METHODS AND TECHNIQUES

A comparison of paraffin and resin-based techniques used in bark anatomy

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Abstract Bark anatomy is an unappreciated discipline in plant systematics, despite its great potential to reveal systematically informative features. In this paper, main reasons for the lack of detailed bark anatomical data in many plant families are identified, including problems with sectioning, terminological issues, and difficulties in observation of dilated stems. We deal with these problems by focusing on two aspects: (1) compare, discuss and improve existing sectioning and maceration techniques using two species with soft and hard bark tissues; and (2) discuss the best way to collect stem bark samples. We hope that this paper will stimulate inclusion of bark anatomical data in future systematic studies.

Keywords bark anatomy; light microscopy; macerations; plant systematics; phloem; sectioning

Supplementary Material Score sheet and a filled-out example of *Davidia involucrata* are available in the Electronic Supplement to the online version of this article (http://www.ingentaconnect.com/content/iapt/tax).

■ INTRODUCTION

Although bark is one of the most conspicuous features of woody plants in the field, and despite several studies having shown its potential for improving phylogenetic relationships in many plant families (e.g., Metcalfe & Chalk, 1950a, b; Zahur, 1959; Esau, 1969; Richter, 1981; Roth, 1981; Esau & Cheadle, 1984; Archer & Van Wyk, 1993; Liu & Gao, 1993; Costa & al., 1997; Carlquist, 1991, 1996, 1998, 2005; De Magistris & Castro, 2001; Castro & al., 2005; Olson, 2005; Schweingruber, 2006; Junikka & Koek-Noorman, 2007; Oskolski & al., 2007, 2010), bark is still one of the most poorly known plant tissues and therefore rarely included in systematic studies. There are three major reasons for the fragmentary knowledge of bark anatomical data. Firstly, bark often has a combination of soft parenchymatous tissues and very thick-walled cells, making thin (10–15 µm) high-quality sections problematic and causing the sections to be easily damaged during the treatment procedure afterwards. Different types of embedding techniques have been used to overcome this problem (e.g., celloidin, Johansen, 1940; polyethyleneglycol (PEG), Richter, 1981; paraffin, Carlquist, 1982; freezing microtome sections, cf. Ruzin, 1999; resin, Melzer & al., 2008; polystyrene foam, Barbosa & al., 2010), but obtaining good bark sections often remains difficult. Secondly, many bark anatomical papers were published before the standardized-terminology paper of Trockenbrodt (1990), and consequently they lack a unified terminological framework, which can lead to interpretation issues and makes comparisons difficult (see also Esau, 1969; Trockenbrodt, 1990). Despite useful additions to Trockenbrodt's paper by Ley-Yadun (1991) and Junikka (1994), a standardized explanatory list similar to the "IAWA List of Microscopic Features for Hardwood Identification" (IAWA Committee, 1989) has never been published. Thirdly, many bark anatomical studies lack crucial information on potentially informative characters, such as the position of the initiation of the periderm. Furthermore, information on how bark tissue changes over time is commonly missing: dilatation events, development of (fiber)sclereids, and rhytidome formation can cause dramatic changes to the original position of particular cells in the bark, which again can lead to incorrect interpretations.

This article was written because we encountered all of the problems mentioned above during the first author's M.Sc. thesis work (Hamann, 2009). However, by adding minor but crucial adjustments to the ethylenediamine protocol of Carlquist (1982) using species having soft as well as hard tissues in their bark, we managed to obtain better bark anatomical sections as compared to the original Carlquist method. In this paper we discuss how to (1) collect stems prior to sectioning, and (2) facilitate the making of high-quality thin bark sections for light microscopy using paraffin and resin embedding. We hope that this article will stimulate future bark anatomical research.

■ MATERIALS AND METHODS

How to collect bark specimens. — Generally, there are three possible sources of suitable specimens, in order of preference: (1) fresh material collected in the field, (2) existing fixed collections in alcohol or FAA, and (3) dried wood collections present in xylaria and herbaria.

(1) When many species need to be collected, fieldwork can be time-consuming and expensive, but self-collecting has major advantages over existing collections made by others. The most important one is being able to carefully select suitable plants with sufficiently mature stems, which are free of diseases and/or physical damage. Furthermore, it is possible to sample stems of different diameters of the same plant, including shoot tips and young twigs, allowing observation of the ontogenetical variation of the various bark tissues due to dilatation and other development processes (e.g., position of phellogen initiation). A suitable saw (e.g., Gardena folding saw, Gardena, Ulm, Germany) can be used to carefully remove stem material from a living plant. It is also possible to use core increments to obtain samples from thick stems, but there is a risk of damaging the bark tissues when inserting and removing the cutting tube, or when removing the sample from the cutting tube (Forster & al., 2000; Rossi & al., 2006; Boura & De Franceschi, 2008). If desired, tissues can be put immediately after collection in an appropriate fixative, such as Craff III, IV, or V for small and relatively soft samples and FAA for larger and harder samples (Sass, 1968), but this will not affect the quality of the sections. Evidently, each collected specimen should be accompanied by a label including the exact collection location and detailed ecological information. Photographs of the bark morphology in the field may be helpful too. Using this strategy, it is possible to discuss the impact of ecological parameters on bark structure, which remains an unexplored field in bark anatomy (compare vestured pits in xylem; Jansen & al., 2004). Botanical gardens and greenhouses can be a viable alternative for collecting in the wild, but cultivars and unusually fast growing plants in greenhouses should be avoided (Lens & al., 2008).

(2) Most stem material in alcohol collections only includes young twigs. However, when mature material is present, it is almost as suitable for bark anatomical studies as self-collected material. Depending on the age, material from alcohol collections faces a few issues, such as the hardening effect of alcohol, which may cause difficulties during sectioning, and dissolving of some cellular contents, especially pigments and lipids. Properly fixating material before storage in 70% alcohol may prevent some of these issues.

