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Bark of *Dirca* L.: Tensile properties, anatomy, and utility for handmade Asian-style bark paper

by

Zachary Hudson

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Co-Majors: Horticulture; Ecology and Evolutionary Biology

Program of Study Committee:

William Graves, Major Professor

Grant Arndt

Lynn Clark

Christopher Currey

Robert Wallace

Mark Widrlechner

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2019

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ABSTRACT

Dirca (Thymelaeaceae) is a genus comprising four species known as leatherwoods. All are characterized by strong fibrous bark that resists tearing perpendicular to its axis. Several Native American peoples are known to have used the bark of *Dirca palustris* for cordage, but the physical properties and anatomy of all four species has not been reported. I address these voids in the literature. Techniques were developed to determine the ultimate tensile strength and the modulus of elasticity of bark of all species of *Dirca* and of additional species from the Thymelaeaceae and other plant families. Ultimate tensile strength of *Dirca* spp. was similar to or greater than that of all other species evaluated. Modulus of elasticity of *Dirca* spp. was intermediate among species. Bark tissue softened in ethylenediamine, embedded in polyethylene glycol 1500, and supported during sectioning with a particular brand of tape led to sections of quality to allow observation of anatomical traits for analysis. Fiber diameter and length were similar among all species of *Dirca*. *Dirca mexicana* and *D. palustris* share non-lignified fibers. Sieve-tube elements, axial parenchyma, and phellem cells differed between *Dirca mexicana* and other species of *Dirca*, but cellular traits cannot be used to differentiate all species. Finally, I used the bark of cultivated plants of *D. mexicana* to create paper that was evaluated for its durability and potential for use as a medium for printmaking. Paper made from *D. mexicana* withstood bending, folding, and creasing better than did gampi paper made from the bark of species of *Wikstroemia* (Thymelaeaceae) native to Japan. The length of fibers of *D. mexicana* are 2.5 times longer than species of *Wikstroemia*. Printmakers found my paper to be a suitable medium for relief, intaglio, lithography, screen, and digital printmaking. Long non-lignified fibers contribute to the use of *Dirca* as cordage and paper. I conclude *D. mexicana* is a

North American source of fibers with properties similar to those of Japanese members of the Thymelaeaceae used to create specialty papers. Bark of *Dirca* should be collected only from cultivated plants due to the vulnerability of wild populations.

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

Introduction

The Thymelaeaceae is a cosmopolitan plant family especially prevalent in South Africa and Australia (Motsi 2009). Members of the family are characterized by their distinct stem anatomy. All but a few genera develop intraxylary phloem and fibers in young wood [primary xylem], and abundant fibers characterize the external bast [secondary phloem] (Solereeder 1908; Herber 2003). Breaking stems in the field can be used to identify members of the family, causing fibers to jut out conspicuously (Herber 2003), resembling a frayed rope. As some of the common names for the Thymelaeaceae (fiber-bark and rope-bark family) suggest, many of its species have been used to create clothing, cordage, decorations, and paper.

The only genus of the Thymelaeaceae native to the continental United States is *Dirca* L., which comprises four species (*D. palustris* L., *D. occidentalis* Gray, *D. mexicana* Nesom & Mayfield, and *D. decipiens* Floden) (Floden *et al.* 2009). Commonly known as leatherwoods, identification of these deciduous shrubs is aided by their occurrence in shaded understory niches, as well as their yellow flowers in winter and early spring, yellow autumnal foliage, and arborescent forms (Fig. 1).



Figure 1. Photographs of *Dirca L.* cultivated in Ames, IA. Left: April anthesis preceding leaf emergence; right: yellowing autumnal color; bottom: arborescent shape.

Like other members of the family, *Dirca spp.* have flexible wood and strong, fibrous bark that resists tearing perpendicular to its axis. The bark, defined as all tissues external to the vascular cambium, of *D. palustris* has been used for cordage by several Native American peoples (Moerman 1998). Bark of *D. occidentalis* is used by the dusky-footed wood rat (*Neotoma*

fuscipes Baird) to construct its nest (Vestal 1938). Paper made from the fibers of bark of *Edgeworthia chrysantha* Lindl., abaca (*Musa textilis* Née) pulp, and other fibers is used to create Japanese banknotes (National Printing Bureau n.d.). The bark of *Lagetta lagetto* (Sw.) Nash can be used to create cordage, and it can be teased apart with fingers to form a lace-like barkcloth that is used to create decorations and clothing in Jamaica (Pearman & Prendergast 2000).

Though it is a comparatively poorly studied plant tissue (Hamann *et al.* 2011), bark is a conspicuous visual feature of woody plants that contributes to a notable trait of identification for Thymelaeaceae. Functional quantitative traits of bark, such as fiber density, vary within clades, while qualitative traits, such as color of the inner bark, can characterize taxonomic groups (Rosell *et al.* 2014). Because the bark of species of Thymelaeaceae have been used to create items that require adequate strength depending on its use, and because the family has been characterized based on quantitative traits of its stem, such as intraxylary and abundant bast fibers (Solereeder 1908; Herber 2003), we sought to determine the tensile properties of the bark of representative taxa of the family.

Anatomy

Accrual of knowledge of comparative bark anatomy and bark's ecological functions lags behind that of closely associated wood tissue (Rosell *et al.* 2014; Angyalossy *et al.* 2016). Bark's heterogeneous composition is the main reason why bark has been neglected (Barbosa *et al.* 2010; Hamann *et al.* 2011; Angyalossy *et al.* 2016). Bark performs a myriad of functions, such as transport of photosynthates (Esau 1960), mechanical support (Niklas 1999), and protection from fire (Harmon 1984). Such functions are possible due to various cell types that range from soft,

non-lignified parenchyma to hard sclerified fibers. The mixture of hard and soft cell types makes sectioning of bark tissue difficult without tearing and crushing cell walls.

Various histological techniques have been developed to address the difficulty. Selected agents have been reported to soften lignified or hard tissues. Hydrofluoric acid demineralizes woody tissue (Langdon 1920) and ethylenediamine acts as a swelling agent which reduces the density of cell walls (Kukachka 1977). Materials such as polystyrene foam solution and adhesive tape (Barbosa *et al.* 2010), and cellulose tape (Bonga 1961) have been reported to act as an anti-tearing support when applied to tissue during sectioning. Despite reported use of agents and materials, researchers continue to be challenged by uneven thickness of sectioned tissue (Kukachka 1977) and separation of tissue and tearing of cell walls (Carlquist 1982).

Differentiation between species of *Dirca* has been based on foliar vestiture, perianth and reproductive morphology, pedicel elongation, and DNA sequencing (Vogelmann 1953; Floden *et al.* 2009). Leaf, stem, and root anatomy of *D. palustris* has been investigated extensively by Van Tieghem (1893), Holm (1921), Choquette (1925), Leandri (1930), and Metcalfe and Chalk (1950). Mature wood anatomy of *D. occidentalis* has been characterized with by McMinn and Foderhase (1935). No anatomical study of *D. mexicana* or *D. decipiens* has been performed. To describe for the first time the structure of bark of *D. occidentalis*, *D. mexicana*, and *D. decipiens*, histological methods were investigated to identify a technique that leads to slides of sectioned tissue of the quality required for anatomical analysis.

Asian-style bark paper

Washi is paper made by hand from the bark of shrubs native to Japan (Barrett 1983). The paper is valued for its strength, thinness, semi-transparency, and resistance to insects and aging

(Barrett 1983). These desirable properties are provided by bark fibers from the species of *Broussonetia* L'Her. ex Vent., *Edgeworthia* Meisn., and *Wikstroemia* Endl. native to Japan (Barrett 1983). *Washi* is a common medium used for printmaking, clothing, banknotes, and explosives (Barrett 1983). American artists who have studied *nagashi-zuki*, a sheet-forming method unique to *washi*, often import Japanese fibers used to make *washi* because alternative species native to the United States with similar properties have not been evaluated (Barrett 1983). *Edgeworthia*, *Wikstroemia*, and *Dirca* belong to the same plant family, Thymelaeaceae. I made paper from the bark of *D. mexicana* using the *nagashi-zuki* method because species of Thymelaeaceae have been used to make bark paper, and its ethnobotanical use implied strong bark tissue. I evaluated its physical properties and response to various printmaking techniques compared to paper made from the bark of species of *Edgeworthia* and *Wikstroemia*. I am not the first investigator to identify a plant for papermaking based on familial affiliation and ethnobotanical use. Mitnan (*Thymelaea hirsute* [L.] Endl.) was chosen as a local bark fiber for paper production in Beer Sheva, Israel because it is a member of the Thymelaeaceae, and the bark of the shrub is used by Bedouin people to make rope (Schmidt & Stavisky 1983). Although there is no documentation of *Dirca* spp. used by Native Americans to make handmade paper, artists and researchers have successfully made paper from the bark of *D. palustris*, but the paper was not evaluated for use (Barrett 1983; Bell 1995).

Dissertation Structure & Objectives

The Introduction and Literature Review is followed by four manuscripts in which I report the tensile and anatomical characteristics of the bark of species of *Dirca* to understand the underlying properties which have allowed the bark to be used to create cordage by several

Native American peoples (Moerman 1998), by a species of dusky-footed wood rat (*Neotoma fuscipes* Baird) to construct their nests (Vestal 1938), and as an Asian-styled fine art paper. Each chapter is formatted based on the intended journal for publication.

Chapter 2 describes my examination of the tensile properties of species of Thymelaeaceae, including all four species of *Dirca*. I sought to determine the tensile properties of the bark of representative taxa of the family by comparing one species representing each of 11 genera of the Thymelaeaceae, and six additional species of woody perennials, each from a different family. Although *D. palustris* is the only species of *Dirca* with documented use of bark by humans, the similar flexibility and tear responses observed led to a hypothesis that all species of *Dirca* share similar tensile properties. Moreover, because the history of uses of bark from many species of the family, and because abundant phloem fibers characterize the family (Solereder 1908; Herber 2003), I hypothesized that all the species of Thymelaeaceae studied would share tensile properties distinct from those of plants from other families.

Fresh, wax-based, and resin-based histological techniques used are described in Chapter 3. My goal was to identify a technique that would result in high-quality slides of sectioned bark of *D. mexicana*. All species of *Dirca* were expected to respond similarly to histological techniques because the bark of all four species of *Dirca* peels off in uniform, continuous strips, and all species were found to share similar ultimate tensile strength and modulus of elasticity of their bark.

In Chapter 4, I provide the first anatomical observation of bark of all species of *Dirca*. The histological techniques used allowed me to measure the diameter and length of fibers, sieve-tube elements, and axial parenchyma, and the tangential length of phellem.

In Chapter 5, the potential of Asian-style handmade *D. mexicana* bark paper was examined to be used as a printmaking medium. Artists who have studied *nagashi-zuki* often import Japanese fibers because alternatives with similar properties have not been evaluated. I conclude that *D. mexicana* could serve as a source of fibers with properties similar to those of plants used to make *washi*. Fold endurance tests and relief, intaglio, lithography, screen, and digital printmaking techniques were used to evaluate the paper.

In Chapter 6, I present general conclusions of my work, identify complementary studies to build on the results of my research, and ask for readers to not collected tissues of species of *Dirca* from wild populations.

In Appendix A, I have included collection permits obtained to legally and morally obtain samples of species examined. Personal information such as names and signatures have been redacted.

Anatomical data collected for all species of *Dirca* is presented as a table in Appendix B.

In Appendix C, origami artist James Lucas evaluated my handmade *D. mexicana* bark paper for use as an origami paper. Comments received from James Lucas are summarized and include a description of his origami Jacana.

Literature Cited

- Angyalossy V, Pace MR, Evert RF, Marcati CR, Oskolski AA, Terrazas T, Kotina E, Lens F, Mazzoni-Viveiros SC, Angeles G, Machado SR, Crivellaro A, Rao KS, Junikka L, Nikolaeva N, Baas P. 2016. IAWA list of microscopic bark features. IAWA Journal 37: 517--615.
- Barbosa ACF, Pace MR, Witovisk L, Angyalossy V. 2010. A new method to obtain good anatomical slides of heterogeneous plant parts. IAWA Journal 31: 373--383.
- Barrett T. 1983. Japanese papermaking: Traditions, tools, and techniques. New York: Weatherhill, Inc.

- Bell LA. 1995. Plant fibers for papermaking. McMinnville, Oregon: Liliaceae Press.
- Bonga JM. 1961. A method for sectioning plant material using cellulose tape. Canadian Journal of Botany 39: 72--730.
- Carlquist S. 1982. The use of ethylenediamine in softening hard plant structures for paraffin sectioning. Stain Technology 57: 311--317.
- Choquette L. 1925. Contribution a l'étude du *Dirca palustris* L. ou "bois de plomb." Ph.D. Thesis, Université de Paris.
- Esau K. 1960. Plant anatomy: Anatomy of seed plants (2nd ed.) New York: John Wiley & Sons.
- Floden AJ, Mayfield MH, Ferguson CJ. 2009. A new narrowly endemic species of *Dirca* (Thymelaeaceae) from Kansas and Arkansas, with a phylogenetic overview and taxonomic synopsis of the genus. Journal of the Botanical Research Institute of Texas 3: 485--499.
- Hamann T, Smets E, Lens F. 2011. A comparison of paraffin and resin-based techniques used in bark anatomy. Taxon 60: 841--851.
- Harmon ME. 1984. Survival of trees after low-intensity surface fires in Great Smoky Mountains National Park. Ecology 65: 796--802.
- Herber BE. 2003. Thymelaeaceae. In: K Kubitzki and C Bayer (eds.), The families and genera of vascular plants, Vol 5: Flowering plants dicotyledons Malvales, Capparales, and non-betalain caryophyllales: 373--396. Berlin: Springer-Verlag.
- Holm T. 1921. Internal structure of the vegetative organs of *Dirca palustris*. American Journal of Science, Fifth Series 2, 177--82.
- Kukachka BF. 1977. Sectioning refractory woods for anatomical studies. U.S.D.A Forest Service Research Note FPL-0236: 1--9.
- Langdon LM. 1920. Sectioning hard woody tissue. Botanical Gazette 70: 82--84.
- Leandri J. 1930. Recherches anatomiques sur la Thyméléacées. Annales des Sciences Naturelles 12:125--237.
- McMinn HE, Forderhase B. 1935. Notes on western leatherwood, *Dirca occidentalis* Gray. Madroño 3, 117--120.
- Metcalfe CR, Chalk L. 1950. Anatomy of the dicotyledons, Vol 2. Oxford: The Clarendon Press.
- Moerman DE. 1998. Native American ethnobotany. Portland, OR: Timber Press.
- Motsi MC. 2009. Origin and diversification of the Australasian genera *Pimelea* and *Thecanthes* (Thymelaeaceae). Ph.D. Dissertation, University of Johannesburg.

- National Printing Bureau. n.d. Characteristics of banknotes. Retrieved from <https://www.npb.go.jp/en/intro/tokutyou/index.html>
- Niklas KJ. 1999. The mechanical role of bark. American Journal of Botany 86: 465--469.
- Pearman G, Prendergast HDV. 2000. Items from the lacebark tree [*Lagetta lagetto* (W. Wring) Nash: Thymelaeaceae] from the Caribbean. Economic Botany 54: 4--6.
- Rosell JA, Gleason S, Méndez-Alonso R, Chang Y, Westoby M. 2014. Bark functional ecology: evidence for tradeoffs, functional coordination, and environment producing bark diversity. New Phytologist 201: 486--497.
- Schmidt J, Stavisky N. 1983. Uses of *Thymelaea hirsuta* (Mitnan) with emphasis on hand papermaking. Economic Botany 37: 310--321.
- Solereder H. 1908. Systematic anatomy of the dicotyledons, Vol. 2 Monochlamydeae. Oxford: Clarendon Press.
- Van Tieghem MP. 1893. Recherches sur la structure et les affinités des Thyméléacées et des Pénéacées. Annales des Sciences Naturelles 7: 185--294.
- Vestal EH. 1938. Biotic relations of the wood rat (*Neotoma fuscipes*) in the Berkeley Hills. Journal of Mammalogy 19: 1--36.
- Vogelmann H. 1953. A comparison of *Dirca palustris* and *Dirca occidentalis* (Thymelaeaceae). Asa Gray Bulletin 2: 77--82.

CHAPTER 2. TENSILE PROPERTIES OF BARK OF *DIRCA* L. (LEATHERWOOD) AND OTHER SPECIES OF THYMELAEACEAE

A paper to be submitted to International Association of Wood Anatomists Journal

Zachary J. Hudson, William R. Graves

Department of Horticulture, Iowa State University, Ames, Iowa, 50011, United States of America

Abstract

Abundant bast fibers in stems of many species in the Thymelaeaceae (often called the Daphne, Mezereum, fiber-bark, or rope-bark family) account for the historical use of their bark for clothing, cordage, decorations, and paper. We sought to assess the extent to which physical properties of bark are consistently distinct within the Thymelaeaceae by measuring the ultimate tensile strength and modulus of elasticity of bark of species from the Thymelaeaceae and other families chosen from local cultivated populations. Although our hypothesis that unusual tensile properties would characterize all genera of the Thymelaeaceae was not supported, all four species of *Dirca* L. had among the greatest ultimate tensile strength (51.8–107.7 MPa) and modulus of elasticity (1945.7–2859.8 MPa) of the taxa we tested.

Introduction

The Thymelaeaceae is a cosmopolitan plant family especially prevalent in South Africa and Australia (Motsi 2009). Members of the family are characterized by their distinct stem anatomy. All but a few genera develop intraxylary phloem and fibers in young wood, and abundant fibers characterize the external bast (Solereder 1908; Herber 2003). Breaking stems in the field can be used to identify members of the family, causing fibers to jut out conspicuously (Herber 2003), resembling a frayed rope.

