic & epoxy resins	Thin sections mounted on slides	2 mm	Entire cones, even larger ones Cone scale arrangement and coarse anatomy (vascular strand distribution, resin canals) Positioning and coarse anatomy of seeds	Easy to obtain good quality sections with no tearing or missing partsCan section large specimens (limited, however, by the size of microscope slides)	Lowest Z-stack resolution: too low for 3D rendering, not suitable for small specimens Lower cell-level resolution Tissues difficult to distinguish Not ideal for staining Small vascular bundles not always resolved Not applicable to specimens with fleshy tissues Multiple specimens required for multiple planes of section
Histological paraffin sectioning	>9 days	Rotary microtome Diverse reagents and stainsIce bath	Thin sections stained and mounted on slides	8–12 µm	Fine anatomical features: cell types, high resolution cellular detail Differentiating tissues and o ther structures Detailed vascular architecture and resin canal geometry	High Z-stack resolution: 3D rendering possible, good for small specimens High cell-level resolution Allows staining for cell types, tissues, and chemistry Applicable to fleshy tissues	Difficult to obtain good quality sections with hard material (which requires lengthy, tedious processing) Suitable only for small specimens 3D rendering is work-intensive- Multiple specimens required for multiple planes of section
Microcomputed tomography (micro-CT)	2–4 hours	MicroCT scannerPowerful computer(s) Software High data storage capacities	Digital files of scan slices, leading to volume renderings, videos	<1µm to >10 µm	Entire cones, even large3D morphology Cone scale arrangement and coarse anatomy (vascular strand distribution, resin canals) Positioning and coarse anatomy of seeds Finer anatomical features	Non-destructive, can be followed by other methods Fastest processing Highest Z-stack resolution High cell-level resolution No physical work required Easiest to produce 3D renderings Virtual sectioning in any desired plane A single specimen allows viewing of multiple section planes	Difficult to distinguish tissues; often requires ground-truthing by other methods (ideally paraffin sections) Can be time consuming with higher resolution or 3D rendering of complex features Requires expensive equipment

tioning. For the same reasons, the presence and distribution of transfusion tissue, which provides important characters in taxonomic comparisons, but is usually difficult (and sometimes impossible) to document in micro-CT and petrographic thin sections, is resolved well in histological paraffin sections. The same is true for specialized cells secreting compounds other than resin, which are readily differentiated by histological stains but cannot be observed in micro-CT or petrographic thin sections. Additionally, like micro-CT but unlike petrographic thin sectioning, histological paraffin sectioning can be performed on specimens that combine hard and fleshy tissues.

CONCLUSIONS

Each of the three methods compared here provides important information on seed cone anatomy and morphology. Overall, petrographic thin sectioning produces good quality sections of large specimens, histological paraffin sectioning yields sections that can be stained for tissue and cell-type differentiation, and micro-CT provides abundant data at high resolution in different planes from only one specimen; additionally, 3D morphology can best be portrayed with micro-CT data. In an ideal chain of operations, the same specimen can first be documented using micro-CT and, following that, sectioned with one of the physical sectioning methods. Identification of tissues, cells, and other structures in physical sections using petrographic thin sections for larger features and histological paraffin sections for smaller details, should precede the analysis of digital data yielded by micro-CT, in order to best complement them. Histological paraffin sections remain the most accurate method for documenting histology, but once a general understanding of cone anatomy is achieved, micro-CT can be used for faster documentation of the three-dimensional structures of the cones.

ACKNOWLEDGMENTS

We thank Dave Baston for maintenance and access to the Cal Poly Humboldt – Biological Sciences Core Facility, and Steve Tillinghast and Lewis McCrigler for access to and maintenance of petrographic sectioning and histology equipment. This study includes data produced in the CTEES facility at the University of Michigan, supported by the Department of Earth and Environmental Sciences and College of Literature, Science, and the Arts. Ari Iglesias and an anonymous reviewer are thanked for comments and suggestions that improved the manuscript.