(3) In herbarium specimens and a considerable number of wood specimens stored in xylaria, the bark is still attached. Although hundreds of these samples can be easily collected in a short time, most of them will not be usable for high-quality bark anatomical observations due to drying and degradation of the soft tissues. This is especially the case in specimens collected more than ten years ago. Consequently, the fragile, non-lignified cell types collapse (e.g., sieve elements) and the bark may detach from the wood, resulting in destruction of the vascular cambium and adjacent cells. Moreover, the resulting sections may degrade further during the treatment process. An additional problem with herbarium samples is that the vast majority consists of juvenile branches with poorly developed bark. In general, mature, dried wood specimens not older than ten years that still have the bark firmly attached provide satisfactory bark sections.

Preparation for light microscopy. — Several steps are required to prepare slides suitable for detailed bark observations. We have used Carlquist's (1982) ethylenediamine softening and paraffin method, suggest some modifications for improvement, and compare it with a commonly used LR White hard grade resin-based technique (London Resin Company, London, U.K.; Tables 1–2; Figs. 2–3). Other resins like Epon (Hexion, Rotterdam, The Netherlands) or Technovit (Heraeus-Kulzer, Wehrheim, Germany) also work, but our experience has learned that LR White impregnates the tissues better.

Suggestions for refining the embedding process using Carlquist's (1982) protocol. — Carlquist's (1982) method consists of softening small bark tissue samples in 4% ethylenediamine for three to four days, followed by rinsing in water, dehydration using alcohol and Johansen's tertiary butanol series (Johansen, 1940), and embedding in high-grade paraffin with a 59°C–61°C melting point. Because we could not obtain paraffin with this melting point, we used Peel-a-wax paraffin (Electron Microscopy Sciences, Hatfield, Pennsylvania, U.S.A.) with a 62°C–64°C melting point (paraffin with a melting point lower than 60°C should not be used). Suggestions for improvement are detailed in bold in Table 1. Although Carlquist (1982) recommends that the tissue samples should not be larger than $5 \times 5 \times 1$ mm, we have successfully embedded and sectioned tissue samples of $8 \times 8 \times 1$ mm and also succulent stem segments up to $35 \times 10 \times 5$ mm. Generally, less-lignified samples can be larger than heavily lignified material, but they should never be larger than necessary to observe all informative characters. The different rinsing steps in water, alcohol, and tertiary butanol are notably improved during the embedding process by using a slowly moving orbital shaker (Ika-Vibrax-VXR, Janke & Kunkel, Staufen, Germany) to keep the fluids moving. The paraffin infiltration is further improved by extending the time each rinsing step takes and by always exsiccating the paraffin before use. When embedding a tissue sample for tangential sections, it is placed in the mold with the wood part facing downwards to ensure no bark material is lost during sectioning. We have found that single-use peel-away plastic ice cube molds are perfect for molding small paraffin blocks. For larger blocks, molds may be made out of paper or aluminum foil (Echols, 1955; Sass, 1968). The embedded paraffin samples are first hardened at room temperature (20°C) for one to several hours depending on size. Use of a cold plate (Adamas Instrumenten, Rhenen, The Netherlands) is suggested to accelerate the hardening. Then samples are transferred to a refrigerator and hardened further at low temperature (0°C–5°C) for several days (Sass, 1968).

Sectioning, staining and mounting of paraffin embedded tissues. — Procedures for sectioning, slide preparation, staining, and mounting are detailed in Table 2. Before sectioning starts, the paraffin blocks are trimmed into a trapezium shape to make them suitable for use in a rotary microtome. A few sections are cut to expose the surface, and the blocks are placed face down in a dish with 2–3 mm of water in a refrigerator overnight (Carlquist, 1982). Transverse, tangential, and radial sections 5–10 µm thick are cut from their respective paraffin blocks using a rotary microtome (Leitz, Wetzlar, Germany). From each of the transverse and radial tissue blocks only about a dozen

Table 1. Procedures for Carlquist's (1982) ethylenediamine method with our modifications in bold, and for LR White embedding.