As some of the common names for the Thymelaeaceae (fiber-bark and rope-bark family) suggest, many of its species have been used to create clothing, cordage, decorations, and paper. The species from which such products have been made represent multiple genera, including *Dais continifolia* L. (Koekemoer *et al.* 2014); *Daphne* L. spp. (Polunin & Stainton 1984; Paul *et al.* 2006); *Dirca palustris* L. (Dellinger 1936); *Edgeworthia* Meisn. and *Wikstroemia* Endl. spp. (Barrett 1983); *Eriosolena composita* (L. f.) Tieghem, *Gnidia linearis* (Leandri) Z. S. Rogers, and *Gyrinops walla* Gaertn. (Gambler 1902); *Lagetta lagetto* (Sw.) Nash (Pearman & Prendergast 2000), *Linodendron cubanum* (A. Rich) Griseb. (Record & Hess 1943); *Peddiea africana* Hook (Pooley 2006); *Pimelea villosa* Sm. (Crowe 1981); and *Thymelaea hirsuta* (L.) Endl. (Schmidt & Stavisky 1983).

The only genus of the Thymelaeaceae native to the continental United States is *Dirca* L., which comprises four species (*D. palustris* L., *D. occidentalis* Gray, *D. mexicana* Nesom & Mayfield, and *D. decipiens* Floden)(Floden *et al.* 2009). Commonly known as leatherwood, identification of these deciduous shrubs is aided by their occurrence in shaded understory niches, as well as their yellow flowers in winter and early spring, yellow autumnal foliage, and small-arborescent forms. Like other members of the family, *Dirca* spp. have flexible wood and strong fibrous bark that resists tearing perpendicular to its axis. The bark, defined as all tissues external to the vascular cambium, of *D. palustris* has been used for cordage by several Native American peoples (Moerman 1998). Baby cradles used archeologically were constructed with bark of *D. palustris* and were found during an archaeological dig at Montgomery Shelter 3, Ozark Bluffs, Arkansas (Dellinger 1936). A population of *D. decipiens* in Arkansas occurs within the range of *D. palustris*. Although the bark was considered to be *D. palustris* at the time, it may

actually have been *D. decipiens* (Floden *et al.* 2009). Bark of *D. occidentalis* is used by the dusky-footed wood rat (*Neotoma fuscipes* Baird) to construct its nest (Vestal 1938). Our work has been focused on *Dirca* because of its relative isolation from other species in its family, and because of observations that cordage and other practical products were made from *Dirca* (Dellinger 1936; Mottiar 2012). Although uses of bark from the Thymelaeaceae have been documented for centuries (Hughes 1978; Mottiar 2012), we were unable to find reports on the physical properties underlying their uses.

Though it is a comparatively poorly studied plant tissue (Hamann *et al.* 2011), bark is a conspicuous visual feature of woody plants that contributes to a notable trait of identification for Thymelaeaceae. Functional quantitative traits of bark, such as fiber density, vary within clades, while qualitative traits, such as color of the inner bark, can characterize taxonomic groups (Rosell *et al.* 2014). Because the Thymelaeaceae has been characterized based on stem traits (Solereder 1908; Herber 2003), we sought to determine the mechanics of the bark of representative taxa of the family. Our findings for the Thymelaeaceae were contextualized through comparable measures of bark from plants in other families. Tensile tests apply force to a material to determine its tensile properties, a subset of physical and mechanical properties. We measured the ultimate tensile strength (UTS), the maximum force applied to a material before it fails; and the modulus of elasticity (MOE), the resistance of a material to deformation, to quantify the mechanics of bark (Tensile Testing n.d.).

We compared all four species of *Dirca*, one species representing each of ten other genera of Thymelaeaceae, and six additional species each from a different family. Although *D. palustris* is the only *Dirca* species with documented use of bark by humans, the similar flexibility and tear

responses we observed led us to hypothesize that all species of *Dirca* share similar tensile properties. Moreover, because the history of uses of bark from many species of the family, and because abundant phloem fibers characterize the family (Solereeder 1908; Herber 2003), we hypothesized that all the species of Thymelaeaceae we studied would share tensile properties distinct from those of plants from other families.

Based on a report from Kohan *et al.* (2012) and Instron, a division of Illinois Tool Works, Inc. (Testing Solutions n.d.), there are no standard procedures to test physical properties of bark. The closest protocol is the American Society for Testing and Materials (ASTM) D-638 standard for plastics, used to test composites of bark and synthetic polymers (Yemele *et al.* 2010). Our procedure was developed based on preliminary testing and methods reported by Xu *et al.* (1997) and Kohan *et al.* (2012). Kohan *et al.* (2012) studied the effect that specimen geometry has on tensile testing of wood strands. The authors concluded dog-bone-shaped samples provide a more accurate measurement of strength than do rectangular samples, because of the removal of stress concentrations in the grips of the tensile-testing machine. Dog-bone-shaped specimens have a gradual taper in the middle of the sample (Fig. 1), also called the gauge area. The taper allows for a smooth transition of load distribution from the edge of the sample to the center, which serves to focus sample failure in the gauge area.

Materials & Methods

Collection and drying

Segments at least 10 cm long of four-, five-, and six-year-old stem were collected for all species of *Dirca*. Stem age was determined by counting conspicuous bud-scale scars. For species without discernable bud-scale scars, stem segments at least 10 cm long with diameters

similar to those of age-identified species of *Dirca* were collected. Representative taxa for families other than Thymelaeaceae were chosen based on their shrub growth form, stem diameter, and whether bark could be removed as a whole piece. Species collected but not analyzed because their bark could not be removed as a whole piece were *Viburnum lentago* L., *Hamamelis virginiana* L., *Euonymum atropurpureus* Jacq., *Staphylea trifolia* L., *Cephalanthus occidentalis* L., *Diervilla lonicera* Mill., and *Calycanthus floridus* L. Because bark properties are affected by the structure of the tissue and environment during development (Hudgins *et al.* 2003; Rosell *et al.* 2014; Rosell 2016), we anticipated variation in bark properties within species (Martin & Crist 1968). Replicates from each plant were collected for testing in case failure did not occur in the gauge area of the specimen samples (Fig. 1).

Stem segments were scored vertically, and bark was peeled away from the wood at a 45° angle. Bark was dried flat to prevent curling; drying between pages of heavy books proved more effective than was drying in a plant press. Bark was placed in the cleft of a book and held flat with one hand while pages were couched over the bark in a rolling motion with another hand. Once the bark samples were dry, determined when bark stood flat and erect when held, they were stored in a plant press. Familial affiliation and species analyzed, collection location, number of replicates, and number of independent samples are listed in table 1. The cultivated *Dirca*, *Leitneria*, and *Peddiea* were grown from seeds collected from populations indigenous to Mexico, United States of America, and South Africa, respectively.

Table 1. Familial affiliation and species analyzed, collection location, number of replicates, and number of independent samples (N). One-hundred eighty combined replicates were collected for all species. Sixty-two of the 180 replicates were not used for analysis due to improper measurement or failure in the gauge area.

Species	Collection Location	Replicates	(N)
Thymelaeaceae			
<i>Dais continifolia</i>	Cape Town, South Africa^	11	4
<i>Daphne ×burkwoodii</i> 'Carol Mackie'	Ames, Iowa, United States of America*	6	2
<i>Dirca decipiens</i>	Overland Park Arboretum, Overland Park, Kansas, United States of America ^	4	1
<i>Dirca mexicana</i>	Ames, Iowa, United States of America *	9 (2015) 16 (2018)	2 (2015) 5 (2018)
<i>Dirca occidentalis</i>	San Mateo County, California, United States of America ^	7	4
<i>Dirca palustris</i>	Potter County, Pennsylvania, United States of America ^	4	3
<i>Edgeworthia chrysantha</i>	Washington Park Arboretum, Seattle, Washington, United States of America ^	4	1

Table 1 continued

<i>Gnidia squarrosa</i>	Harold Porter National Botanical Garden, Betty's Bay, South Africa^	10	3
<i>Gonystylus punctatus</i>	Lyon Arboretum, Honolulu, Hawaii, United States of America ^	5	0
<i>Passerina corymbosa</i>	Harold Porter National Botanical Garden, Betty's Bay, South Africa^	6	2
<i>Peddiea africana</i>	Ames, Iowa, United States of America *	5	4
<i>Phaleria disperma</i>	Harold Porter National Botanical Garden, Betty's Bay, South Africa^	3	2
<i>Struthiola myrinites</i>	Harold Porter National Botanical Garden, Betty's Bay, South Africa^	20	4
<i>Wikstroemia oahuensis</i>	Lyon Arboretum, Honolulu, Hawaii, United States of America ^	3	1
Cornaceae			
<i>Cornus racemosa</i>	Ames, Iowa, United States of America *	13	3
Betulaceae			

Table 1 continued

<i>Corylus americana</i>	Ames, Iowa, United States of America *	6	2
Simaroubaceae			
<i>Leitneria floridana</i>	Ames, Iowa, United States of America *	10	3
Myricaceae			
<i>Morella pensylvanica</i>	Ames, Iowa, United States of America *	7	1
Anacardiaceae			
<i>Rhus aromatica</i>	Ames, Iowa, United States of America *	17	3
Adoxaceae			17
<i>Sambucus nigra</i> ssp. <i>canadensis</i>	Ames, Iowa, United States of America *	14	2

Samples collected from a native population are denoted with (^). Samples collected from cultivated plants are denoted with (*).

Sample preparation and conditioning

Sample dimensions (Fig. 1) were based on those used by Xu *et al.* (1997), and Kohan *et al.* (2012). Paper stencils were used to trace dog-bone shapes onto the samples of dried, flat bark, which were cut into shape with a precision knife and scissors. Bark samples were cut with the 90 mm dimension (Fig. 1) parallel to the stem axis. Knots, scars, and other defects were avoided.

Because different species have different equilibrium moisture contents, conditioning at a specific relative humidity and temperature is standard (ASTM 2010). We designed a conditioning chamber (Fig. 2) based on a description from Hoadley (1980). A tray of saturated sodium nitrite solution (820 g L^{-1} at 20°C) maintained relative humidity at $57.2 \pm 5.0\%$ in 2015 and $59.5 \pm 0.6\%$ in 2018 within an enclosed chamber at $20.4^\circ\text{C} \pm 0.3^\circ\text{C}$ in 2015 and $19.8^\circ\text{C} \pm 0.2^\circ\text{C}$ in 2018. Excess undissolved sodium nitrite was added to the solution to maintain a saturated solution during fluctuations in temperature. Bark samples were randomly placed between perforated ceramic plates and conditioned. Samples of different species were brought to equilibrium based on weight, at which point the bark neither gained nor lost moisture to the surrounding air. Samples were weighed every other day until the direction of weight change became random which signals equilibrium was reached (ASTM 2010). Samples remained in the conditioning chamber up to 4 days after equilibrium until tension tests were performed.

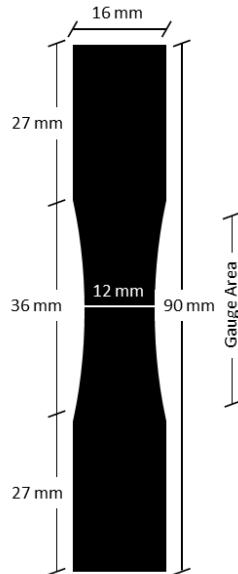


Figure 1. Dimensions of dog-bone-shaped bark samples. Dimensions used were based on those used by Xu *et al.* (1997), Kohan *et al.* (2012), and a preliminary experiment.



Figure 2. Desiccator conditioning chamber within a growth chamber set at 20°C. (Left) Relative humidity was controlled by using a saturated solution of sodium nitrite (820 g L^{-1} at 20°C). A data logger (Watchdog, Spectrum Technologies, Inc., Aurora, IL) was used to monitor temperature and relative humidity. (Rear) An aquarium air pump was used to circulate air between the three desiccators. (Right) Samples of bark were placed between ceramic plates to prevent curling.

Tensile testing

Axial tension tests were conducted on 180 bark samples with a universal testing machine (Fig. 3) (Instron 4500, Instron Industrial Products, Grove City, PA). The frame was equipped with a 10 kN load cell and a computer-controlled, screw-drive crosshead. Serrated grips were used to reduce slippage. We used an elongation rate of 7.00 mm min⁻¹. An extensometer was not used due to unavailability. A sample is loaded between the grips. The top grip applies force to the sample by moving upwards. Applied force divided by the change in length of the sample is the modulus of elasticity. Ultimate tensile strength is the maximum force a sample sustains during the test (Tensile Testing n.d.).



Figure 3. Instron 4500 Universal Testing Machine with a sample loaded in grips above an array of already assessed samples.

Because species differ in equilibrium moisture contents (ASTM 2010), the samples were grouped by species into individual plastic bags once removed from the conditioning chamber. Samples were randomized to determine sampling order. Samples were used for analysis only if

horizontal breakage occurred in the gauge area during testing (Fig. 1). Sixty-two bark samples were omitted from our analysis due to breakage elsewhere.

Analysis

Two comparisons of tensile properties were performed (2015 and 2018), with *D. mexicana* used in both. *Cornus racemosa* Lam., *Corylus Americana* Walter, *Daphne ×burkwoodii* Turill 'Carol Mackie', *D. decipiens*, *D. mexicana*, *D. occidentalis*, *D. palustris*, *Edgeworthia chrysantha* Lindl., *Leitneria floridana* Chapm., *Morella pensylvanica* (Mirb.) Kartesz, *Rhus aromatica* Aiton, and *Sambucus nigra* ssp. *canadensis* (L.) R. Bolli were analyzed in 2015. *Dais continifolia* L., *D. mexicana*, *Gnidia squarrosa* (L.) Druce, *Gonystylus punctatus* A. C. Sm., *Passerina corymbosa* Eckl. ex C. H. Wright, *Peddiea africana* Hook., *Phaleria disperma* (G. Frost.) Baill, *Struthiola myrsinoides* Lam., and *Wikstroemia oahuensis* (A. Gray) Rock were analyzed in 2018.

Data from 2015 and 2018 were pooled and analyzed with JMP (Statistical Analysis System, Cary, NC) statistical software. For the four species of *Dirca*, UTS and MOE data were analyzed with Welch's ANOVA because of small unequal sample sizes (Liu 2015). A pooled analysis of all species of *Dirca* and all non-Thymelaeaceae were analyzed with a Welch's t-test. For species of Thymelaeaceae, and species from families other than Thymelaeaceae, UTS and MOE data were analyzed with Welch's ANOVA because of small unequal sample sizes (Liu 2015) and Tukey-Kramer HSD because of small sample size ($\alpha = 0.05$) (McHugh 2011). Data for MOE violated the equal variance assumption and were, thus, log-transformed. Transformation did not create equal variance, so data was analyzed with Welch's ANOVA for unequal variance, and small unequal sample sizes (Liu 2015). All values are reported herein without transformation.

Ultimate tensile strength and MOE data of a pooled analysis of all genera of Thymelaeaceae and other families were analyzed with Welch's ANOVA because of small unequal sample sizes (Liu 2015) and Tukey-Kramer HSD because of small sample size ($\alpha = 0.05$) (McHugh 2011).

Results & Discussion

Among species of *Dirca*

Species of *Dirca* share similar ultimate tensile strength ($p = 0.191$, $\alpha = 0.05$) and modulus of elasticity ($p = 0.332$, $\alpha = 0.05$) (Fig. 4). The mean UTS ranged from 51.8 ± 11.8 to 107.7 ± 41.9 MPa. The mean MOE ranged from 1945.7 ± 327 to 2859.8 ± 829.5 MPa. Despite *D. palustris* as the only species with documented use of bark for cordage, similarities in the bark properties of all four species suggest they could be used in this way.

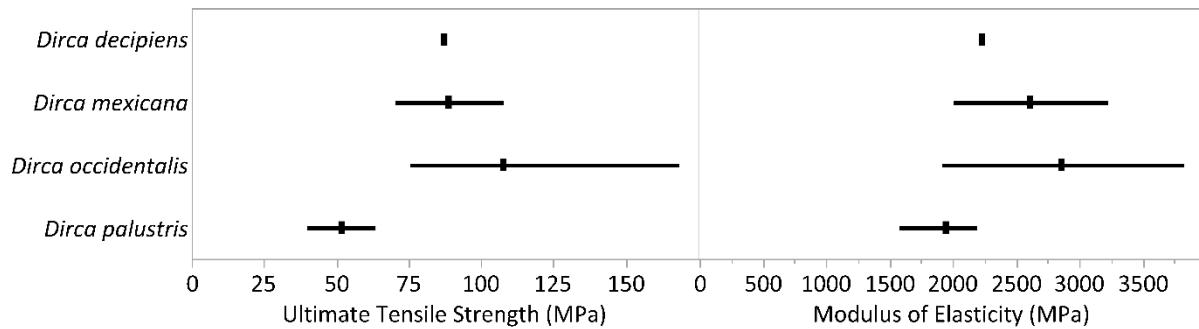


Figure 4. Ultimate tensile strength and modulus of elasticity measured for species of *Dirca*. Horizontal lines represent the range of values. Vertical marks denote the mean. *Dirca decipiens* is represented by a single measurement. All species of *Dirca* share similar UTS and MOE properties based on Tukey-Kramer HSD.

Among Thymelaeaceae

Because the bark of all species of *Dirca* had similar tensile properties, one species, *D. mexicana*, was chosen to represent the genus in comparisons with species of other genera. Bark among members of the Thymelaeaceae did not differ significantly in resistance to breakage, except for *D. mexicana* and *P. africana* (Fig. 5). *Dirca mexicana* had the greatest mean UTS (82.7

± 8.5 MPa), while *Peddiea africana* had the second lowest (32 ± 4.4 MPa). *Edgeworthia chrysanthra* had the lowest UTS, but the sample size of N=1 increased the probability of a Type II error. Species of Thymelaeaceae differed in resistance of bark to stretching. *Struthiola myrsinithes* had the greatest resistance to stretching (mean MOE of 3062.2 ± 296.7 MPa), in contrast to *P. africana*, which had the lowest resistance (667.88 \pm 50.61 MPa).