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doi: 10.5710/PEAPA.18.09.2022.425

Recibido: 24 de abril 2022
Aceptado: 18 de septiembre 2022
Publicado: 7 de diciembre 2022

APPENDIX 1. HISTOLOGICAL PARAFFIN SECTIONING PROTOCOL DRAFT

Dehydration and paraffin embedding protocol

Following fixation in FPA (formalin - propionic acid - 50% ethanol; 1:1:18), we used the standard paraffin embedding protocol for plant microtechnique, with the following times in each solution:

Day 1: covered vial, room temperature		Day 2: covered vial, room temperature	
50% ethanol	4 h	isopropanol	4 h
70% ethanol	4 h	isopropanol	4 h
1:1 70% ethanol: isopropanol	overnight	isopropanol	overnight

Day 3: oven (58°C)		Day 4–6: open vial, oven (58°C)	
3:1 isopropanol: paraffin	4 h (covered vial)	paraffin	24 h
1:1 isopropanol: paraffin	4 h (covered vial)	paraffin	24 h
1:3 isopropanol: paraffin	overnight (remove cap)	paraffin	24 h

The paraffin used is 56°C Paraplast (Oxford Labware/ Sherwood Medical, St. Louis, Missouri).

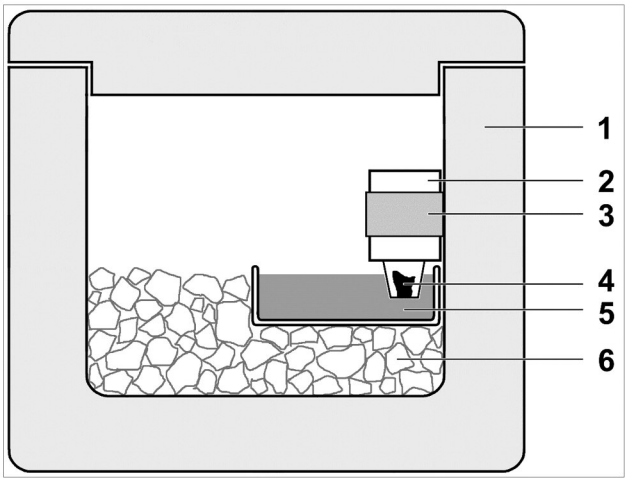

Figure S1. Setup for softening specimens for histological paraffin sectioning. **1,** Styrofoam box; **2,** wood block with paraffin block (4) glued to it; **3,** tape fastening the wood block to the Styrofoam box; **4,** paraffin block with embedded specimen; **5,** container with softening agent; **6,** ice.

Softening and sectioning protocol for hard, sclerified paraffin-embedded material

Specimens are included in paraffin blocks using the standard plant microtechnique procedures for fixing, dehydrating, and embedding plant material in paraffin (*e.g.*, Chamberlain, 1932; Johansen, 1940; Sass, 1958; Berlyn & Miksche, 1976; Ruzin, 1999). Section the paraffin block on the rotary microtome until tissue of the specimen is exposed. Remove the specimen from the microtome and place face-down in the softening agent; we used 5% Downy fabric softener (Proctor & Gamble, Cincinnati, Ohio). The softening agent is placed in a shallow ice bath to stay chilled (Fig. 1). This initial soaking takes 24–48 h, depending on specimen size (the larger the specimen, the longer the time). Replace/replenish the ice as needed; it is important the specimen is cold when sectioning, as that increases the integrity of the sections obtained.

Observe carefully the quality of sections while sectioning the softened, chilled specimen. Stop sectioning when the ribbon of sections begins to tear, shred, or has missing parts. This is because the sectioning has removed the softened and chilled tissue to the depth of softener penetration reached by the initial softening. Return the specimen to the softening setup with fresh ice, to continue infiltrating the newly exposed tissues with softening agent. Soak for 30 min–1 h (depending on specimen size), then resume sectioning, repeating as needed.

The mounting of sections on slides and staining follow regular procedures. We followed Walker's Sam quadruple staining protocol (Appendix 2) and we used Eukitt mounting medium (O. Kindler, ORSAtec GmbH, Freiburg, Germany) for mounting the coverslips.