	Carlquist's ethylenediamine method (our modifications in bold)	LR White
Preparation	cut bark tissue samples ("chips") of about 5 × 5 × 1 mm	cut small bark tissue samples, preferably smaller than 5 × 5 × 5 mm
	make 1 chip per plane in transverse, tangential, and radial plane-orientation	make 1 chip per plane in transverse, tangential, and radial plane-orientation
	include a small piece of wood attached to the vascular cambium	include a small piece of wood attached to the vascular cambium
	cut multiple samples to cover entire bark thickness if required	small-sized samples generally facilitate impregnation and are favored over larger samples (up to 10 × 10 × 10 mm for fragile nonlignified stems)
Softening	soften in 4% ethylenediamine for 3–4 days at room temp.	not required
	exsiccate samples	
	keep ethylenediamine in motion	
	rinse 2–3 times in pure water for 2 h	
Embedding	rinse in alcohol series: 5%–11%–18%–30%, 2 h/step	when dried or immersed in water-based fixative: rinse in alcohol series: 30%–50%–70%–96%, 2 h/rinsing step, last step 1 night
	rinse in 50 : 40 : 10 pure water : 96% alcohol : 100% tertiary butanol solution, 2 h	rinse in 100% alcohol for 1 h
	rinse in 30 : 50 : 20 solution, 1 night	all LR White steps in refrigerator and exsiccate at each resin step
	rinse in 15 : 50 : 35 solution, 1 h	rinse in 3 : 1 100% alcohol : 100% LR White for 8 h
	rinse in 45 : 55 96% alcohol : 100% tertiary butanol, 1 h	rinse in 2 : 1 solution, 1 night
	rinse in 25 : 75 100% alcohol : 100% tertiary butanol, 1 h	rinse in 1 : 1 solution, 8 h
	rinse twice in 100% tertiary butanol, 1 h/step, then once overnight	rinse in 1 : 2 solution, 1 night
	rinse in 2 : 1 100% tertiary butanol : paraffin (59°C–61°C) solution, 2 h	rinse in 1 : 3 solution, 8 h
	rinse in 1 : 1 solution, 2 h	rinse in 100% LR White for 1 night
	rinse in 1 : 2 solution, 2 h	embed tissue in 100% LR White in capsule (ensuring that tissue sample is properly oriented), add sample and label in capsule (preferably smaller than 1 cm in diameter), and close capsule
	rinse twice in pure paraffin for 1 h, exsiccate every time, then rinse in pure paraffin overnight	put slide holder with closed capsules on top in oven and harden at 60°C for 48 h
	extend time of rinsing steps in pure paraffin to 1–2 days for better penetration in tissues	
	embed in pure paraffin in mold	
	embed tangential tissue samples so that wood part will be sectioned first	
	use cold plate when pouring paraffin blocks for rapid cooling	
	first harden paraffin blocks at room temp. for 1 to several h, then transfer to refrigerator and harden for at least 4–5 days, keep in refrigerator until sectioning	
	always use paraffin with a melting point of 60°C or more	
	keep fluids in motion as often as possible	
	always exsiccate paraffin before use	
	always pour paraffin carefully to avoid air bubbles	
	large samples: extend time for rinsing steps in water : alcohol : tertiary butanol series to 3–4 hours or longer; also extend time for rinsing steps with pure paraffin to 2–4 days/step	

Table 2. Procedures for sectioning, mounting and staining for paraffin embedding and LR White hard-grade embedding.

	Paraffin embedding	LR White hard-grade embedding
Sectioning and slide preparation	trim paraffin blocks into trapezium shape for sectioning	dissolve 2 g of gelatin in 500 ml of water at 50°C, then add and dissolve 0.2 g chromium(III) potassium sulfate
	knife inclination: 1°	dip slides in cooled chrome alum-gelatin adhesive, then dry for 1 night at 32°C
	use extra-hard disposable microtome knives	use tungsten carbide D-knife, knife inclination: 5° (glass knives also work)
	make several sections until the surface of the bark sample is exposed, then place the paraffin block surface down in a dish with 1–2 mm of pure water, soak overnight in refrigerator	trim block until desired region of tissue is reached
	cut sections 5–10 µm thick	cut sections 4–8 µm thick
	place sections in storage tray in same order as they were cut	put 40% aceton to the slides, add sections and place on a heating element at 60°C
	place marked slides on a heating element at 40°C and cover them with Riopel's adhesive followed by a thin layer of pure water	heat fix sections to slides for 1 h at 60°C–70°C
	place sections on slides in same order as they were cut	
	heat fix sections to slides at 40°C for at least 1 h	
Staining and mounting	**Safranin/alcian blue**	**Toluidine blue**
	rinse slides with sections three times in xylene, 5 min	stain sections on slides in 1% toluidine blue for 20 s
	rinse in alcohol series: 100%–96%–70%–50%–30%, 2 min/step	rinse slides once or twice in pure water for 2 min
	rinse in pure water, 2 min	dry slides at room temp.
	stain in alcian blue/safranin (2 : 1 1% alcian blue in water : 1% safranin in ethanol 50%), 20 min	mount sections in Depex mounting medium and add cover slip
	rinse twice in pure water, 2 min	dry slides for 4–7 days at room temp.
	rinse in alcohol series: 30%–50%–70%–96%–100%, 2 min/step	
	rinse three times in xylene, 5 min/step	
	mount sections in Depex resin, apply coverslip	
	dry slides for 4–7 days at room temp.	
	Phloem stain according to Cheadle & al. (1953)	
	rinse slides with sections three times in xylene, 5 min	
	rinse in alcohol series: 100%–96%–70%–50%–30%, 2 min/step	
	rinse in pure water, 2 min	
	stain in 1% tannic acid, 5–10 min	
	rinse three times in pure water, 2 min	
	stain in 1%–2% iron chloride, 5 min	
	rinse three times in pure water, 2 min	
	check if sufficiently stained	
	rinse in 1% $NaHCO_3$ in 25% alcohol, at least 30 min	
	stain in saturated solution of lacmoid in 30% alcohol with a few milliliter of the 1% $NaHCO_3$ solution added for 18 h or longer, keep lacmoid solution in motion while staining	
	rinse in 1% $NaHCO_3$ in 50% alcohol, 20 s	
	rinse in alcohol series: 70%–96%–100%, 2 min/step	
	rinse three times in xylene, 5 min	
	mount sections in Depex resin, apply coverslip	
	dry slides for 4–7 days at room temp.	

sections need to be cut. However, serial sections covering the entire bark thickness should be cut from the tangential block. Extra-hard Feather N35 disposable microtome knives (Feather, Osaka, Japan) are used, with a knife inclination of 1°. The slides are covered with a thin layer of Riopel's adhesive (Riopel, 1962), followed by a thin layer of water on which the sections float. It is important to use as few Riopel's adhesive as possible on the slides to minimize staining of the adhesive later onwards. If a strong staining of the Riopel adhesive still occurs, it is also possible to use the subbed slides used for LR White sections, but the sections are much more likely to fall off the slides during the staining process. The tangential sections on each slide should be arranged in the same order as they were sectioned; subsequent (strips of) sections are placed on numbered slides. Staining of all sections occurs using two different stains: (1) safranin/alcian blue stain which is a standard double stain for wood (Lens & al., 2005, 2007; even numbered slides) and (2) the phloem stain (Cheadle & al., 1953; odd numbered slides). Other stains that can be used are acridin red-chrysoidin (Junikka & Koek-Noorman, 2007) or cresyl-violet (Keating, 1996). To improve the staining of callose by the lacmoid solution, the containers are kept in motion during the entire 18 hour period by placing them on a slowly moving orbital shaker. Finally, the sections are mounted in Depex mounting medium (VWR International, Amsterdam, The Netherlands). The excess Depex mounting medium can be removed from the slides using a razor blade after 4–7 days of drying in a fume hood. Then a voucher label listing species name, collector name and number, collection location, type of section, and slide number is glued to each slide using bookbinder glue.