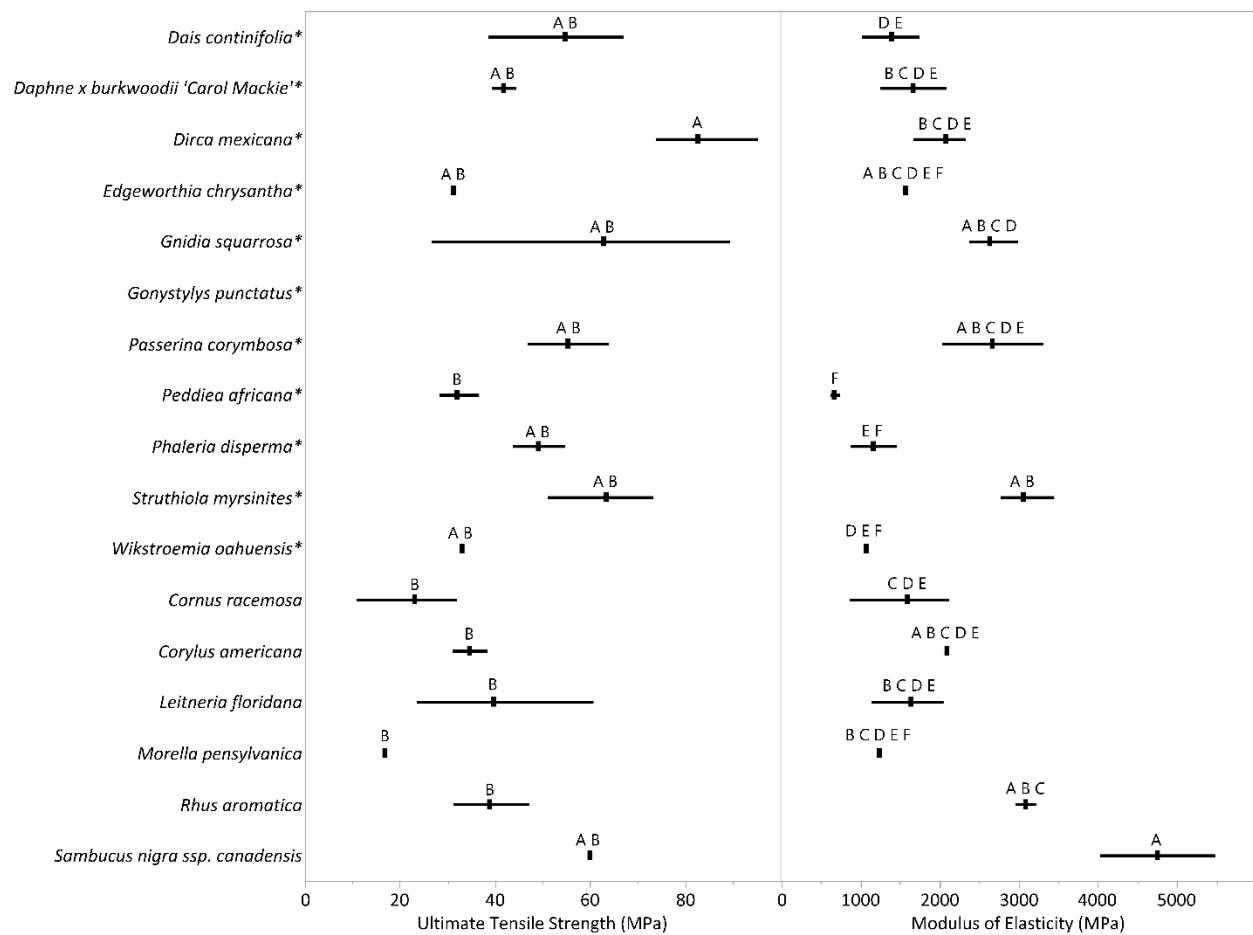


Figure 5. Ultimate tensile strength and modulus of elasticity measured for species within and outside of the Thymelaeaceae. Members of Thymelaeaceae are marked with *. Horizontal lines represent the range of values. Vertical marks denote the mean. Letters above mean represent Tukey-Kramer HSD comparisons. Means marked with the same letter were not significantly different ($\alpha = 0.05$). *Edgeworthia chrysanthra*, *Morella pensylvanica*, and *Wikstroemia oahuensis* are each represented by a single measurement. All replicates of *Gonystylus punctatus* failed outside of the gauge area resulting in a sample size of 0.

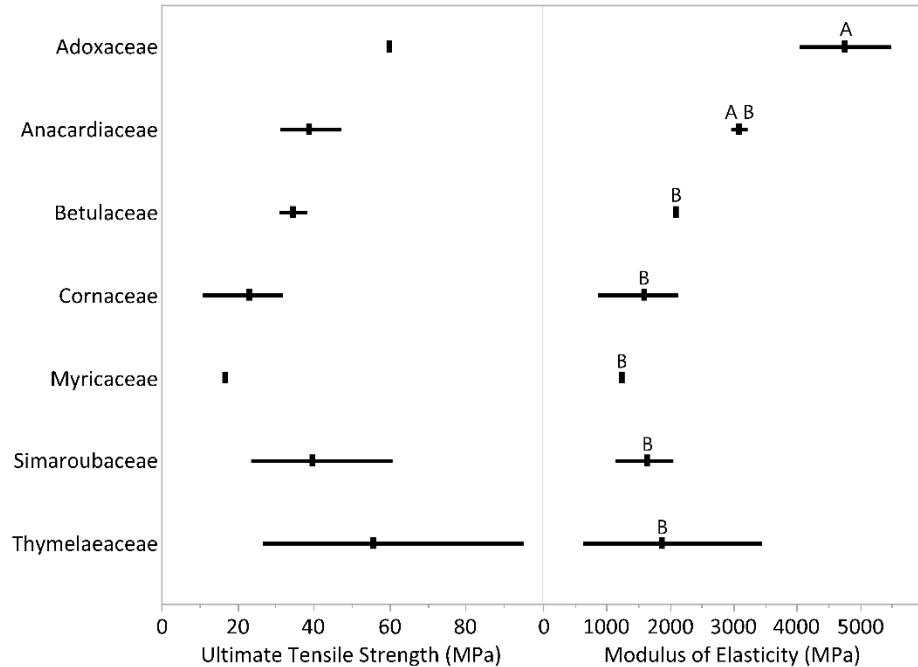


Figure 6. Ultimate tensile strength and modulus of elasticity measured for families analyzed. Horizontal lines represent the range of values. Vertical marks denote the mean. Letters above mean represent Tukey-Kramer HSD comparisons. Means marked with the same letter were not significantly different ($\alpha = 0.05$). Myricaceae is represented by a single measurement.

Zhang *et al.* (2013) found correlations between lignin content and the UTS and MOE of wood fibers. As lignin content decreased, UTS increased and MOE decreased. Van Tieghem (1893) noted, and Choquette (1925) confirmed, that the bark fibers of *D. palustris* lack lignin. As part of a concurrent study, we found that bark fibers of *D. mexicana* lack lignin. A possible explanation for the high UTS and moderate MOE of *D. mexicana* (Fig. 5) is the lack of lignin in its bark fibers. Van Tieghem (1893), and Metcalfe and Chalk (1950) reported that some species of Thymelaeaceae contain lignified bark fibers, while others, like *Daphne caucasica* Pall. and *D. cneorum* L., have lignin-free bark fibers. Hemicellulose content of fibers of wood of *Cunninghamia lanceolata* (Lamb.) Hook (Zhang *et al.* 2013) and size of fibers of bark of *Picea mariana* Mill. and *Populus tremuloides* Michx. (Yemele *et al.* 2010) have been linked to UTS and MOE.

All species evaluated

Our hypothesis that bark from various genera of the Thymelaeaceae have tensile properties distinct from those of bark of plants from other families was not supported. Although the bark of *D. mexicana* has greater resistance to breakage than all other species tested from outside of the Thymelaeaceae except *Sambucus nigra* ssp. *canadensis*, *S. nigra* ssp. *canadensis* shared similar resistance to breakage with all tested species of Thymelaeaceae, with a mean UTS of 59.9 ± 0.5 MPa compared to the greatest mean of 82.7 ± 8.5 MPa for *D. mexicana* (Fig. 5). A pooled analysis of all species of *Dirca* and all non-Thymelaeaceae supports *Dirca* having greater UTS ($p = <0.001$, $\alpha = 0.05$) and similar MOE ($p = 0.113$, $\alpha = 0.05$).

There was no clear pattern with resistance to stretching (Fig. 5). *Sambucus nigra* ssp. *canadensis* and *Rhus aromatica* have greater resistance to stretching than *Dais continifolia*, *P. africana*, and *Phaleria disperma*, yet *S. nigra* ssp. *canadensis* and *R. aromatica* share resistance to stretching with *Daphne ×burkwoodii* ‘Carol Mackie,’ *D. mexicana*, *Edgeworthia chrysanthia*, *Gnidia squarrosa*, and *Passerina corymbosa*. *Sambucus nigra* ssp. *canadensis* had the greatest resistance to stretching with mean MOE of 4755.9 ± 1029.7 MPa, while *P. africana* had the lowest of 667.9 ± 50.6 MPa. High resistance to breakage and stretching of bark of *S. nigra* ssp. *canadensis* is consistent with a report by Welch (2013), who noted that bark fiber from *S. nigra* spp. *caerulea* (Raf.) Bolli and possibly *S. racemosa* L. have been used by the Potter Valley Pomo for unspecified purposes as a tool.

A pooled analysis of all genera of Thymelaeaceae compared to other families further does not support our hypothesis (Fig. 6). No difference was observed for resistance to breakage. Adoxaceae had greater resistance to stretching than the Thymelaeaceae. A pooled analysis of all

genera of Thymelaeaceae compared to all other species measured half supports our hypothesis. Thymelaeaceae had significantly greater UTS ($p < 0.001$, $\alpha = 0.05$) and no difference is observed for MOE ($p = 0.266$, $\alpha = 0.05$).

Conclusion

Certain morphological and anatomical traits of bark are used to characterize and identify taxonomic groups. Rosell *et al.* (2014) reported functional quantitative traits of bark, such as density of fibers, are highly variable within clades. Tensile properties are quantitative traits. Based on our results, we conclude UTS and MOE of the bark of members of the Thymelaeaceae are not distinct characteristics of the family; however, bark of *Dirca* is highly resistant to breakage.

Lignin and hemicellulose have differing effects on the UTS and MOE of fibers (Zhang *et al.* 2013). Van Tieghem (1893), and Metcalfe and Chalk (1950) reported that some species of Thymelaeaceae contain lignified bark fibers, while others, like *Daphne caucasica* and *D. cneorum*, have lignin-free bark fibers. A plausible explanation for the differences within the family could be found in differences in the lignin and hemicellulose contents of the bark of different species.

All but two of the species of Thymelaeaceae that we tested shared similar resistance to breakage, but there was no pattern of shared resistance to stretching. Chemical analyses of lignin and hemicelluloses, and fiber dimensions of species tested would complement our study and shed light on whether chemical composition and size of bark fibers correlate with our findings and explain variation among species of Thymelaeaceae.

Despite reports that the bark of many species of Thymelaeaceae is used for clothing, cordage, decorations, and paper, evidence does not support the family as having greater tensile

properties than those of species we investigated of Cornaceae, Betulaceae, Simaroubaceae, Myricaceae, Anacardiaceae, and Adoxaceae. Because the UTS and MOE of species of Thymelaeaceae were intermediate among species from other families, we conclude that the so-called rope-bark family cannot be defined by the extent of resistance to breakage and stretching of its bark. Other physical properties such as fiber size, and lignin and hemicellulose content of the bark of species of Thymelaeaceae must account for its variable uses. Bark of species of *Broussonetia* (Moraceae), *Edgeworthia*, and *Wikstroemia* are valued for papermaking because of their long, slender, thin-walled fibers and high amounts of hemicelluloses. These properties of their fibers yield some of the thinnest and strongest paper (Barrett 1983).

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Literature Cited

- ASTM Standard D4933-99, 1989. 2010. Standard guide for moisture conditioning of wood and wood-based materials. West Conshohocken, PA: ASTM International.
- Barrett T. 1983. Japanese papermaking: Traditions, tools, and techniques. New York: Weatherhill, Inc.
- Choquette L. 1925. Contribution a l'étude du *Dirca palustris* L. ou "bois de plomb." Ph.D. Thesis, Université de Paris.

- Crowe A. 1981. A field guide to the native edible plants of New Zealand. Birkenhead, Auckland, New Zealand: Godwit Publishing.
- Dellinger SC. 1936. Baby cradles of the Ozark Bluff dwellers. American Antiquity 1: 197--214.
- Floden AJ, Mayfield MH, Ferguson CJ. 2009. A new narrowly endemic species of *Dirca* (Thymelaeaceae) from Kansas and Arkansas, with a phylogenetic overview and taxonomic synopsis of the genus. Journal of the Botanical Research Institute of Texas 3: 485--499.
- Gambler JS. 1902. A manual of Indian timbers. London: Sampson Low, Marston & Company.
- Hamann T, Smets E, Lens F. 2011. A comparison of paraffin and resin-based techniques used in bark anatomy. Taxon 60: 841--851.
- Herber BE. 2003. Thymelaeaceae. In: K Kubitzki and C Bayer (eds.), The families and genera of vascular plants, Vol 5: Flowering plants dicotyledons Malvales, Capparales, and Non-betalain caryophyllales: 373--396. Berlin: Springer-Verlag.
- Hoadley RB. 1980. Understanding wood: A craftsman's guide to wood technology. Newton, CT: The Taunton Press.
- Hudgins JW, Krekling T, Franceschi VR. 2003. Distribution of calcium oxalate crystals in the secondary phloem of conifers: A constitutive defense mechanism? New Phytologist 159: 677-690.
- Hughes S. 1978. Washi: The world of Japanese paper. Tokyo: Kodansha International.
- Koekemoer M, Steyn HM, Bester SP. 2014. Guide to plant families of southern Africa. Pretoria: South African National Biodiversity Institute.
- Kohan N, Via BK, Taylor S. 2012. A comparison of geometry effects on tensile testing of wood strands. Forest Products Journal 62: 167--170.
- Liu H. 2015. Comparing welch anova, a kruskal-wallis test, and traditional anova in case of heterogeneity of variance. Master Thesis. Virginia Commonwealth University.
- Martin RE, Crist JB. 1968. Selected physical-mechanical properties of eastern tree barks. Forest Products Journal 18: 54--60.
- McHugh ML. 2011. Multiple comparison analysis testing in ANOVA. Biochemia Medica 21: 203--209.
- Metcalfe CR, Chalk L. 1950. Anatomy of the dicotyledons, Vol 2. Oxford: The Clarendon Press.
- Moerman DE. 1998. Native American ethnobotany. Portland, OR: Timber Press.

- Motsi MC. 2009. Origin and diversification of the Australasian genera *Pimelea* and *Thecanthes* (Thymelaeaceae). Ph.D. Dissertation, University of Johannesburg.
- Mottiar Y. 2012. On the discovery of eastern leatherwood (*Dirca palustris*). Canadian Field Naturalist 126: 86--88.
- Paul A, Arunachalam A, Khan ML, Arunachalam K. 2006. *Daphne papyraceae* Wall. ex Steud. ó [sic] a traditional source of paper making in Arunachal Pradesh. Natural Product Radiance 5: 133--138.
- Pearman G, Prendergast HDV. 2000. Items from the lacebark tree [*Lagetta lagetto* (W. Wring) Nash: Thymelaeaceae] from the Caribbean. Economic Botany 54: 4--6.
- Polunin O, Stainton A. 1984. Flowers of the Himalaya. Delhi: Oxford University Press.
- Pooley E. 2006. Forest plants in the forest and in the garden. Durban: The Flora Publication Trust.
- Record SJ, Hess RW. 1943. Timbers of the new world. New Haven: Yale University Press.
- Rosell JA, Gleason S, Méndez-Alonso R, Chang Y, Westoby M. 2014. Bark functional ecology: Evidence for tradeoffs, functional coordination, and environment producing bark diversity. New Phytologist 201: 486--497.
- Rosell JA. 2016. Bark thickness across the angiosperms: More than just fire. New Phytologist 211: 9--102.
- Schmidt J, Stavisky N. 1983. Uses of *Thymelaea hirsuta* (Mitnan) with emphasis on hand papermaking. Economic Botany 37: 310--321.
- Solereder H. 1908. Systematic anatomy of the dicotyledons, Vol. 2 Monochlamydeae. Oxford: Clarendon Press.
- Tensile Testing. (n.d.). Retrieved March 03, 2015, from <https://www.instron.us/en-us/our-company/library/test-types/tensile-test>
- Testing Solutions. (n.d.). Retrieved March 03, 2015, from <https://www.instron.us/en-us/testing-solutions>
- Van Tieghem MP. 1893. Recherches sur la structure et les affinités des Thyméléacées et des Pénéacées. Annales des Sciences Naturelles 7: 185--294.
- Vestal EH. 1938. Biotic relations of the wood rat (*Neotoma fuscipes*) in the Berkeley Hills. Journal of Mammalogy 19: 1--36.
- Welch JR. 2013. Sprouting valley: Historical ethnobotany of the northern Pomo from Potter Valley, California. Denton, TX: Society of Ethnobiology.

Xu X, Schneider E, Chien AT, Wudl F. 1997. Nature's high-strength semitransparent film: The remarkable mechanical properties of *Prunus serrula* bark. *Chemistry of Materials* 9: 1906--1908.

Yemele MCN, Koubaa A, Cloutier A, Soulounganga P, Wolcott M. 2010. Effect of bark fiber content and size on the mechanical properties of bark/HDPE composites. *Composites: Part A* 41: 131--137

Zhang S, Wang G, Fei B, Yu Y, Cheng H, Tian G. 2013. Mechanical function of lignin and hemicelluloses in wood cell wall revealed with microtension of single wood fiber. *BioResources* 8: 2376--2385.