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APPENDIX 2. SAM-WALKER QUADRUPLE STAINING PROTOCOL

The protocol was developed by Sterling Sam and perfected by Dennis K. Walker (both of Humboldt State University, currently California State Polytechnic University - Humboldt).

The steps of the protocol, with solutions and durations of immersion are as follows:

1. 100% xylene; 4 minutes.
2. 100% xylene; 4 minutes or until all wax is removed.
3. 1:1 100% xylene:100% ethanol; 4 minutes.
4. 100% ethanol; 3 minutes.
5. 95% ethanol; 3 minutes.
6. 70% ethanol; 3 minutes.
7. 50% ethanol; 3 minutes.
8. Weigert's Iron Hematoxylin (see notes below); 60 seconds.
9. Tap water; dip repeatedly and replace the water, until slides are clean of all dye.
10. 35% ethanol; 2 minutes.
11. Bismark Brown (see notes below); 6–15 hours.
12. 70% ethanol; dip repeatedly and replace the solution until slides are clean of all dye.
13. Phloxine (see notes below); 20 minutes.
14. 100% ethanol; 2 minutes.
15. 100% ethanol; dip repeatedly until slides are clean of all dye.
16. Fast Green-Orange G (see notes below); 5 minutes.
17. 1:1 100% ethanol: 100% xylene; dip repeatedly to remove most of the dye from the slides.
18. 1:1 100% ethanol: 100% xylene; dip repeatedly until slides are clean of all dye.
19. 100% xylene; 4 minutes.
20. 100% xylene; 4 minutes.
21. Mount coverslips with xylene-soluble adhesive of choice (*e.g.*, Eukitt; O. Kindler, ORSAtec GmbH, Bobingen, Germany) right out of the 100% xylene solution.

Results

Chromatin and nucleoli can stain black to purple with Weigert's Iron Hematoxylin and pink to red with Phloxine. Cuticle and lignified cell walls such as those of xylem tracheary elements should stain brown with Bismark Brown. Unlignified cell walls will stain green with Fast Green. Tannins may stain brown with Bismark Brown and green with Fast Green. Cytoplasm, chloroplasts, spindle apparatus, and some cell walls may stain pink with Phloxine. Thick cell walls may stain gray to blue to green with Hematoxylin and Fast Green if they are unlignified.

Notes

Weigert's Iron Hematoxylin. Dissolve 2 g hematoxylin in 20 ml 100% ethanol and dilute to 200 ml with water.

Weigert's Iron Hematoxylin loses potency over time and should not be used after it has aged for one week. Judge the optimum staining duration by the staining of nuclei. The final appearance will be determined partly by the staining time in Phloxine at step 13.

Bismark Brown. Dissolve 2 g Bismark Brown Y in 200 ml 50% ethanol.

Bismark Brown staining must be long enough to color the xylem cell walls, but not heavy enough to interfere with Phloxine staining of epidermis or Fast Green staining of paenchyma.

Phloxine. Dissolve 2 g Phloxin B in 200 ml 95% ethanol.

Phloxine staining should be strong enough to be visible in the cell walls of the epidermis and thin-walled tracheids. Long staining times in Phloxine will overpower the Hematoxylin and produce dark red nuclei. Staining times that are short enough to leave the nuclei stained black to purple by Hematoxylin may under-stain the epidermis and thin-walled tracheids with Phloxine. The best differential nuclear staining consists of Hematoxylin-stained chromatin and Phloxine-stained nucleoli.

Fast Green-Orange G. Mix equal parts of Fast Green and Orange G saturated in clove oil, prepared as follows:

-Fast Green: 1.5 g Fast Green FCF + 600 ml clove oil + 300 ml 100% ethanol; filter before use.

-Orange G: 2 g Orange G + 150 ml clove oil + 150 ml methyl cellosolve + 150 ml 100% ethanol.

Fast Green-Orange G staining does not seem to improve with longer times, nor with additional Orange G staining.