LR White method. — The procedure for embedding, sectioning, staining and mounting tissues embedded in LR White resin is detailed in Tables 1 and 2. Properly orienting the tissue samples in the capsules is crucial, as corrections of obliquely orientated samples during sectioning are limited. Before sectioning, LR White blocks can be trimmed in a trapezium shape using a trimming device (Dremel Europe, Breda, The Netherlands) to remove superfluous LR White (dust is carcinogenic—wear respiratory protection). To improve the adhesion of the sections, the slides are subbed with chrome alum-gelatin adhesive (Appelhans, pers. comm.; based on Pappas, 1971) and left to dry at 60°C for 1 night before use. All sections were cut using a Microm HM360 automatic rotary microtome (Thermo Fisher Scientific, Walldorf, Germany), using a tungsten carbide D-knife, with a knife angle of 5° and a section thickness of 4–8 µm. The sections can be stained with toluidine blue (1 g sodium tetraborat (Borax) and 1 g toluidine in 100 ml pure water) (Mercer, 1963; Burns, 1978) or safranin (but not with alcian blue or lacmoid, which cannot penetrate LR White resin).

Bark samples and techniques used. — Fresh bark material of *Impatiens niamniamensis* Gilg (Ericales, Balsaminaceae) was obtained from the living collections of the National Botanic Garden of Belgium (accession number 19770093), while fresh bark material of *Davidia involucrata* Baill. (Cornales, Nyssaceae) was retrieved from the living collections of the Hortus Botanicus Leiden (accession number A95059). The two species were selected based on their bark structure: *I. niamniamensis* is characterized by a soft, semisucculent stem without thick-walled sclerified cells, while the bark of *D. involucrata* contains large zones of very thick-walled sclereids. To show the difference between dried and fresh material, some samples of both species were dried at 60°C for five days in an oven. Fresh and dried material of both species was treated using both Carlquist's (1982) ethylenediamine protocol and our adjusted method, and according to the LR White hard-grade technique. Sections were cut, stained, and mounted using the methods described above, and pictures were taken with a Colorview IIIu digital camera connected to a PC running Cell^D imaging software, attached to a Olympus BX-51 microscope (Olympus Nederland B.V., Zoeterwoude, The Netherlands).

To illustrate the advantages of each stain, fresh material of *Rhododendron ponticum* (Ericales, Ericaceae), a species that has very narrow sieve elements with very small sieve plates, was obtained from the Bos van Bosman park in Leiden and embedded using our improved paraffin method.

Macerations. — Fresh material of *Davidia involucrata* from the Hortus Botanicus Leiden (accession number A95059) was compared with 40-year-old dried material of the same species obtained from the NHN-L xylarium (Basel, Bot. Gart. 130/H, Lw 0708156). Fresh and dried bark slivers of *D. involucrata* were macerated using different protocols: (1) Jeffrey's maceration fluid (cf. Johansen, 1940; Table 3), (2) Franklin's

Table 3. Summary of maceration procedure according to Jeffrey's method (Johansen, 1940) with our improvements in bold.

Preparation	divide bark into slivers thinner than 300 µm
	put slivers in numbered vials filled with pure water **and exsiccate to remove all air**
Maceration and rinsing	macerate slivers in Jeffrey's maceration fluid at room temp., 24 h
	centrifuge material for 3–5 min, rinse with pure water by centrifuging material for 3–5 min to remove all traces of maceration fluid (repeat until water is clear)
Staining and mounting	**stain with astra blue (0.5 g astra blue and 2 g tartaric acid in 100 ml of pure water) at room temp., 1 night**
	put a few drops of glycerin-gelatin on a slide on a heating element, then place some macerated tissue on slide
	carefully tease tissue apart with dissection needles and swirl around until evenly spread on slide
	place cover slip
	let glycerin-gelatin solidify at room temp.

wood maceration fluid (Franklin, 1945), (3) Gifford's fragile tissue maceration fluid (cf. Ruzin, 1999), (4) Mahlberg's parenchyma and collenchyma maceration fluid (cf. Ruzin, 1999), and (5) Schmid's improved Jeffrey's method (Schmid, 1982). For each rinsing step the material was centrifuged at 4000 rpm for three to five minutes in pure water to avoid losing any macerated tissue and to speed up the process. Pipettes were used instead of decanting to further avoid loss of macerated material. Different staining methods were tried in combination with Jeffrey's original method: (1) astra blue in water (cf. Table 3), (2) 1% safranin O in water and destaining in water (cf. Johansen, 1940), and (3) 1% safranin O in 50% alcohol preceded by dehydration (cf. Johansen, 1940). When making slides, the macerated tissue is carefully pulled apart using dissecting needles, and then swirled through the glycerin-gelatin, after which a cover slip is applied.

Observations. — To standardize our descriptions, we created a score sheet listing the characters as defined by Trockenbrodt (1990) and Junikka (1994). At the top of the score sheet, the observer writes down the specimen information, including stem diameter and number of growth rings, and voucher data. This is followed by a list of measurements of main bark anatomical tissues. Our score sheet, as well as a filled-out example of *Davidia involucrata*, is available in the Electronic Supplement on the *Taxon* website. A standardized description format is dependent on the group of study, however, and should not be used at a high taxonomic level. Consequently, observations that do not fit any of the pre-defined categories are written down separately on the score sheet, and categories may be marked as 'not applicable' if required.