CHAPTER 3. SECTIONING OF LIGNIN-FREE HETEROGENEOUS BARK OF *DIRCA MEXICANA* (THYMELAEACEAE)

A paper to be submitted to International Association of Wood Anatomists Journal

Zachary J. Hudson¹, Tracey M. Stewart², William R. Graves¹

¹Department of Horticulture, Iowa State University, Ames, Iowa, 50011, United States of America

²Roy J. Carver High Resolution Microscopy Facility, Iowa State University, Ames, Iowa, 50011,

United States of America

Abstract

Techniques to overcome the difficulty of sectioning heterogeneous plant tissues focus on softening lignified tissues mixed among non-lignified tissues. Such techniques may not be adequate for heterogeneous tissues of non-lignified, thick-walled and thin-walled cells. Fresh, wax-based, and resin-based techniques were investigated to obtain high-quality slides of sectioned bark tissue of *Dirca mexicana* Nesom & Mayfield. Herein, we present modifications of published methods to section lignin-free bark tissue. Tissue softened in ethylenediamine, embedded in polyethylene glycol 1500, and sectioned with the support of a particular brand of commercially available packaging tape provided anatomical sections of the highest acuity.

Introduction

Bark may be the one of the most conspicuous visual features of a woody plant, yet the accrual of knowledge of comparative bark anatomy (Angyalossy *et al.* 2016) and the ecological functions of bark (Rosell *et al.* 2014) lags behind that of closely associated wood tissue. Its heterogeneous composition is the main reason why bark has been neglected (Barbosa *et al.* 2010; Hamann *et al.* 2011; Angyalossy *et al.* 2016). Bark, defined as all tissues external to the

vascular cambium, performs a myriad of functions, such as transport of photosynthates (Esau 1960), mechanical support (Niklas 1999), and protection from fire (Harmon 1984). Such functions are possible due to various cell types that range from soft, non-lignified parenchyma to hard sclerified fibers. The mixture of hard and soft cell types makes sectioning bark tissue difficult. Various histological techniques have been developed to address the difficulty, yet researchers continue to be challenged by uneven thickness of sectioned tissue (Kukachka 1977) and separation of tissue and tearing of cell walls (Carlquist 1982). Selected agents have been reported to soften lignified or hard tissues. Hydrofluoric acid demineralizes woody tissue (Langdon 1920) and ethylenediamine acts as a swelling agent which reduced the density of cell walls (Kukachka 1977).

Dirca L. of Thymelaeaceae comprises four woody species. Van Tieghem (1893), and Metcalfe and Chalk (1950) reported that some species of Thymelaeaceae contain lignified bark fibers, while others have lignin-free bark fibers. Van Tieghem (1893) noted, and Choquette (1925) confirmed, that the bark fibers of *Dirca palustris* L. lack lignin. Donaldson (1995) noted that a reduction of lignin concentration between the middle lamella and adjacent S1 and S2 layers of cells was associated with the fracture of cell walls during sectioning of wood of *Pinus radiata* D. Don.

Van Tieghem (1893) and Choquette (1925) described the bark anatomy of mature *D. palustris* as consisting of concentric rings of thick-walled fibers, which lack lignin, separated by rings of thin-walled parenchyma. When we sectioned un-softened bark of *Dirca mexicana* Nesom & Mayfield, the thick-walled fibers resist cutting and were pushed into the thin-walled parenchyma layer, causing tearing and crushing of the bark tissue. We hypothesize that the

difficulties we have encountered are due to the heterogeneous composition of the bark and the lack of lignin within the cell wall of the phloem fibers. Following the assessment of various techniques for sectioning bark tissue of *Dirca*, this report describes modifications to the methods of Barbosa *et al.* (2010) that were needed to generate high-quality sections of *D. mexicana*.

Materials & Methods

Sample collection and preparation

Segments varying between 0.5–1.5 cm in diameter of four-, five-, and six-year-old stems were collected in summer 2015 from cultivated plants of *D. mexicana* in Ames, Iowa, United States of America. Segments were immediately placed into formalin-acetic-alcohol fixative (Johansen 1940) for at least three months.

Fresh-, wax-, and resin-based methods

Common histological techniques for un-fixed fresh material, and for fixed material embedded in paraffin wax or LR White resin (Electron Microscopy Sciences, Hatfield, Pennsylvania, United States of America) were evaluated.

Un-fixed stem segments were sectioned 100 µm thick with a vibratome (Vibratome 3000, Technical Products International, Maryland Heights, Missouri, United States of America). Tissue was stained with a one second dips in aqueous 1% (w/v) fast green and carbol fuchsin. Sections were mounted in Permount (Thermo Fisher Scientific, Waltham, Massachusetts, United States of America).

Paraffin wax embedded stem segments were dehydrated in an aqueous ethanol series set in a tissue rotator to keep solution in motion. Samples were dehydrated in an aqueous 50% ethanol solution for five hours, followed by an aqueous 70% ethanol solution for eight hours.

Tissues were then dehydrated for 96 hours, 24 hours each in aqueous 85, 95, and two 100% ethanol solutions. Next, tissue was washed in 1:1 (xylanes (Thermo Fisher Scientific, Waltham, Massachusetts, United States of America):100% ethanol), followed by two 100% xylanes solutions, set in a tissue rotator to keep solution in motion. Tissue was washed for three hours per solution. Paraffin wax infiltrated the bark tissue by use of serial solutions of xylanes and paraffin, set in a 60°C oven. Tissue was placed in a 3:1 (xylanes:paraffin) solution for 24 hours, followed by a 1:1, 1:3, and two 100% paraffin solutions, 12 hours per solution. Lastly, tissue was embedded in a paraffin mold. Embedded tissue was sectioned 10 µm thick using a rotary microtome (Spencer 820, American Optical Manufacturing Company, Southbridge, Massachusetts, United States of America). Sections were mounted on Haupt's adhesive-subbed slides and stained with an aqueous 1% (w/v) solution each of safranin and fast green. Slides were dehydrated in serial aqueous 100, 95, 70, and 50% ethanol, dipped five times followed by a one minute wash for each ethanol solution. Tissue was stained with safranin for eight hours, dipped twice in deionized water, and 100% ethanol, followed by a 30 seconds dip in fast green. Next, tissue was dipped ten times in 0.5% clove oil and xylanes solution, and 1:1 100% ethanol: xylanes. Coverslips were mounted with Permount. Two additional steps were employed to improve the quality of sectioned tissue. Paraffin embedded tissue was frozen for 12 hours or hardened in ice water prior to sectioning.

LR White resin embedded stem segments were dehydrated in an aqueous ethanol series set in a tissue rotator to keep solution in motion. Tissue was dehydrated in 50% for five hours, 70% for 8 hours, and 85, 95, and two 100% ethanol solutions for 24 hours per solution. LR White Resin infiltrated the bark tissue by use of serial solutions of ethanol and LR White resin, set in a

tissue rotator to keep solution in motion. Infiltration was over 72 hours, 12 hours each at ratios of 3:1, 1:1, 1:3 (100% ethanol: LR White) and three 100% resin solutions. Tissue was embedded in a resin foil tray, covered to eliminate as much air as possible, and placed into a 60°C oven for 24 hours. Embedded tissue was cut 2 µm thick using an ultramicrotome (Leica EM UC7, Leica Microsystems, Wetzlar, Germany). Sections were stained with Epoxy Tissue Stain (Electron Microscopy Sciences, Hatfield, Pennsylvania, United States of America) by submerging the tissue in a single drop. Tissue was placed on a hotplate until a silver rim forms on the drop of stain. Last, tissue was washed in deionized water and mounted in Permount on a Haupt's adhesive-subbed slide.

Ethylenediamine and paraffin wax method

We evaluated two techniques suggested for difficult-to-section tissues, including modifications proposed by Hamann *et al.* (2011) to the use of ethylenediamine (Carlquist 1982) to soften hard plant structures for paraffin sectioning.

Bark tissue was softened in vials of an aqueous 4% (v/v) solution of ethylenediamine for three days at room temperature (23°C), set in a tissue rotator to keep solution in motion. Tissues were rinsed in deionized for six hours; the water was replaced after the second and fourth hours. Samples were dehydrated in an aqueous ethanol series of 5, 11, 18, and 30%. The samples were held for two hours in each of the four ethanol concentrations. Samples were then rinsed four eight hours in a 50:40:10 (deionized water: 96% ethanol: 100% tertiary butanol) solution, and a one hour in a 15:50:35 solution. Next, tissue was rinsed for one hour in a 45:55 solution of 96% ethanol: 100% tertiary butanol, followed by one hour in a 25:75 solution of 100%

ethanol: 100% tertiary butanol. Lastly, tissue was rinsed in 100% tertiary butanol, first for an hour, then for eight hours after the tertiary butanol was changed.

Tissue was transferred to capped glass vials and placed in a 60°C oven for additional processing. Paraffin wax infiltrated the bark tissue by use of serial solutions of 100% tertiary butanol: paraffin. The infiltration was over six hours, two hours each at ratios of 2:1, 1:1, and 1:2. Tissue was rinsed in 100% paraffin wax twice for one hour per rinse. This was followed by a 48-hour rinse before tissue was embedded in a paraffin mold. Molds were hardened in a refrigerator for four days prior to sectioning. Embedded tissue was sectioned 10 µm thick with a rotary microtome (American Optical Spencer 820).

Sectioned tissue was mounted on Haupt's adhesive-subbed slides and dried on a slide warmer at 40°C. Tissue was stained with an aqueous 1% (w/v) solution of fast green. Slides were washed in 100% xylenes three times for 5 minutes per wash. Next, slides were dipped five times and washed for one minute per aqueous solution of 100, 95, 70, and 50% ethanol. Tissue was stained in fast green for 30 seconds followed by 10 dips in a solution of 0.5% clove oil and xylenes, and 10 dips in 1:1 100% ethanol: xylenes. Coverslips were mounted with Permount.

Ethylenediamine and polyethylene glycol 1500 method

Our second evaluated technique was described by Barbosa *et al.* (2010) for sectioning heterogeneous plant parts by using ethylenediamine and polyethylene glycol 1500.

Bark tissue was placed in capped glass vials in a 60°C oven in either an aqueous 4 or 10% solution of ethylenediamine for four days. Samples were rinsed in deionized water for two hours. In a 60°C oven, polyethylene glycol infiltrated the tissue by use of serial 10% aqueous solutions for 24 hours per step, ending with 100% wax. Samples were embedded in a

polyethylene glycol mold and hardened at room temperature (23°C). Embedded tissue was sectioned 15 µm thick with a rotary microtome (American Optical Spencer 820).

Barbosa *et al.* (2010) proposed optional steps to improve sectioning and prevent tearing by using a polystyrene foam solution and adhesive tape. Solid foam was dissolved in 2 ml of n-butyl acetate to create a saturated solution. The solution was applied to tissue using a paint brush and allowed to solidify before sectioning. Barbosa *et al.* (2010) did not provide details for using adhesive tape. We modified the method described by Bonga (1961) for use with cellulose tape. A strip of tape was pressed against the exposed surface of the sample to prevent damage during sectioning. We found adhesive tape (Barbosa *et al.* 2010) and cellulose tape (Bonga 1961) to be too ambiguous as descriptive terms because they are not specific to a type of tape. We compared six different tapes (Table 1) for how well they prevented crushing and tearing of tissue and aided with mounting. A solvent is needed to remove the tape from the tissue; we compared different solvents because Barbosa *et al.* (2010) suggested n-butyl acetate, whereas Bonga (1961) suggested xylene. Scotch Greener packaging tape (3M, St. Paul, Minnesota, United States of America), 3M Transpore (3M, St. Paul, Minnesota, United States of America), and Walgreens athletic tape (Walgreens Company, Deerfield, Illinois, United States of America) were placed in xylenes and n-butyl acetate. Scotch Transparent tape (3M, St. Paul, Minnesota, United States of America) was placed in xylenes. Scotch Greener Magic tape (3M, St. Paul, Minnesota, United States of America) was placed in xylenes, 100% ethanol, deionized water, acetone, and n-butyl acetate. Universal masking tape (Universal Office Products, Nostell, Wakefield, UK) was placed in xylenes. One slide was used for each tape/solvent combination tested. Slides were placed in a solvent and checked every 8 hours until the tape separated from the tissue. We

found cutting strips of tape 1 mm wider than the embedded tissue and 7–10 cm long eased handling while sectioning. One end of the tape was pressed on the exposed tissue block with a finger; the other end was held while sectioning.

To mount the sectioned tissue, Barbosa *et al.* (2010) suggested the use of an albumin adhesive over a gelatin adhesive like Haupt's (Haupt 1930) because albumin adheres tissue to slides better than gelatin and it does not cause background staining. Adhesive was made fresh of equal parts glycerin and fresh egg white. A drop of the albumin adhesive was spread over the slide. Barbosa *et al.* (2010) notes sectioned tissue should be stained and dried before using an albumin adhesive but does not provide details on staining unmounted tissue. We also tried the mounting method described by van Horne and Zopf (1951). An eyedropper was used to place a single drop of Haupt's adhesive (Haupt 1930) and two drops of an aqueous 5% formaldehyde solution on cleaned slides. Drops are mixed and spread over the slide. Tape-backed sections were trimmed and mounted on slides of either albumin adhesive, Haupt's adhesive-subbed, or the adhesive described by van Horne and Zopf (1951). Slides were dried for 24 hours with the tape side down on top of filter paper on a slide warmer at 42°C. Pressure was applied to the slides by using lead weights. Mounted tissue was stained with an aqueous 1% (w/v) solution each of safranin and fast green. Slides were dehydrated in serial aqueous 100, 95, 70, and 50% ethanol, dipped five times followed by a one minute wash for each ethanol solution. Tissue was stained with safranin for eight hours, dipped twice in deionized water, and 100% ethanol, followed by a 30 seconds dip in fast green. Next, tissue was dipped ten times in 0.5% clove oil and xylenes solution, and 1:1 100% ethanol: xylenes. Coverslips were mounted with Permount.

Histochemistry

Sections of *D. mexicana* were stained with toluidine blue-O to identify the presence of lignin within the middle lamella and cell walls of fibers according to O'Brien *et al* (1964). Slides of sectioned tissue were placed into an aqueous 0.05% toluidine blue-O (w/v) solution for two minutes and then washed in tap water for one minute. Coverslips were mounted with Permount.

Results & Discussion

Fresh-, wax-, and resin-based methods

None of these techniques provided the desired quality of sectioned tissue (Fig. 1). Our additional steps did not improve the quality of sectioned tissue (Fig. 1C). Use of LR White resin provided more support than did paraffin wax and permitted cellular morphology to be viewed. However, the limitations due to cost, embedded sample size, infiltration, and section size were substantial, leading us to abandon this approach.

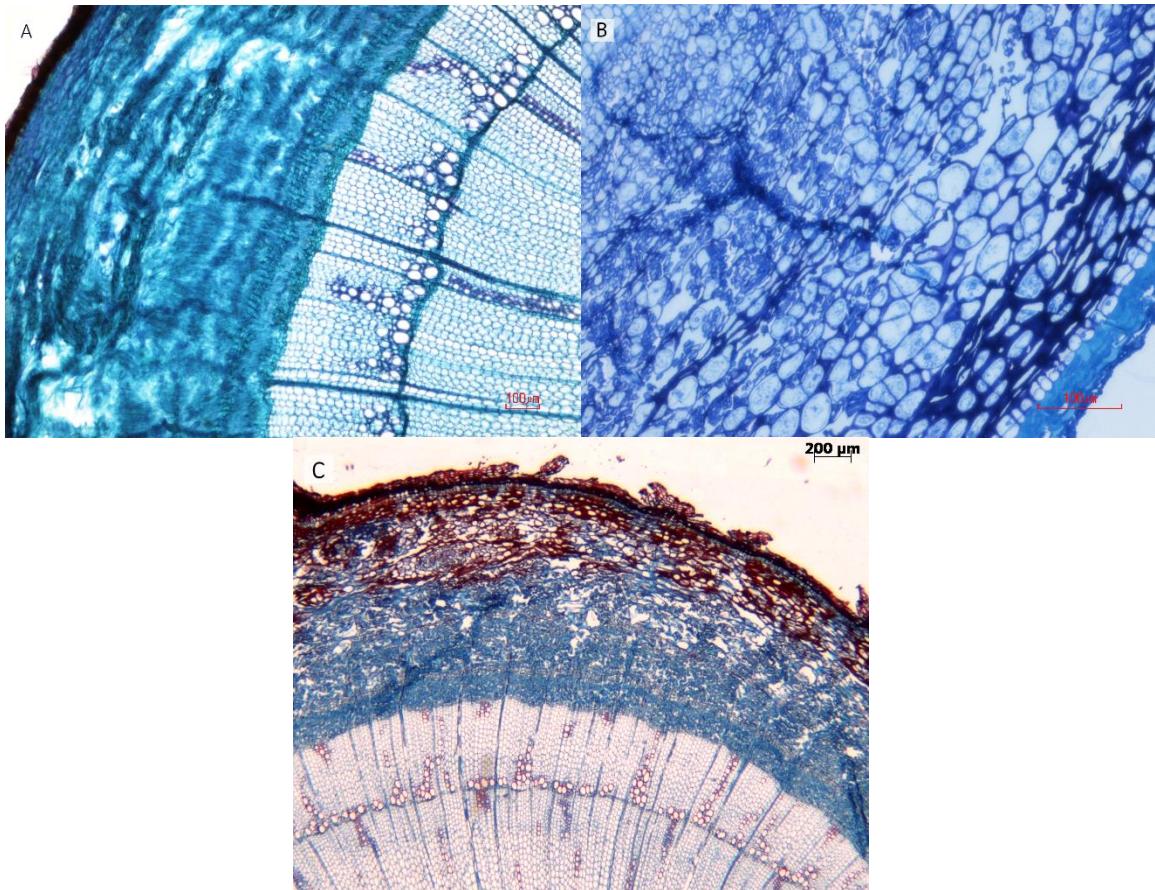


Figure 1. Transverse sections of secondary xylem and phloem of *Dirca mexicana*. (A) Fresh, unfixed stem tissue, 15 μm thick, stained with carbol fuchsin and fast green, tissue torn and crushed. -- Scale bar = 100 μm . (B) Fixed, LR White resin embedded stem tissue, 2 μm thick, stained with Epoxy tissue stain (Electron Microscopy Sciences, Hatfield, Pennsylvania, United States of America), tissue torn and crushed. -- Scale bar = 100 μm . (C) Fixed, frozen paraffin wax embedded tissue, 10 μm thick, stained with safranin and fast green, tissue torn and crushed. -- Scale bar = 200 μm .

Ethylenediamine and paraffin wax method

We were unable to produce high-quality sections of softened, paraffin-embedded tissue by using the method described by Hamann *et al.* (2011) (Fig. 2).

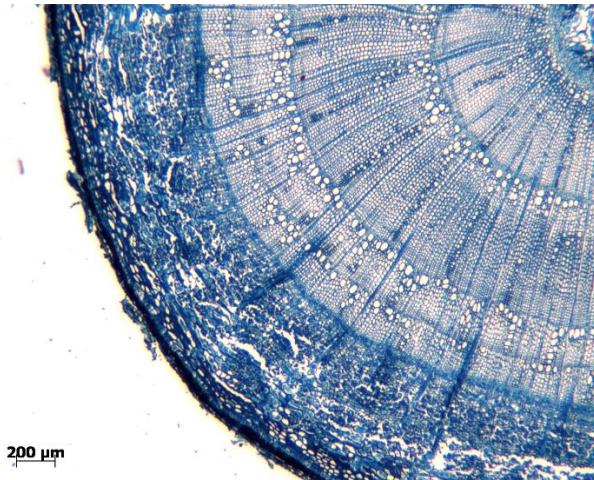


Figure 2. Transverse section of secondary xylem and phloem of *Dirca mexicana*. Ethylenediamine softened and paraffin wax embedded tissue, 10 μm thick, stained with fast green, tissue torn and crushed. -- Scale bar = 200 μm .

Ethylenediamine and polyethylene glycol 1500 method

We were unable to produce high-quality sections of tissue by following methods and additional recommendations provided by Barbosa *et al.* (2010). This may have been due in part to the lack of details concerning the procedural steps, type of solvent used, and whether percentages were based on weight or volume. Further modifications we made resulted in high-quality sections of bark tissue (Fig. 3). Barbosa *et al.* (2010) suggested placing samples in a closed jar containing a 4 or 10% solution of ethylenediamine, and then placing the jar in a 50–60°C oven. We obtained the best results by placing samples in an aqueous 10% ethylenediamine (v/v) solution at room temperature (23°C) for four days. Placing the solution in a 60°C oven caused the bark tissue to separate from the adjacent wood before the sample could be embedded (Fig. 4). Although the bark tissue we studied lacks lignin, we believe softening the tissue with ethylenediamine at room temperature was a beneficial step to reduce tearing and crushing of cells when sectioning. Qin *et al.* (2015) concluded that ethylenediamine penetrates crystalline cellulose and breaks and reforms hydrogen bonds at ambient pressure. Changes in

the chemical structure of cellulose within the cell wall of thick-walled fibers may have resulted in better sectioning of the bark tissue.

We do not recommend the use of polystyrene foam solution as an anti-tearing agent as suggested by Barbosa *et al.* (2010). When we applied the polystyrene solution to our polyethylene glycol 1500 embedded samples, the n-butyl acetate solvent caused the polyethylene glycol to soften and adhere to the blade, thereby preventing sectioning.

We do not recommend the use of an albumin adhesive as suggested by Barbosa *et al.* (2010). It did not reliably fix sectioned tissue to our slides, and it caused uneven staining with safranin and fast green. We also do not recommend use of Haupt's adhesive-subbed slides due to poor adhesion of sectioned tissue to the slide. Use of Haupt's adhesive described by van Horne and Zopf (1951) resulted in 100% adhesion of sectioned tissue.

Scotch Greener packaging tape provided support that reduced crushing and tearing of tissue during sectioning. Use of the tape as an anti-tearing support resulted in the sectioned tissue of the highest acuity. We found soaking the slides in a solution of xylenes for eight hours removed the tape and adhesive from the sectioned tissue (Table 1). Although Walgreens brand athletic tape was successfully removed with n-butyl acetate and xylenes (Table 1), its anti-tearing support was insufficient when sectioning tissue.

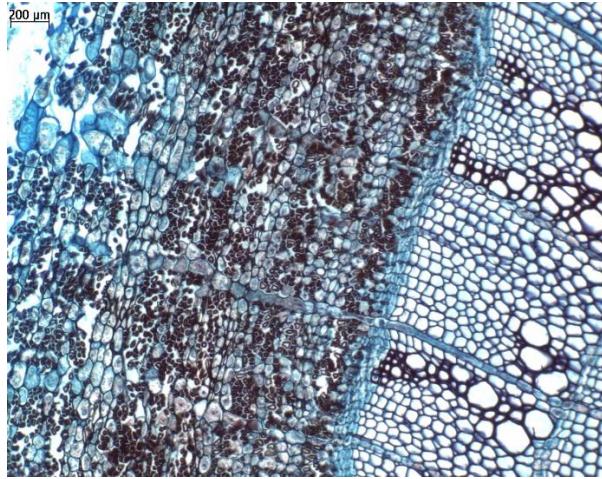


Figure 3. Transverse section of secondary xylem and phloem of *Dirca mexicana*. The tissue was minimally torn and crushed by using ethylenediamine to soften, polyethylene glycol 1500 to embed, and packaging tape as anti-tearing support during sectioning. Stained with safranin and fast green, 15 μm thick, strata of fibers and parenchyma cells are evident. -- Scale bar = 200 μm .



Figure 4. Whole and partial stem segments of *Dirca mexicana*, approximately 3 mm thick, soaked in an aqueous 10% ethylenediamine solution (v/v) for four days in a 60°C oven. The phloem and xylem tissue separated along the vascular cambium of the stem.

Although softening with ethylenediamine improved the quality of sectioned tissue, use of Scotch Greener packaging tape removed with xylenes optimized anti-tearing support for sectioned tissue (Table 1). However, we were not able to use the tape following the methods described by Barbosa *et al.* (2010) or Bonga (1961).

Table 1. Adhesive tapes used as anti-tearing support for sectioning polyethylene glycol 1500 embedded bark tissue of *Dirca mexicana*. Brand, type, solvent used to separate tape from sectioned tissue and time placed in solution, and result are reported. All slides were dried for 24 hours before being placed into a solution. One slide was used for each tape/solvent combination tested. Slides were placed in a solvent and checked every 8 hours until the tape separated from the tissue. Results: X= tape remained fixed to slide; Y= tape backing removed but adhesive remained fixed to tissue; Z= tape and adhesive separated from tissue, tissue remained fixed to slide.

Brand	Type (Product Number)	Solvent/Time	Result
Scotch	Packaging Tape, Greener (3750)	Xylenes/8 hours	Z
Scotch	Packaging Tape, Greener (3750)	n-butyl acetate/8 hours	X
Scotch	Transparent (600)	Xylenes/24 hours	X
Scotch	Magic, Greener (812)	Xylenes/48 hours	Y
Scotch	Magic, Greener (812)	200 proof Ethanol/32 hours	Y
Scotch	Magic, Greener (812)	Deionized Water/24 hours	X
Scotch	Magic, Greener (812)	Acetone/8 hours	Y
Scotch	Magic, Greener (812)	n-butyl acetate/8 hours	Y
Universal	Masking Tape (51312)	Xylenes/8 hours	X
3M	Transpore (1527-1)	Xylenes/24 hours	Y
3M	Transpore (1527-1)	n-butyl acetate/24 hours	Y
Walgreens	Athletic Tape (382865)	Xylenes/8 hours	Z
Walgreens	Athletic Tape (382865)	n-Butyl acetate/8 hours	Z

Histochemistry

Treatment with toluidine blue-O identified an un-lignified compound middle lamella and cell wall of fibers of *D. mexicana* (Fig. 5) (O'Brien *et al.* 1964). A red-purple color indicates an un-

lignified compound middle lamella. A green or blue color indicating lignified walls did not appear for the cell wall of fibers (arrows) (O'Brien *et al.* 1964). The lack of lignin within the middle lamella and cell wall of fibers may explain the tendency of sectioned tissue of *D. mexicana* to be crushed or torn based on the conclusions of Donaldson (1995).

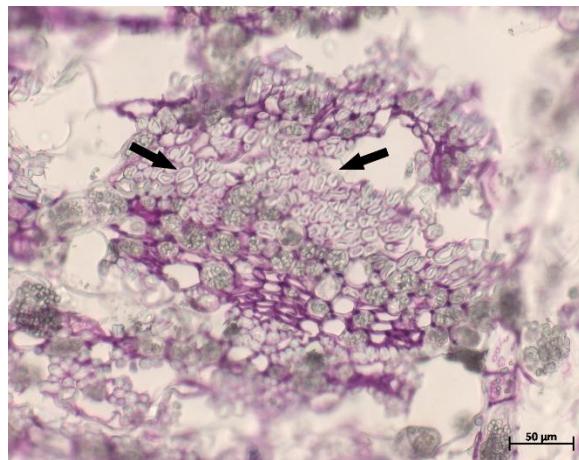


Figure 5. Transverse sections of secondary phloem of *Dirca mexicana* stained with toluidine blue-O. Red-purple color indicates an un-lignified compound middle lamella. A green or blue color indicating lignified walls did not appear for the cell wall of fibers (arrows). -- Scale bar = 50 μ m.

Conclusions

Techniques to address the difficulty of sectioning heterogeneous tissues have focused on softening lignified tissues mixed among non-lignified tissues. Such techniques may not be adequate for heterogeneous tissues comprising non-lignified cells that vary in wall thickness. Perhaps because of this, we were unable to obtain high-quality sections of bark tissue by using published methods as described, but our modifications of protocols reported by Barbosa *et al.* (2010) led to slides of satisfactory quality (Fig. 3). A lack of lignin identified between the middle lamella and cell wall of fibers of *D. mexicana* may explain the tendency of sectioned tissue of *D. mexicana* to be crushed or torn.

Although the bark tissue we studied lacks lignin, we believe softening the tissue with ethylenediamine at room temperature (23°C) helped reduce tearing and crushing of cells when sectioning. Softening with an aqueous 10% (v/v) solution of ethylenediamine improved the quality of sectioned tissue compared to a 4% solution or none, but use of Scotch Greener packaging tape was essential for obtaining high-quality sections of the bark tissue of *Dirca*.

Polyethylene glycol is a water-soluble wax that prohibits the use of aqueous solutions during sectioning and mounting. However, use of tape as an anti-tearing support permitted use of an aqueous solution of Haupt's adhesive and formaldehyde as the mounting tissue adhesive.

By modifying existing techniques, we were able to embed, section, and mount bark tissue of *Dirca* with satisfactory quality to describe the anatomy of the bark in detail. Our recommended adjustments could have potential for application for species with lignin-free bark fibers both within and outside of the Thymelaeaceae, including *Daphne caucasica* and *D. cneorum* (Van Tieghem 1893).

Acknowledgements

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Literature Cited

- Angyalossy V, Pace MR, Evert RF, Marcati CR, Oskolski AA, Terrazas T, Kotina E, Lens F, Mazzoni-Viveiros SC, Angeles G, Machado SR, Crivellaro A, Rao KS, Junikka L, Nikolaeva N, Baas P. 2016. IAWA list of microscopic bark features. IAWA Journal 37: 517--615.
- Barbosa ACF, Pace MR, Witovisk L, Angyalossy V. 2010. A new method to obtain good anatomical slides of heterogeneous plant parts. IAWA Journal 31: 373--383.
- Bonga JM. 1961. A method for sectioning plant material using cellulose tape. Canadian Journal of Botany 39: 72--730.

- Carlquist S. 1982. The use of ethylenediamine in softening hard plant structures for paraffin sectioning. *Stain Technology* 57: 311--317.
- Choquette L. 1925. Contribution a l'étude du *Dirca palustris* L. ou "bois de plomb." Ph.D. Thesis, Université de Paris.
- Donaldson LA. 1995. Cell wall fracture properties in relation to lignin distribution and cell dimensions among three genetic groups of radiate pine. *Wood Science and Technology* 29: 51--63.
- Esau K. 1960. Plant anatomy: Anatomy of seed plants (2nd ed.) New York: John Wiley & Sons.
- Hamann T, Smets E, Lens F. 2011. A comparison of paraffin and resin-based techniques used in bark anatomy. *Taxon* 60: 841--851.
- Harmon ME. 1984. Survival of trees after low-intensity surface fires in Great Smoky Mountains National Park. *Ecology* 65: 796--802.
- Haupt AW. 1930. A gelatin fixative for paraffin sections. *Stain Technology* 5: 97--98.
- Johansen DA. 1940. Plant microtechnique. New York: McGraw-Hill.
- Kukachka BF. 1977. Sectioning refractory woods for anatomical studies. U.S.D.A Forest Service Research Note FPL-0236: 1--9.
- Langdon LM. 1920. Sectioning hard woody tissue. *Botanical Gazette* 70: 82--84.
- Metcalfe CR, Chalk L. 1950. Anatomy of the dicotyledons, Vol 2. Oxford: Clarendon Press.
- Niklas KJ. 1999. The mechanical role of bark. *American Journal of Botany* 86: 465--469.
- O'Brien TP, Feder N, McCully ME. 1964. Polychromatic staining of plant cell walls by toluidine blue O. *Protoplasma* 59: 367--373.
- Qin L, Li WC, Zhu JQ, Liang JN, Li BZ, Yuan YJ. 2015. Ethylenediamine pretreatment changes cellulose allomorph and lignin structure of lignocellulose at ambient pressure. *Biotechnology for Biofuels* 8: 174--189.
- Rosell JA, Gleason S, Méndez-Alonso R, Chang Y, Westoby M. 2014. Bark functional ecology: Evidence for tradeoffs, functional coordination, and environment producing bark diversity. *New Phytologist* 201: 486--497.
- Van Horne RL, Zopf LC. 1951. Water-soluble embedding materials for botanical microtechnique. *Journal of Pharmaceutical Sciences* 40: 31--34.
- Van Tieghem MP. 1893. Recherches sur la structure et les affinités des Thyméléacées et des Pénéacées. *Annales des Sciences Naturelles* 7: 185--294.

CHAPTER 4. ANATOMY OF BARK OF SPECIES OF *DIRCA* L. (THYMELAEACEAE)

A paper to be submitted to International Association of Wood Anatomists Journal

Zachary J. Hudson, William R. Graves

Department of Horticulture, Iowa State University, Ames, Iowa, 50011, United States of America

Abstract

Aspects of anatomy of bark of *Dirca occidentalis* Gray, *Dirca mexicana* Nesom & Mayfield, and *Dirca decipiens* Floden is observed for the first time. Anatomical characters were examined to identify differences between all four species of *Dirca* L. The length of fibers was similar between all four species ($p = 0.162$) ranging from 0.526–7.151mm. Differences between species was identified for the diameter and length of sieve-tube elements, axial parenchyma, and tangential length of phellem cells. The occurrence of xylem tissue in the cortex and adjacent phloem is described for the first time. Methods chosen herein to section and stain stem tissue did not result in slides of tissue of quality needed to describe the structure of bark in detail.

Introduction

Dirca L. is a genus of four species (*D. palustris* L., *D. occidentalis* Gray, *D. mexicana* Nesom & Mayfield, and *D. decipiens* Floden) and the only extant member of the cosmopolitan family Thymelaeaceae native to the continental United States. *Dirca occidentalis* is endemic to the wooded hills around the San Francisco Bay, California, United States of America (Vogelmann 1953). *Dirca mexicana* is endemic to the Sierra Madre Oriental of Tamaulipas, Mexico (Nesom & Mayfield 1995). *Dirca decipiens* is endemic to Johnson County, Kansas and Ozark Highlands, Carroll County, Arkansas, United States of America (Floden *et al.* 2009). *Dirca palustris* has the greatest distribution ranging from southern Ontario, Canada to northern Florida, United States

of America, west to eastern North Dakota, eastern Oklahoma, and central Louisiana, United States of America (Floden *et al.* 2009).