■ RESULTS AND DISCUSSION

Stains. — Safranin stains lignified cell walls red, as well as the cells' nucleus, while alcian blue colors cellulose in the non-lignified cell walls blue and also stains the cells' cytoplasmic details (Fig. 1A). Due to this specific double staining the various cell types and cellular features are simple to recognize. However, sieve plates and lateral-wall sieve areas are very hard to distinguish in this species with very narrow sieve elements (arrows in Fig. 1A). The phloem stain by Cheadle & al. (1953) colors cellulose walls, cytoplasm, P-proteins, and nuclei light brown to grayish brown, while callose and lignified cellulose are clear blue to greenish-blue (Fig. 1B). So, using the Cheadle & al. stain, sieve plates and lateral-wall sieve areas are more easily recognizable due to their distinctive blue color, but other cellular features are more difficult to observe than with the safranin/alcian blue stain. The two stains used are complimentary, and allow better observation of cellular features and contents than using only one type of stain.

Embedding. — In this paper, we suggest several adjustments to Carlquist's (1982) protocol for paraffin embedding that improve the section quality of bark using model species with (1) soft bark cells without sclerified cell walls (*Impatiens niamniamensis*; Fig. 2) and (2) a heterogenous combination of soft and hard bark tissues (*Davidia involucrata*; Fig. 3). The most important adjustments are to (1) keep the fluids in motion during embedding and staining, (2) exsiccate the paraffin during embedding, (3) extend rinsing times for better penetration of the tissues, (4) use a slide adhesive that minimizes the risk of loosing sections after mounting, and (5) use extra-hard disposable microtome knives during sectioning. It is evident that fresh samples (Figs. 2B, D, F, 3B, D, F) yield much better sections than dried samples (Figs. 2A, C, E, 3A, C, E) when using the same technique. In dried *Impatiens* samples the secondary phloem cells are usually crushed, and the dilatated cortex cells often damaged, while in *Davidia* the axial and ray parenchyma cells, and to a lesser degree the sieve elements often collapse. When dried samples are investigated, bark tissues embedded in paraffin (Fig. 2A, C, 3A, C) generate much better quality sections than LR White-embedded tissue (Figs. 2E, 3E). Probably, this not only relates to the rehydration of the paraffin blocks

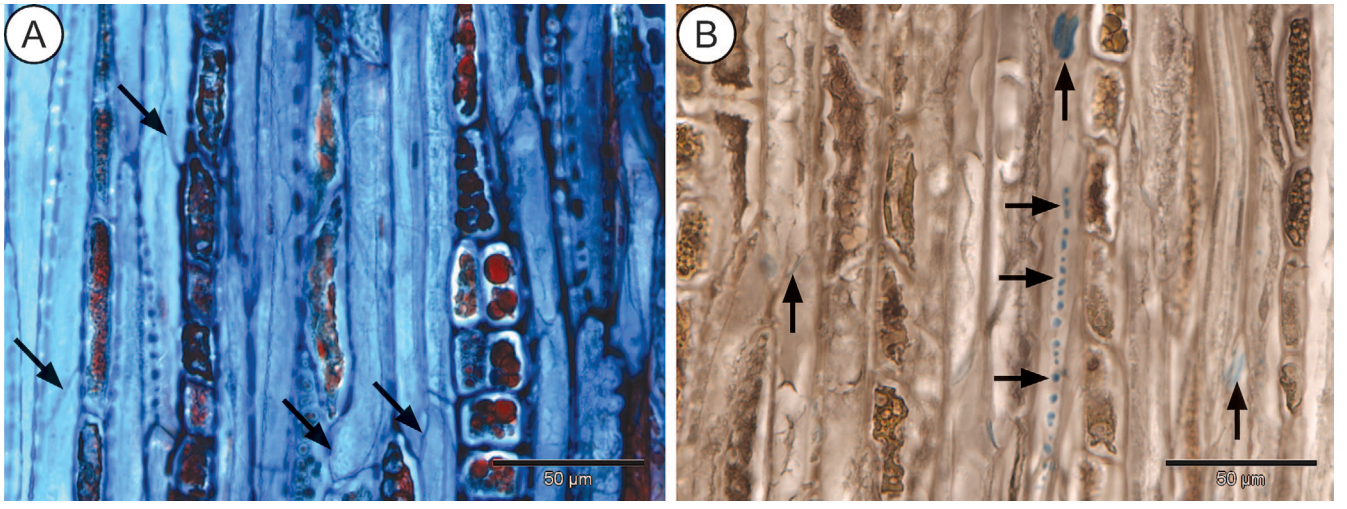

Fig. 1. Light microscope pictures of tangential bark sections of *Rhododendron ponticum*. **A,** Safranin/alcian blue stain; sieve plates and lateral sieve areas are difficult to distinguish (arrows). **B,** Stain according to Cheadle & al. (1953) colors sieve plates (vertical arrows) and lateral sieve areas (horizontal arrows) blue.

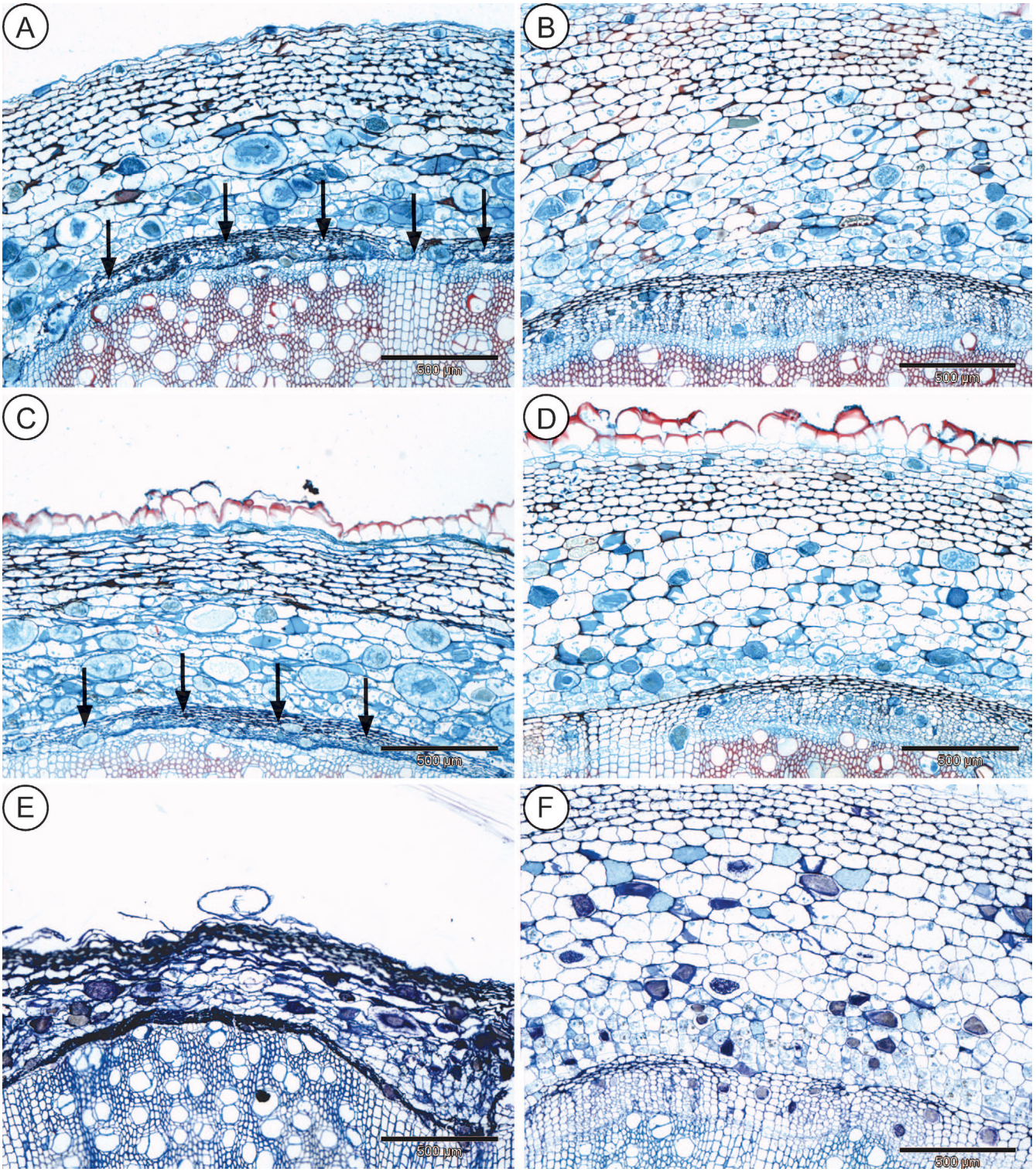

Fig. 2. Light microscope pictures of transverse bark sections of a species with soft bark (*Impatiens niamniamensis*), using Carlquist's original paraffin method **(A–B)**, our adjusted method **(C–D)** and LR White embedding **(E–F)**. **A, C, E,** Dried stems; **B, D, F,** fresh stems. **A,** Secondary phloem collapsed (arrows); **B,** secondary phloem intact, some damaged cell walls in the collenchyma and parenchyma; **C,** secondary phloem collapsed (arrows); **D,** secondary phloem and remaining bark tissues intact; **E,** severe cell wall collapse in entire bark; **F,** secondary phloem and remaining bark tissues intact.