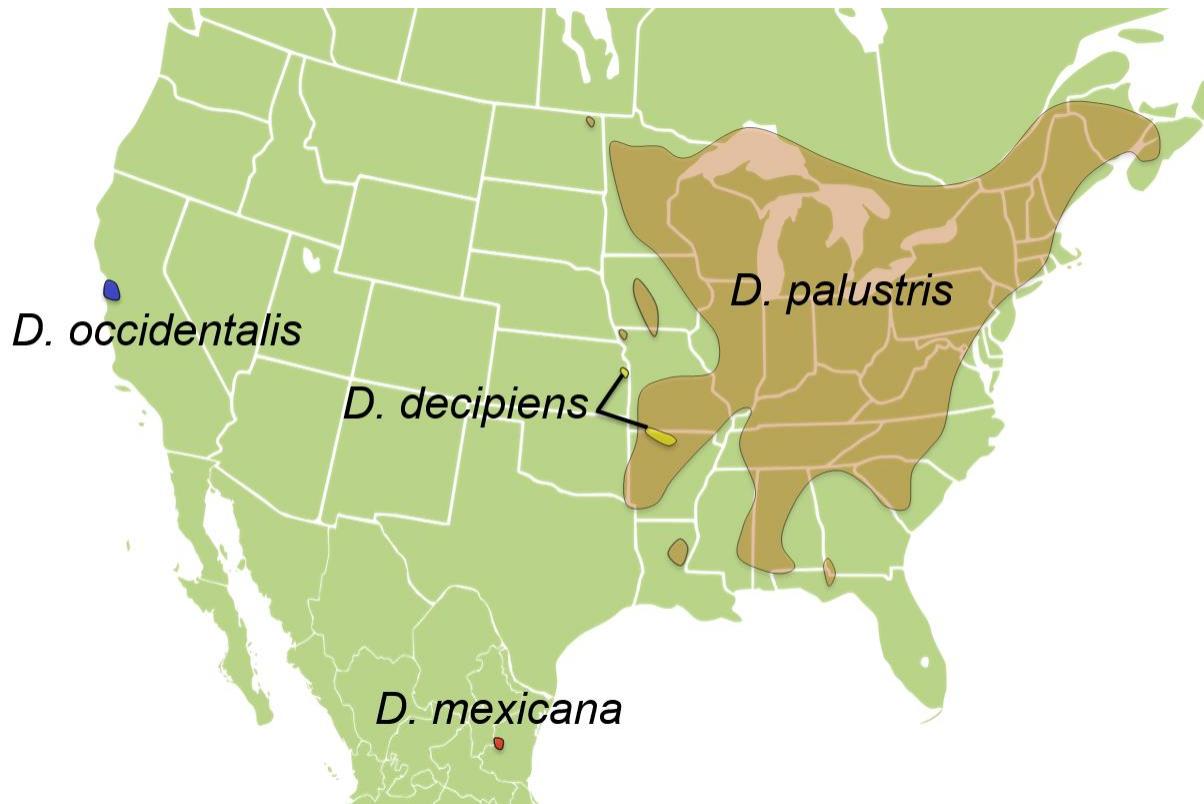


Figure 1. Geographical range of species of *Dirca*. Map courtesy of Bryan Peterson.

Commonly known as leatherwoods, identification of these deciduous shrubs is aided by their occurrence in shaded understory niches, as well as their yellow flowers in winter and early spring, yellow autumnal foliage, and small-arborescent forms. At maturity, the species reach up to two meters in height (Norris 2011).

Dirca spp. are characterized by flexible wood and a strong fibrous bark that resists tearing perpendicular to its axis. Branches can be tied into a bowknot (Fig. 2), without breaking, while attached to the body of the plant. If one tried to snap off a branch, the wood may break, but the bark would remain attached to the body of the plant and can be peeled all the way down to the

base of the plant (Anderson 1933). These peculiar characteristics have been acknowledged for centuries (Mottiar 2012). The bark, defined as all tissues external to the vascular cambium, of *D. palustris* has been used for cordage by several Native American peoples (Moerman 1998). Baby cradles used archeologically were constructed with bark of *D. palustris* and were found during an archaeological dig at Montgomery Shelter 3, Ozark Bluffs, Arkansas, United States of America (Dellinger 1936). A population of *D. decipiens* in Arkansas occurs within the range of *D. palustris*. Although the bark was considered to be *D. palustris* at the time, it may actually have been *D. decipiens* (Floden *et al.* 2009). Coincidentally, humans are not the only animal to use the bark of *Dirca* spp. Vestal (1938) reported nests of dusky-footed wood rats (*Neotomo fuscipes* Baird) are made with the bark of *D. occidentalis*. Photographic evidence of putative harvesting by these rodents of bark and wood is presented in figure 3.



Figure 2. One year old branch of *Dirca occidentalis* tied into a knot.



Figure 3. Putative harvesting of bark of *Dirca occidentalis* by the dusky-footed wood rat (*Neotoma fuscipes* Baird).

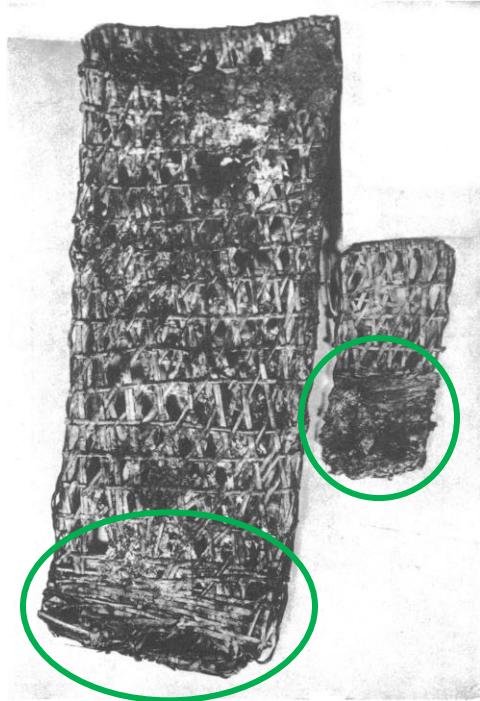


Figure 4. Baby cradles constructed with the bark of *Dirca palustris* (circled) recovered from Montgomery Shelter 3, Ozark Bluffs, Arkansas, United States of America.

Differentiation between species of *Dirca* has been based on foliar vestiture, perianth and reproductive morphology, pedicel elongation, and DNA sequencing (Vogelmann 1953; Floden *et al.* 2009). Leaf, stem, and root anatomy of *D. palustris* has been investigated extensively by Van Tieghem (1893), Holm (1921), Choquette (1925), Leandri (1930), and Metcalfe and Chalk (1950). Mature wood anatomy of *D. occidentalis* has been characterized with transverse sections by McMinn and Foderhase (1935). To date, no anatomical study has been performed for *D. mexicana* or *D. decipiens*.

Anatomy of bark of *Dirca palustris*

Only the anatomy of bark of *D. palustris* has been described, thus the description is used to generalize the anatomy of the bark of the genus. The anatomy of secondary bark of *D. occidentalis*, *D. mexicana*, and *D. decipiens* will be described and compared to *D. palustris*. Choquette (1925) describes the phellem tissue as a layer significantly thinner than the remaining cortex and phloem tissue, with an exfoliating outermost layer. Collenchyma present in the primary stem are replaced with average-sized, tangentially elongated, thin-walled cells which increase in size as we advance towards the pith. Choquette (1925) uses the term “pericycle” to refer to the outermost layer of phloem fibers of a mature stem which are difficult to distinguish from bast [secondary phloem] fibers. The International Association of Wood Anatomists defines pericycle fibers as fibers located at the periphery of the vascular cylinder inside the innermost cortical layer and outside the primary phloem of a stem or branch (Angyalossy *et al.* 2016). We conclude the use of pericycle by Choquette (1925) to identify fibers which originated during primary growth. Van Tieghem (1893) and Choquette (1925) describes primary and secondary fibers to consist of three layers; primary wall, S1 and S2. None of the layers are lignified. Van

Tieghem (1883) notes as a fiber matures, the S2 layer thickens at the cost of the S1 layer which eventually disappears and then only two layers can be identified; primary wall and secondary wall. However, Choquette (1925) was able to identify three layers of equal thickness in mature fibers. Fibers are described to have a length of 3–5 mm, a diameter of 5–10 µm, and blunt or sharp terminal ends (Choquette 1925). Phloem is described as stratified rings which separate into cones that terminate in points towards the periphery (Fig. 5) (Choquette 1925). The inverted complementary cones of the phloem consist of parenchyma with uni-/multiseriate rays (Fig. 5) (Choquette 1925). In transverse section, the stratified rings of phloem are separated by stratified rings of phloem fibers, the later rings having a thickness 2–3 times that of the rings of phloem (Fig. 5).

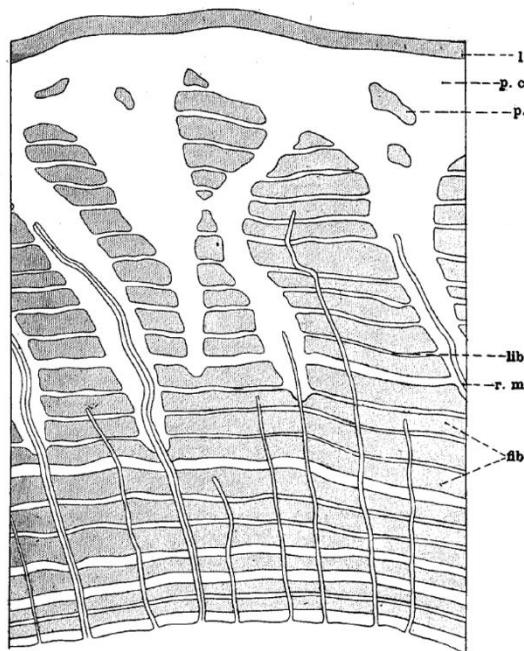


Figure 5. Transverse schematic of mature bark of *Dirca palustris*. l., phellem; p. c., cortical parenchyma; p., pericyclic fiber; lib., secondary phloem; r. m., ray; fib., phloem fiber.

Figure taken from Choquette, 1925.

In tangential section, rays are 1–4 cells thick and 5–20 cells in length, fibers and phloem parenchyma elongate in the axial direction with undulating wall thickenings, and large quantities of thin, prismatic calcium oxalate crystals (Fig. 6) (Choquette 1925).

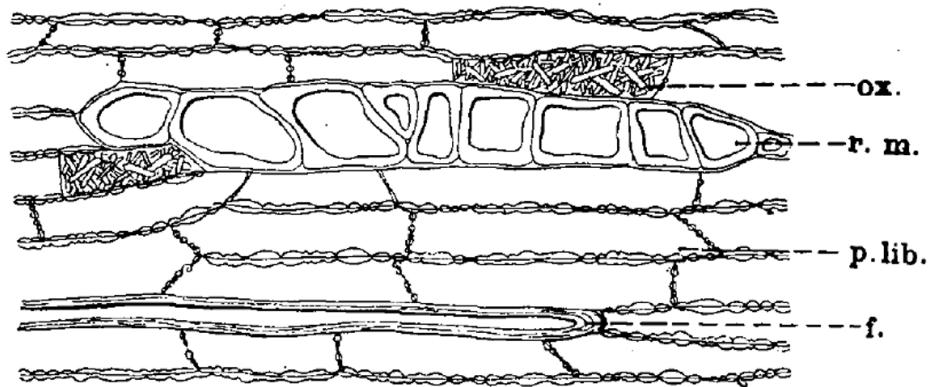


Figure 6. Tangential schematic of mature bark of *Dirca palustris*. ox., oxalate crystal; r. m., ray; p. lib., phloem parenchyma; f., phloem fiber. Figure taken from Choquette, 1925.

Our study aims to present, for the first time, a detailed description of the anatomy of the bark of *D. occidentalis*, *D. mexicana*, and *D. decipiens*. We will address the discrepancy regarding the number of cell wall layers of mature phloem fibers of *D. palustris*. Additionally, anatomical characteristics of bark will be examined to identify, if any, characters which differentiate the species.

Materials & Methods

Collection

A single four-year-old stem segment, at least 1 cm in length, was collected from 3, 4, or 5 individual plants of a single population. Species, location, number of individual plants sampled, tissue age, and date collected are listed in table 1. All species, except *D. mexicana*, were collected from native populations. *Dirca mexicana* was collected from cultivated populations in Ames, Iowa and Kingston, Rhode Island, United States of America. Segments were fixed in

formalin-acetic-alcohol (FAA). The age of a stem was determined by skipping the current year's growth and counting back to the fifth bud-scale scar.

Table 1. Location, number of individual plants sampled, tissue age, and date collected for species of *Dirca*. All species, except *Dirca mexicana*, were collected from native populations in the United States of America. Cultivated *Dirca mexicana* were grown from seeds collected from Sierra Madre Oriental of Tamaulipas, Mexico.

Species	Location	Individual Plants Sampled	Tissue Age	Date Collected MM/DD/YYYY
<i>Dirca palustris</i>	Jersey Valley County Park, Wisconsin	4	4	09-26-2016
<i>Dirca palustris</i>	Rainbow Lake Wilderness, Wisconsin	4	4	09-26-2016
<i>Dirca palustris</i>	Androscoggin County, Maine	3	4	06-12-2016
<i>Dirca palustris</i>	Torreya State Park, Florida	3	4	08-11-2016
<i>Dirca palustris</i>	Cavalier County, North Dakota	5	4	08-20-2016
<i>Dirca occidentalis</i>	San Mateo County, California	5	4	02-24-2016
<i>Dirca mexicana</i>	Ames, Iowa	5	4	09-25-2016
<i>Dirca mexicana</i>	Kingston, Rhode Island	4	4	05-25-2016
<i>Dirca decipiens</i>	Overland Park Arboretum, Kansas	4	4	03-12-2016
<i>Dirca decipiens</i>	Carroll County, Arkansas	4	4	03-13-2016

Sectioning and staining

Tissues were sectioned using modifications to the ethylenediamine and polyethylene glycol 1500 (PEG) technique described by Barbosa *et al.* (2010). Stem segments 3 mm thick were placed into an aqueous 10% ethylenediamine (v/v) solution at 23°C for four days. Samples were rinsed in deionized water for 2 hours to remove ethylenediamine. Samples were placed in vials of aqueous solutions of increasingly concentrated PEG (w/v) in 10% increments, from 10–100%, for 24 hours in each solution. To keep PEG from solidifying, vials were placed into a 60°C oven. Samples were transferred to a mold and embedded in pure PEG. Molds solidified for 24 hours at 23°C. Transverse, radial, and tangential sections of bark were made with rotary microtome (Reichert-Jung 2050 Supercut, Reichert Technologies, Depew, New York, United States of America). Sections were cut 15 µm thick with the knife set at a 10° angle, at a sectioning speed of 35 mm s⁻¹. Strips of Scotch Greener packaging tape (3M, St. Paul, Minnesota, United States of America) 1 mm wider than the embedded tissue and 7–10 cm long were used as an anti-tearing support. One end of the tape strip was pressed on the exposed tissue block with a finger; the other end was held while sectioning. A single drop of Haupt's adhesive (Haupt 1930) and two drops of an aqueous 5% formaldehyde solution(v/v) were place on cleaned slides, mixed, and spread to cover the entire slide (Van Horne & Zopf 1951). Tape-backed sections were trimmed and placed tape side up on this adhesive solution. Slides were dried for 24 hours with the tape side down on top of filter paper on a slide warmer at 42°C. Pressure was applied to the slides by using lead weights (Bongo 1961).

A preliminary study identified the polychromatic stain toluidine blue O differentiate cell types by color (Fig. 7)(O'Brien 1964). Slides were submerged in xylenes (Thermo Fisher

Scientific, Waltham, Massachusetts, United States of America) for 8 hours to remove tape support. Samples were submerged in an aqueous 0.05% toluidine blue O (w/v, pH 7) solution for 2 minutes followed by a 2 minute rinse in deionized water (O'Brien *et al* 1964). Slides were mounted in Permount (Thermo Fisher Scientific, Waltham, Massachusetts, United States of America). After two days of drying the slides became cloudy inhibiting the view of the stained tissue, so a different technique was chosen. Slides were submerged in xylenes for 8 hours to remove tape support. Samples were hydrated for 1 minute in each of 100%, 95%, 70%, and 50% ethanol, and deionized water. Samples were submerged in an aqueous 0.05% toluidine blue O solution (w/v, pH 7) for 2 minutes followed by 3 serial rinses in tap to remove excess stain. Samples were dehydrated in 70%, 95%, and 100% ethanol, 5 dips in each solution, followed by 10 dips in 1:1 (xylenes:100% ethanol), and submerged in three solutions of xylenes, 5 minutes each. Slides were mounted in Permount.

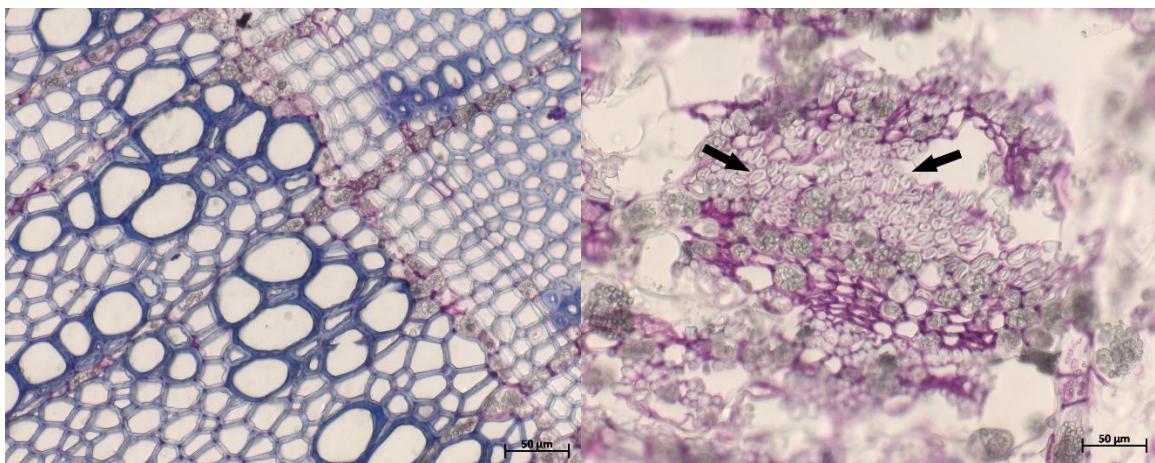


Figure 7. Transverse sections of a mature stem of *Dirca mexicana* stained with toluidine blue O. Secondary xylem (left). Secondary phloem (right), red-purple color indicates an un-lignified compound middle lamellae. Fibers (arrow) are unstained. -- Scale bar = 50 μ m.

Histochemistry

We tested for the presence of lignin within the cell wall of fibers of bark of *D. mexicana* using several histochemical techniques. Phloroglucinol (Wiesner reaction), methyl red, and potassium permanganate (Maule test) reactions described by Johansen (1940) and toluidine blue O reaction described by O'Brien *et al.* (1964) were used to detect the presence of lignin.