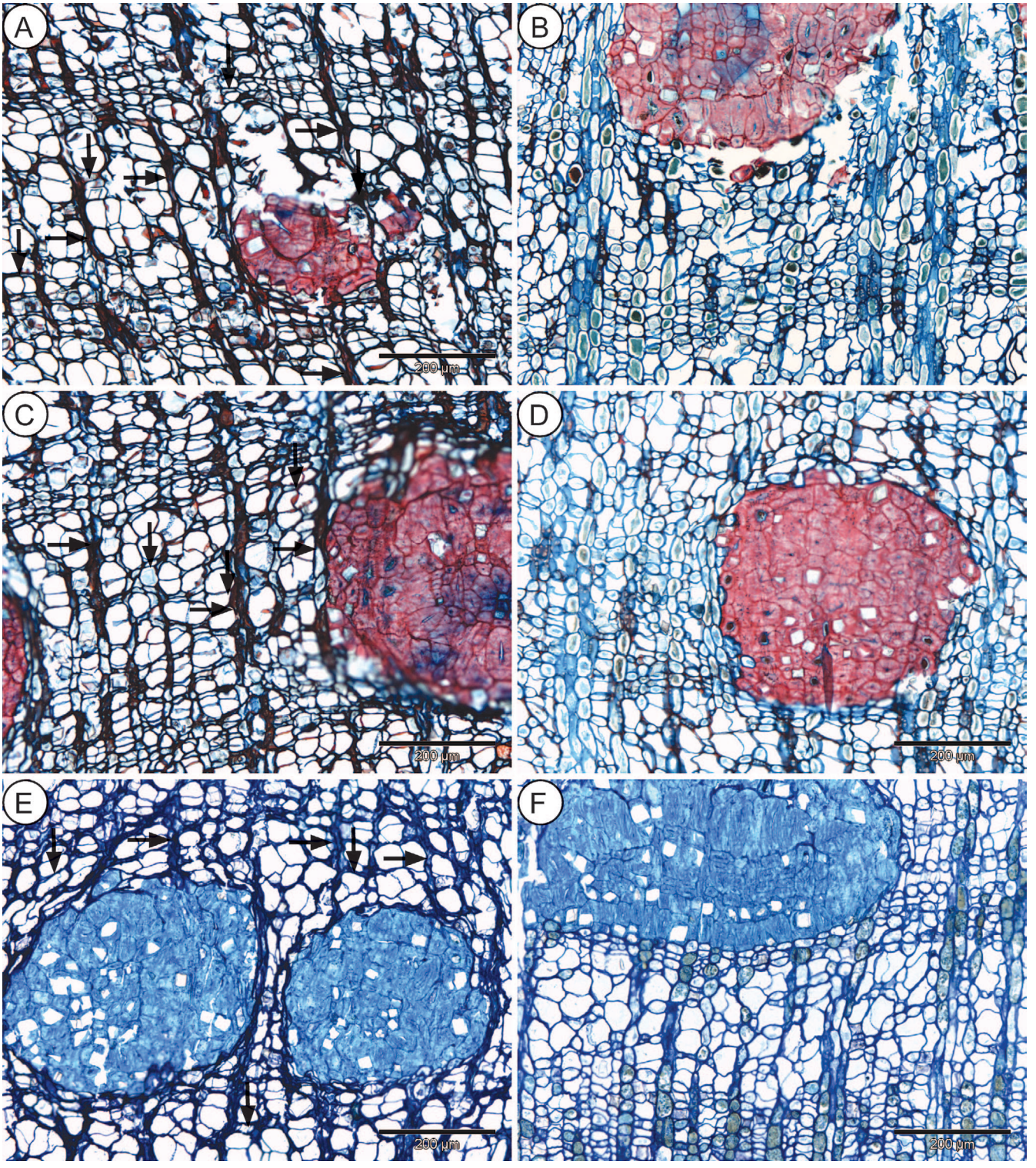

Fig. 3. Light microscope pictures of transverse bark sections of a species with hard bark (*Davidia involucrata*), using Carlquist's original paraffin method **(A–B)**, our adjusted method **(C–D)** and LR White embedding **(E–F)**. **A, C, E,** Dried stems; **B, D, F,** fresh stems. **A,** Walls of thin-walled secondary phloem cells are often crushed and torn, especially in the regions near the thick-walled sclereids, collapsed parenchyma cells indicated by arrows (horizontal arrows: ray parenchyma, vertical arrows: axial parenchyma); **B,** walls of thin-walled secondary phloem cells are frequently crushed and torn in the regions near the thick-walled sclereids; **C,** walls of secondary phloem cells sometimes crushed, collapsed parenchyma cells indicated by arrows (horizontal arrows: ray parenchyma, vertical arrows: axial parenchyma); **D,** secondary phloem intact, rarely crushed; **E,** walls of secondary phloem cells sometimes crushed, collapsed parenchyma cells indicated by arrows (horizontal arrows: ray parenchyma, vertical arrows: axial parenchyma); **F,** secondary phloem intact.

just before sectioning, but also to the longer dehydration and embedding procedures of the paraffin methods. Furthermore, when fresh samples are studied, our improved paraffin-based technique (Figs. 2D, 3D) increases the quality of bark anatomical sections in *Impatiens* and *Davidia,* compared to Carlquist's original method (Figs. 2B, 3B). Using our improved method, sectioning is noticeably easier and smoother, especially in species with a mixture of soft and hard bark cells, such as *Davidia*. With fresh material, the LR White protocol results in comparable (in case of soft bark tissues; Fig. 2F) or even slightly better sections (in case of harder bark tissues; Figs. 3F) than paraffin-based methods (Fig. 2B, D, 3B, D).

Although LR White embedding does not require softening, which leads to a significant reduction of the embedding process time compared to paraffin embedding (1 week vs. 2–3 weeks), and much thinner sections (up to 1–2 µm) are possible, we prefer our adjusted paraffin method to the LR White protocol, because the latter has several major drawbacks compared to the paraffin technique: (1) LR White is much harder and thus more difficult to trim and to section, resulting in smaller-sized samples that are not efficient when the entire radial diameter of mature bark needs to be sectioned; (2) correction of the orientation of the embedded LR White tissue is much more limited during sectioning; (3) serial tangential sectioning of bark is more complicated and much more time-consuming in resin-embedded stems as sections do not stick to each other and may curl strongly; and (4) staining possibilities in LR White are more limited because some specific bark stains cannot be used as they will stain the LR White resin or cannot penetrate the resin.

Bark macerations. — Jeffrey's method (cf. Johansen, 1940) (Fig. 4C, D) is the only maceration method that satisfactorily macerates bark tissue. No obvious difference was noticed between Schmid's improved Jeffrey's method (Schmid, 1982) and Jeffrey's original method. A maceration time of 24 hours is the absolute minimum for Jeffrey's method; tissue samples can be macerated longer if needed. Gifford's and Mahlberg's maceration methods (cf. Ruzin, 1999) are not aggressive enough to macerate the harder tissues, making it impossible to dissect the bark slivers. On the other hand, Franklin's method (Franklin, 1945) (Fig. 4B) is too aggressive, and dramatically damages the most fragile tissues of the phloem, such as sieve elements, while any periderm tissue present remains intact. Macerated tissues stained in 1% safranin in water have excellent contrast,

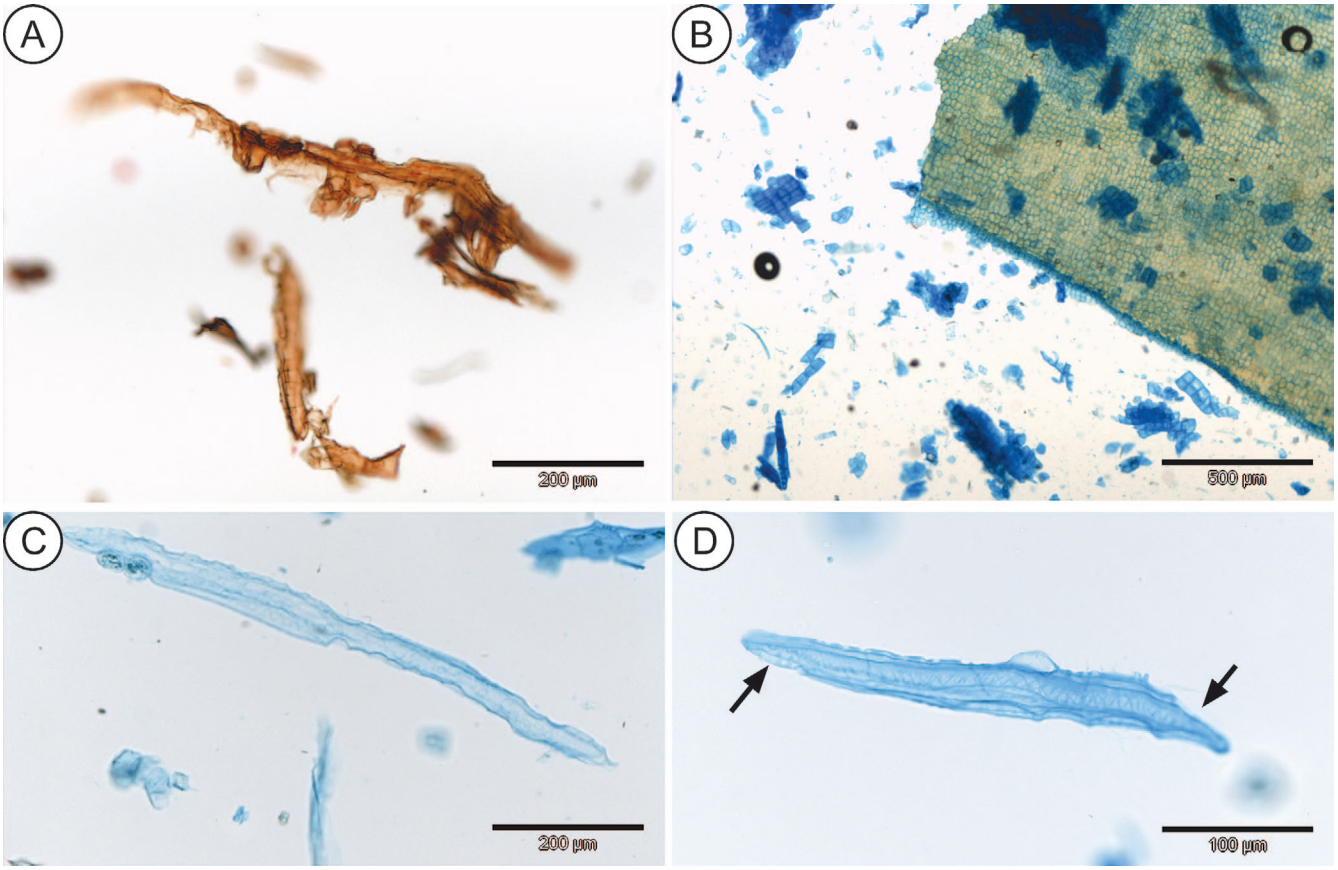

Fig. 4. Light microscope pictures of macerations of *Davidia involucrate*. **A–B,** Failed macerations: **A,** tissue macerated with Jeffrey's method and stained with safranin in alcohol; fragile tissues badly damaged due to dehydration and mounting process; **B,** tissue macerated in Franklin's maceration mix and stained with astra blue; periderm failed to macerate, sieve elements unrecognizable. **C–D,** Successful macerations using Jeffrey's method and astra blue stain: **C,** intact sieve element from dried sample; **D,** intact sieve element from fresh sample, with clearly visible sieve plates (arrows) and lateral wall sieve areas.

but require several dozen rinsing steps in water to prevent safranin residues from strongly staining the glycerin-gelatin. These rinsing steps will damage the macerated material. The dehydration steps required for staining in 1% safranin in 50% alcohol and the subsequent mounting in Depex severely damage the fragile bark tissues as well (Fig. 4A). Astra blue does not require damaging rinsing steps in water to remove excess stain and generally yields good results, and therefore is preferred. Surprisingly, it is possible to make satisfactory maceration slides using dried bark that is otherwise unsuitable for sectioning, although finding intact cells is somewhat difficult (Fig. 4C). However, the best macerations are those that use recently collected fresh material that has been preserved in alcohol or an appropriate fixative, which have many intact cells showing excellent details, including sieve plates and lateral sieve areas (Figs. 4D).

■ CONCLUSIONS

Using our relatively simple adjustments of Carlquist's ethylenediamine method it is possible to improve the quality of bark sections of dried and especially fresh stems collected in the field. We prefer our suggested paraffin protocol over LR White because of the possibility to make more and larger sections in a shorter time frame, and the advantage of having a wider range of applicable stains. Similarly, our modifications to Jeffrey's maceration method make it possible to obtain excellent bark macerations. Based on the bark anatomical literature, we believe that bark anatomy has a great potential as a valuable source of phylogenetically informative characters that should be included in systematic studies. We hope that the present paper will contribute to a revival of bark anatomical research in the systematic community.

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