Maceration

Bark tissue was macerated by boiling in an aqueous 1.3% sodium carbonate solution (w/v). Stem sections 0.5 cm in width were cut from FAA fixed tissue. Bark was peeled away from the adjacent wood tissue and placed into test tubes containing 10ml of an aqueous 1.3% sodium carbonate solution (w/v). Test tubes were placed into a test tube rack submerged in a pot of water. The pot was heated and the bark tissue was boiled until the tissue broke down into a slurry when agitated with a glass stir rod. Tissues were placed into centrifuge tubes, washed with deionized water and decanted. An aqueous 0.5% safranin solution (w/v) was added to centrifuge tubes for 24 hours. Tissue was removed with forceps and washed twice in deionized water. Tissue was mounted as described above (Van Horne & Zopf 1951). Tissue was placed onto a slide, teased apart using two dissection needles. Slides were dried, uncovered, for 1 hour on a slide warmer at 42°C. Once dried, slides were mounted with Permount.

Analysis

Bark terminology used follows that of Angyalossy *et al.* (2016). Cellular dimensions were quantified using ImageJ (National Institutes of Health, Bethesda, Maryland, United States of America) image processing program. Images were spatially calibrated by tracing a line over an image's scale bar to set a pixel to unit ratio. Dimensions for sieve-tube elements, companion

cells, and axial parenchyma were viewed as radial sections. Fibers were viewed as a maceration. Vascular arrangement and phellem were viewed as transverse sections. Rays were viewed as tangential sections. Cellular dimensions were analyzed using R statistical software (R Core Team, Vienna, Austria) RRPP package version 0.3.0 (Collyer and Adams, 2018). Data was analyzed using a perANOVA. Differences between species were determined using pairwise contrasts, $\alpha = 0.05$.

Results & Discussion

Anatomy

Sectioning using modifications to the ethylenediamine and polyethylene glycol 1500 (PEG) technique described by Barbosa *et al.* (2010) did not result in slides of quality required for anatomical study. Crushing and tearing of tissue occurred in transverse, radial, and tangential sections (Fig. 8). Sections stained with toluidine blue O did not differentiate by cell types as reported by O'Brien *et al.* (1964), except phellem cells. All cell types were stained inconsistently blue, with some cells without color (Fig. 8), phellem was colored yellow, green, and blue (Fig. 9)

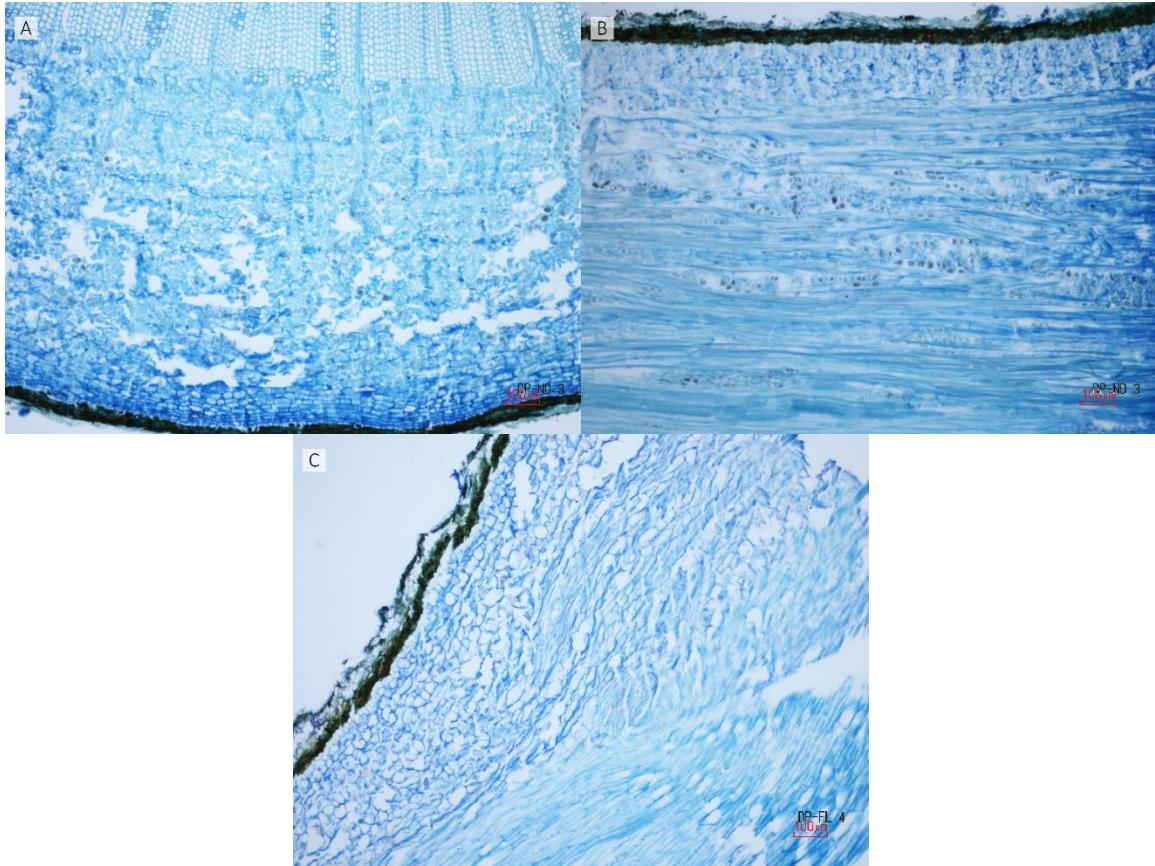


Figure 8. Transverse (A), radial (B), and tangential (C) sections of *Dirca palustris* stained with toluidine blue O. Tissues are damaged, cells are not differentiated by type, and are stained an inconsistent blue. – Scale bar = 100µm.

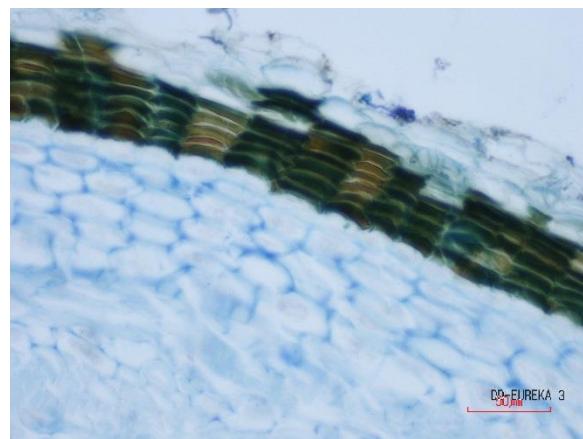


Figure 9. Transverse section of *Dirca decipiens* stained with toluidine blue O. Phellem consists of tangentially elongated cells with a convex shape, 4–7 cell layers thick. – Scale bar = 50µm.

Monochrome staining, and damaged cells caused difficulties identifying cell types. We were able to identify fibers, sieve-tube elements, axial parenchyma, and phellem cells. Individual cells and the arrangement of rays, companion cells, cortical parenchyma, and phelloderm could not be reliably identified because cell wall boundaries could not be identified due to damaged or uncolored tissue. No cellular trait was different among all species of *Dirca*. Observed differences were only between *D. mexicana* and other species. Diameter and length of fiber, sieve-tube element, axial parenchyma, and tangential length of phellem are presented in table 2. Raw anatomical data table is available in Appendix B.

Values are expressed as the mean \pm standard deviation. *Dirca palustris*. *Fibers*. We viewed bundles of fibers with each cell's terminal end to be blunt, but determined cuts made with our razor blade were the cause. Fibers terminated to narrow points typical of intrusive growth (Fig.10). Fiber diameter was $9.78 \pm 2.40 \mu\text{m}$ and length was $2.529 \pm 0.925 \text{ mm}$. *Sieve-tube element*. Sieve-tube elements were observed as a single element or in bundles of 2 or 3. Diameter was $6.66 \pm 1.59 \mu\text{m}$ and length $57.37 \pm 15.66 \mu\text{m}$. *Axial parenchyma*. Diameter was $15.13 \pm 5.23 \mu\text{m}$ and length $34.97 \pm 7.46 \mu\text{m}$. *Phellem*. Phellem cells were tangentially elongated with a convex shape in rows of 4–7 cells; tangential length was $22.76 \pm 3.09 \mu\text{m}$. Primary xylem was present in the cortex and adjacent phloem layers in half of the plants collected from Torreya State Park, Florida, and Rainbow Lake Wilderness, Wisconsin, United States of America (Fig. 11).

Dirca occidentalis. *Fibers*. We viewed bundles of fibers with each cell's terminal end to be blunt, but determined cuts made with our razor blade were the cause. Fibers terminated to narrow points typical of intrusive growth (Fig.10). Fiber diameter was $9.61 \pm 2.27 \mu\text{m}$ and length

was 2.611 ± 0.591 mm. *Sieve-tube element*. Sieve-tube elements were observed in bundles of 3.

Diameter was 6.80 ± 0.93 μm and length 26.13 ± 5.59 μm . *Axial parenchyma*. Diameter was

17.90 ± 5.14 μm and length 35.90 ± 7.55 μm . *Phellem*. Phellem cells were tangentially

elongated with a convex shape in rows of 4–7 cells; tangential length was 21.03 ± 1.52 μm

Dirca mexicana. *Fibers*. We viewed bundles of fibers with each cell's terminal end to be blunt, but determined cuts made with our razor blade were the cause. Fibers terminated to narrow points typical of intrusive growth (Fig.10). Fiber diameter was 10.18 ± 2.54 μm and length was 2.882 ± 0.440 mm. *Sieve-tube element*. Sieve-tube elements were observed in bundles of 3. Diameter was 17.18 ± 13.41 μm and length 107.51 ± 88.53 μm . *Axial parenchyma*. Diameter was 35.75 ± 30.05 μm and length 103.88 ± 91.78 μm . *Phellem*. Phellem cells were tangentially elongated with a convex shape in rows of 4–7 cells; tangential length was 58.31 ± 55.56 μm

Dirca decipiens. *Fibers*. We viewed bundles of fibers with each cell's terminal end to be blunt, but determined cuts made with our razor blade were the cause. Fibers terminated to narrow points typical of intrusive growth (Fig.10). Fiber diameter was 10.76 ± 3.06 μm and length was 2.891 ± 0.702 mm. *Sieve-tube element*. Sieve-tube elements were observed as a single element. Diameter was 8.84 ± 1.6 μm and length 50.41 ± 10.27 μm . *Axial parenchyma*. Diameter was 16.20 ± 5.56 μm and length 46.42 ± 16.47 μm . *Phellem*. Phellem cells were tangentially elongated with a convex shape in rows of 4–7 cells; tangential length was 22.17 ± 7.37 μm . *Xylem*. Primary xylem was present in the cortex and adjacent phloem layers in half of the plants collected from Carroll County, Arkansas, United States of America (Fig. 11).

Van Tieghem (1893), Holm (1921), and Metcalfe and Chalk (1950) identified bicollateral vasculature producing intraxylary phloem around the pith in primary growth of young stems, but no mention of xylem originating from phloem or cortex tissues.

Environmental factors, such as temperature (Thomas *et al.* 2006) and photoperiod (Winstead 1971) have an effect on the cellular characteristics of woody plants. Since samples of *D. mexicana* were collected from cultivated populations grown in atypical conditions than its native population, and anatomical differences were only observed between *D. mexicana* and other species of *Dirca*, observed differences could be due to environment and not genetic difference. Evaluation of environmental effect on anatomical traits for species of *Dirca* is needed to further examine the observed differences.

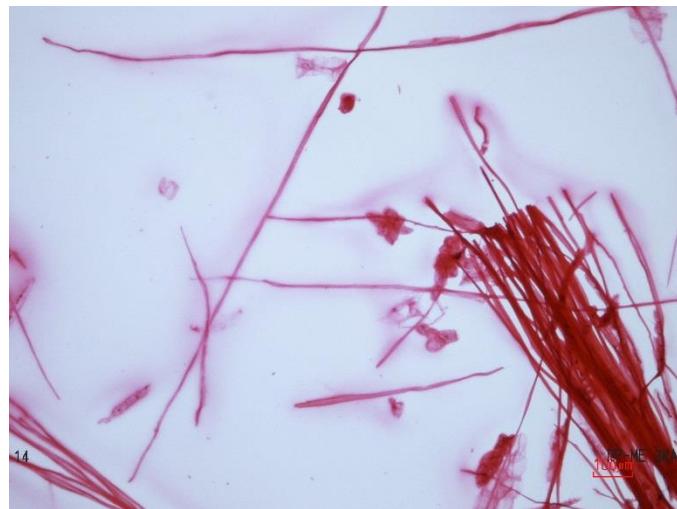


Figure 10. Bark maceration of *Dirca palustris* stained with an aqueous 0.5% safranin solution (w/v). Narrowed terminal ends of fibers. – Scale bar = 100µm

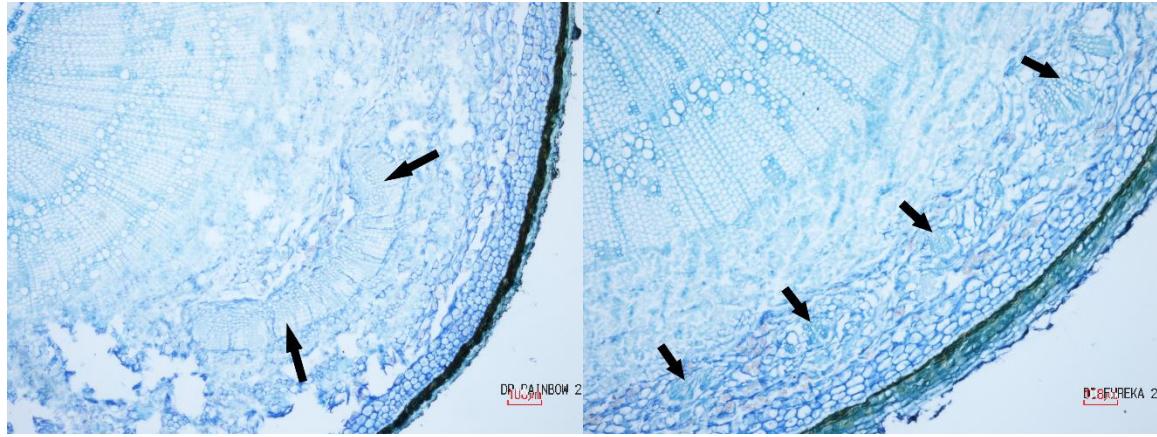


Figure 11. Transverse sections of stems of *Dirca palustris* (left) and *Dirca decipiens* (right) stained with toluidine blue O. Secondary xylem (arrows) present in cortex and adjacent phloem layers. -- Scale bar = 100 μ m.

Table 2. Diameter (D) and Length (L) of fiber, sieve-tube element, axial parenchyma, and phellem for species of *Dirca*. Values are mean \pm standard deviation.

Species	Cell Type			
	Fiber (D: μ m, L:mm)	Sieve-tube Element	Axial Parenchyma	Phellem
<i>Dirca palustris</i>	D: 9.78 ^a \pm 2.40 L: 2.529 ^a \pm 0.925	D: 6.66 ^a \pm 1.59 L: 57.37 ^a \pm 15.66	D: 15.13 ^a \pm 5.23 L: 34.97 ^a \pm 7.46	L: 22.76 ^a \pm 3.09
<i>Dirca occidentalis</i>	D: 9.61 ^a \pm 2.27 L: 2.611 ^a \pm 0.591	D: 6.80 ^{ab} \pm 0.93 L: 26.13 ^a \pm 5.59	D: 17.90 ^{ab} \pm 5.14 L: 35.90 ^a \pm 7.55	L: 21.03 ^{ab} \pm 1.52
<i>Dirca mexicana</i>	D: 10.18 ^a \pm 2.54 L: 2.882 ^a \pm 0.440	D: 17.18 ^b \pm 13.41 L: 107.51 ^b \pm 88.53	D: 35.75 ^b \pm 30.05 L: 103.88 ^b \pm 91.78	L: 58.31 ^b \pm 55.56
<i>Dirca decipiens</i>	D: 10.76 ^a \pm 3.06 L: 2.891 ^a \pm 0.702	D: 8.84 ^{ab} \pm 1.60 L: 50.41 ^{ab} \pm 10.27	D: 16.20 ^a \pm 5.56 L: 46.42 ^{ab} \pm 16.47	L: 22.17 ^a \pm 7.37

Column values with the same superscript letter are not significantly different ($\alpha = 0.05$).

Histochemistry

All reactions used to identify lignin within the cell wall of fibers of bark of *D. mexicana* were negative. Treatments with phloroglucinol and methyl red did not produce a red-violet color indicating the presence of lignin (Johansen 1940). Treatment with potassium permanganate (Fig. 12) did not produce a deep red color indicating lignin (O'Brien *et al.* 1964). However, treatment with potassium permanganate turned xylary elements brown, a result

typically indicating the presence of lignin in coniferous trees (Johansen 1940). Treatment with toluidine blue O (Fig. 12) stained bark tissue reddish-purple indicating collenchyma, parenchyma, and un-lignified compound middle lamellae (O'Brien *et al.* 1964). A negative reaction of any one test does not necessarily mean the absence of lignin, however we conclude the cell wall of fibers of bark of *D. mexicana* do not contain lignin based on multiple histochemical tests.

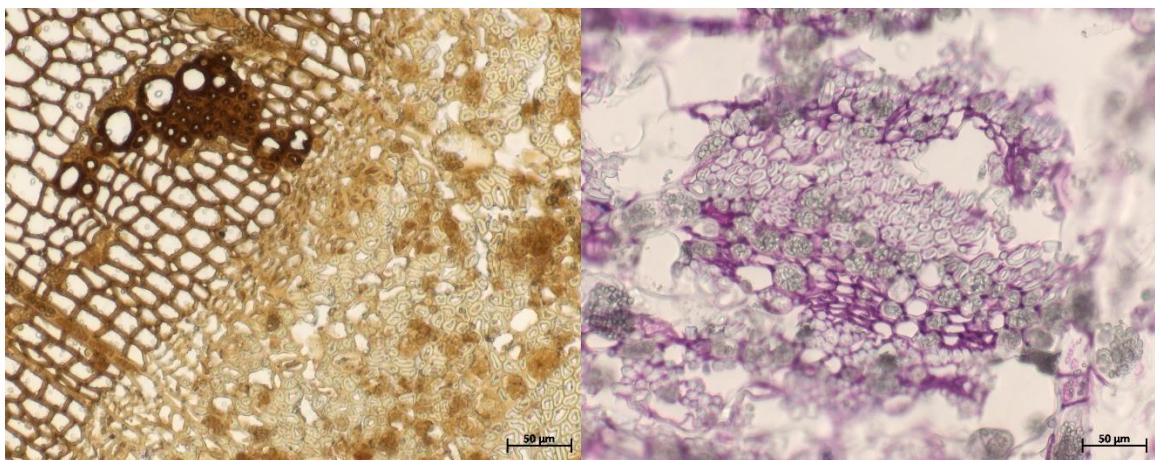


Figure 12. Transverse sections of secondary xylem and phloem of *Dirca mexicana*. Xylem and phloem treated with potassium permanganate (left). A deep-red color indicating lignin did not develop in either xylem or phloem tissue. Phloem treated with toluidine blue O (right). Tissue developed a reddish-purple color indicating collenchyma, parenchyma, and un-lignified compound middle lamellae. -- Scale bar = 50 μ m.

Conclusion

Sectioning using modifications to the ethylenediamine and polyethylene glycol 1500 technique described by Barbosa *et al.* (2010), and staining with toluidine blue O (O'Brien *et al.* 1964) did not result in slides of tissue of quality required for anatomical study. Use of additional histological and staining procedures need to be investigated to describe the structure of the bark of species of *Dirca* in detail. We are unsure why the solution of 0.05% toluidine blue O (w/v, pH 7) differentiated cells by color in our preliminary study (Fig. 7) but did not in the main study (Fig.8). Use of the same stain recipe but different staining procedures may have caused the

difference. However, use of a different staining procedure was required due to water and Permount becoming cloudy as the mounting medium dried.

Fibers, sieve-tube elements, axial parenchyma, and phellem cells are described as representative of the whole genus unless otherwise noted. Fiber diameter was similar between all species ($p = 0.402$) with range of 5.79–21.20 μm . Fiber length was similar between all species ($p = 0.162$) with range of 0.526–7.151 mm. We were unable to view the number of cell wall layers of fibers with our chosen methods. Fibers terminated to narrow points typical of intrusive growth. Choquette (1925) describes the diameter of fibers of *D. palustris* to range 5–10 μm , and length of fibers of *D. palustris* to range 3–5 mm, however we concluded larger ranges for diameter (5.79–21.20 μm) and length (0.53–7.15 mm). The cell wall of fibers of *D. mexicana* do not contain lignin, a feature shared with *D. palustris* (Choquette 1920) and other species of Thymelaeaceae (Van Tieghem 1893).

Sieve-tube elements occurred in bundles of 1–3 with a length ranged of 20.07–249.30 μm , and diameter range of 4.35–39.40 μm . The diameter and length of sieve-tube elements of *D. mexicana* were larger than *D. palustris* ($p = 0.002$ and $p = 0.018$, respectively). *Dirca mexicana* had longer sieve-tube elements than *D. occidentalis* ($p = 0.017$).

Axial parenchyma are rectangular with a diameter range of 8.79–84.09 μm , and length range of 23.14–271.18 μm . The diameter and length of axial parenchyma of *D. mexicana* were larger than *D. palustris* ($p = 0.008$ and $p = 0.001$, respectively). *Dirca mexicana* had larger diameter axial parenchyma than *D. decipiens* ($p = 0.045$) and longer axial parenchyma than *D. occidentalis* ($p = 0.032$).

Phellem stratified, composed of 4–7 rows of tangentially elongated (13.16–150.86 µm), convex shaped cells. Tangential length of phellem cells were different between *D. mexicana* and *D. palustris* ($p = 0.006$), and *D. decipiens* ($p = 0.012$).

We report the occurrence of secondary xylem originating in the cortex and adjacent phloem for the first time for the genus. Dedifferentiation may have occurred in the cortex and phloem tissue to initiate adventitious buds to develop into new shoots. Further investigation is needed to determine the origin and development of the xylem tissue.

Acknowledgments

We thank Dr. Bryan Peterson for collecting samples of *D. palustris* from Androscoggin County, Maine.

Literature Cited

- Anderson E. 1933. Leatherwood (*Dirca palustris*). Arnold Arboretum Bulletin of Popular Information 5, 25--27.
- Angyalossy V, Pace MR, Evert RF, Marcati CR, Oskolski AA, Terrazas T, Kotina E, Lens F, Mazzoni-Viveiros SC, Angeles G, Machado SR, Crivellaro A, Rao KS, Junikka L, Nikolaeva N, Baas P. 2016. IAWA list of microscopic bark features. IAWA Journal 37, 517--615.
- Barbosa ACF, Pace MR, Witovisk L, Angyalossy V. 2010. A new method to obtain good anatomical slides of heterogeneous plant parts. IAWA Journal 31: 373--383.
- Bonga JM. 1961. A method for sectioning plant material using cellulose tape. Canadian Journal of Botany 39: 72--730.
- Choquette L. (1925). Contribution a l'étude du *Dirca palustris* L. ou "bois de plomb." Ph.D. Thesis, Université de Paris.
- Collyer ML, Adams DC. 2018. RRPP: An R package for fitting linear models to high-dimensional data using residual randomization. Methods in Ecology and Evolution 9: 1772--1779.
- Dellinger SC. 1936. Baby cradles of the Ozark Bluff dwellers. American Antiquity 1, 197--214.

- Floden AJ, Mayfield MH, Ferguson CJ. 2009. A new narrowly endemic species of *Dirca* (Thymelaeaceae) from Kansas and Arkansas, with a phylogenetic overview and taxonomic synopsis of the genus. *Journal of the Botanical Research Institute of Texas* 3, 485--499.
- Haupt AW. 1930. A gelatin fixative for paraffin sections. *Stain Technology* 5: 97--98.
- Holm T. 1921. Internal structure of the vegetative organs of *Dirca palustris*. *American Journal of Science, Fifth Series* 2: 177--82.
- Johansen DA. 1940. Plant microtechnique. New York: McGraw-Hill.
- Leandri J. 1930. Recherches anatomiques sur la Thyméléacées. *Annales des Sciences Naturelles* 12:125--237.
- McMinn HE, Forderhase B. 1935. Notes on western leatherwood, *Dirca occidentalis* Gray. *Madroño* 3: 117--120.
- Metcalfe CR, Chalk L. 1950. Anatomy of the dicotyledons Vol. 2. Oxford: Clarendon Press.
- Moerman DE. 1998. Native American ethnobotany. Portland, OR: Timber Press.
- Mottiar Y. 2012. On the discovery of eastern leatherwood (*Dirca palustris*). *Canadian Field-Naturalist* 126: 86--88.
- Nesom GL, Mayfield MH. 1995. A new species of *Dirca* (Thymelaeaceae) from the sierra of northeastern Mexico. *SIDA* 16: 459--467.
- Norris KD. 2011. Horticultural & ecophysiological evaluations of leatherwoods (*Dirca* spp.). Master Thesis. Iowa State University.
- O'Brien TP, Feder N, McCully ME. 1964. Polychromatic staining of plant cell walls by toluidine blue O. *Protoplasma* 59: 367--373.
- Thomas DS, Montagu KD, Conroy JP. 2006. Temperature effects on wood anatomy, wood density, photosynthesis and biomass partitioning of *Eucalyptus grandis* seedlings. *Tree Physiology* 27: 251--260.
- Van Horne RL, Zopf LC. 1951. Water-soluble embedding materials for botanical microtechnique. *Journal of Pharmaceutical Sciences* 40: 31--34.
- Van Tieghem. 1893. Recherches sur la Structure et les affinité des Thyméléacées et des Pénacées. *Annales des Sciences Naturelles* 7: 185--294.
- Vestal EH. 1938. Biotic relations of the wood rat (*Neotoma fuscipes*) in the Berkeley hills. *Journal of Mammalogy* 19: 1--36.

Vogelmann H. 1953. A comparison of *Dirca palustris* and *Dirca occidentalis* (Thymelaeaceae). Asa Gray Bulletin 2: 77--82.

Winstead JE. 1971. Fiber tracheid length and wood specific gravity of seedlings as ecotypic characters in *Liquidambar styraciflua* L. Ecology 53: 165--172.

CHAPTER 5. FROM DIRCA TO DESIGN: PRINTMAKING WITH *DIRCA MEXICANA* BARK PAPER

A paper to be submitted to the Journal of Visual Art Practice

Zachary J. Hudson¹, Andrew J. Zandt², April Katz², William R. Graves¹

¹Department of Horticulture, Iowa State University, Ames, IA 50011

²Department of Art and Visual Culture, Iowa State University, Ames, IA 50011

Abstract

Washi is paper made by hand from the bark of native Japanese shrubs. The paper is strong, semi-transparent, and resistant to insects and aging. *Washi* is a common medium used for printmaking, clothing, banknotes, and explosives. Artists who have studied *nagashi-zuki*, a sheet-forming method unique to *washi*, often import Japanese fibers because alternatives with similar properties have not been identified. We propose *Dirca* L. (leatherwood), a shrub endemic to North America, as a source of fibers with properties similar to those plants traditionally used to make *washi*. A modified double-fold endurance test indicate that *Dirca mexicana* (Mexican leatherwood) Nesom & Mayfield bark paper we made by hand withstands repeated bending, folding, and creasing better than paper made from *Wikstroemia* sp. (Japanese fiber), suggesting an alternative as good as or better for use with various printmaking techniques and paper arts and crafts that involve folding. We engaged emerging and professional printmakers in creating original prints on our *D. mexicana* bark paper, and evaluated how the paper responds to ink, pressure, and chemicals used in various printmaking techniques via rating-scale surveys and linear measurements. We identified *D. mexicana* as a North American source of fibers with similar properties to *Wikstroemia* spp. used to make gampi *washi*. Handmade *D. mexicana* bark paper was successfully used as a paper medium for intaglio,

lithography, relief, digital, and screenprinting printmaking techniques, as well as calligraphy ink, ink markers, gouache, and acrylic paint.

Introduction

Korean Buddhist monks are credited with introducing Chinese paper and the art of papermaking to Japan during the 5th and 6th century. *Washi*, Japanese handmade paper, is a product uniquely Japanese (Hughes 1982) and valued for its strength, semi-transparency, and resistance to insects and aging. These desirable properties are provided by fibers from the barks of shrubs native to Japan in the genera *Broussonetia* L'Her. ex Vent., *Edgeworthia* Meisn., and *Wikstroemia* Endl. The properties have allowed *washi* to become a common medium used for printmaking, clothing, banknotes, and explosives (Barrett 1983).

While teaching the authors how to make *nagashi-zuki* paper, Timothy Barrett, Director, Center for the Book, University of Iowa mentioned *nagashi-zuki* papermakers are generally interested in alternative species as a source of fiber. American artist Winifred Lutz was once told by a Japanese papermaking, Kubota Yasuichi, that he could not understand why Americans who studied *nagashi-zuki* insisted on importing Japanese fibers once they had returned to their home studios because the cost did not make economic sense. Yasuichi suggested there must be a plant native to the United States which would yield fibers comparable to kozo (*Broussonetia* spp.), mitsumata (*Edgeworthia* spp.), and gampi (*Wikstroemia* spp.), and whose use would create a distinctive American style *nagashi-zuki* paper (Barrett 1983). Our search for a North American fiber similar to those native to Japan led us to *Dirca* L. because of its familial affiliation and the documented uses of its bark.

Edgeworthia, *Wikstroemia*, and *Dirca* belong to the same plant family, Thymelaeaceae. A common-name for the family is the fiber-bark or rope-bark family, aptly named because genera such as *Dais* L. (Koekemoer *et al.* 2014), *Daphne* L. (Polunin & Stainton 1984; Paul *et al.* 2006), and *Eriosolena* Blume (Gamble, 1902) are used around the world to create paper. Other genera of the family are used to create clothing and cordage (Gamble 1902; Polunin & Stainton 1984; Pooley 2006; Koekemoer *et al.* 2014). Like other members of the family, *Dirca* spp. have flexible wood and strong, fibrous, tear-resistance bark.

Dirca spp. are characterized by strong, fibrous, tear-resistant bark that has been used by Native Americans to create cordage (Gilmore 1933; Smith 1933a & 1933b; Dellinger 1936). We propose *Dirca* L., a shrub endemic to North America, as a source of fibers similar to those native to Japan.

We are not the first investigators to identify a plant for papermaking based on familial affiliation and ethnobotanical use. Mitnan (*Thymelaea hirsute* (L.) Endl.) was chosen as a local bark fiber for paper production in Beer Sheva, Israel because it is a member of the Thymelaeaceae, and the bark of the shrub is used by Bedouin people to make rope (Schmidt & Stavisky 1983). Although there is no documentation of *Dirca* spp. used by Native Americans to make handmade paper, artists and researchers have successfully made paper from the bark of *D. palustris* (Barrett 1983; Bell 1995).

To evaluate if the bark of *Dirca* spp. yields fibers comparable to fibers from the bark of shrubs native to Japan, we created handmade *D. mexicana* bark paper using the *nagashi-zuki* sheet-forming method unique to washi. The quantity of paper needed for the project required 1.2 kg of dried bark. Some populations of *Dirca* spp. are considered endangered. To avoid

negatively impacting a native population, we chose to use *D. mexicana* due to access to several cultivated populations in Ames, IA and Haymarket, VA.

A modified double-fold endurance test was performed to quantify how the paper withstands repeated bending, folding, and creasing compared to mitsumata and gampi papers. Emerging and professional printmakers from Iowa State University and the city of Ames, IA were recruited to create an original design to print on our paper and a commercially available gampi paper. Printmakers were asked to print their design using at least one printmaking technique such as intaglio, lithography, relief, digital, and screenprinting. Along with the prints, printmakers were asked to write a response comparing their experience using the two papers. A rating-scale survey was used to evaluate the technical aspects of the paper and prints such as ink spread, consistency of ink, and texture. Transparency of the paper was quantified as light transmittance. Lastly, the color of the paper was matched with a Pantone color. Mitsumata and gampi papers were chosen as a control based on availability. We do not declare gampi as the best paper for any printmaking technique, but we know it can be used.

Materials & Methods

Bark collection

Stems of *D. mexicana* were collected from garden populations in Ames, IA and Haymarket, VA. Branches were scored and bark was stripped and hung to dry (Fig. 1). A total of 1.2 kg of dried bark was collected.

Bark preparation and papermaking

Dirca mexicana bark paper was made using the *nagashi-zuki* sheet-forming method. With the assistance of the University Print Society at Iowa State University, the paper was made

at the Center for the Book, University of Iowa papermaking facility. Dried bark was soaked in water for 2 hours until it was no longer rigid. Black bark (outermost layer) was removed by scraping with the blunt side of a knife (Fig. 2). Roots of *Hibiscus manihot* L. were beaten flat with a mallet and introduced to water to create *neri*, a viscous mucilage used to deflocculate bark fibers and control drainage during sheet-formation (Fig. 3). Green and white bark (bast tissue) were boiled in an aqueous 1.3% sodium carbonate solution (w/v) until the tissue was able to be pulled apart in the axial and radial directions, around 2 hours. After cooking, the bark is rinsed in water to remove remaining sodium carbonate solution (Fig. 4). Green and white bark were suspended in water and remaining pieces of black bark, defects, and debris were removed by hand (Fig. 5). Green and white bark were beaten with hand mallets for 30 minutes to further separate fibers (Fig. 6). Small amounts of green and white bark were added to a vat containing water and *neri*, and agitated to create a homogenous fiber solution. Sheet of paper were formed using a *su*, a bamboo woven mat, set inside a deckle mold. The mold was dipped into the vat and sheets formed by moving the mold in an oscillating sloshing motion (Fig. 7). With a sheet of paper adhering to it, the *su* is removed from the mold, inverted, and lowered over felt with a rolling motion. Additional sheets are placed on top of each other, separated by layers of felt (Fig. 8). A stack of sheets of paper were placed into a screw press to remove excess water. Sheets were separated from the layers of felt and brushed onto the surface of a steam-heated dryer using a hand brush (Fig. 9). No steps were used to control the color of the paper. The Pantone studio iphone application version 3.0.19 (Pantone, Carlstadt, New Jersey, USA) was used to identify the color of the paper based on a digital image.



Figure 1. Removed bark of *Dirca mexicana* hung on line to dry.



Figure 2. Removal of black bark by scraping with the blunt side of a knife. Left Bucket: Bark tissue with outermost layer (black bark). Right Bucket: Removal of black bark results in green and white bark.



Figure 3. *Neri*, viscous mucilage formed when smashed roots of *Hibiscus manihot* are introduced to water.



Figure 4. Green and white bark boiled in an aqueous 1.3% sodium carbonate solution (w/v). Bark is pulled apart in the axial direction.



Figure 5. Cooked green and white bark suspended in water. Remaining black bark, defects, and debris is removed from tissue.



Figure 6. Green and white bark is beat with hand mallets to further separate adjacent fibers.



Figure 7. Sheet-forming using a *su* and deckle mold.



Figure 8. Couching of a sheet of *Dirca mexicana* paper onto a sheet of felt.



Figure 9. Drying sheets of *Dirca mexicana* paper hand crushed onto a steam-heated dryer.

Fold endurance and transparency

A modified folding endurance test was performed using a fold endurance tester (M.I.T., Tinius Olsen Testing Machine Company, Willow Grove, PA) according to the Technical Association of Pulp and Paper Industry (TAPPI) T 511 om-02 standard (TAPPI 2006). Samples were not collected in accordance with TAPPI T 400 standard for sampling and accepting a single lot of paper, paperboard, fiberboard, and related products because the paper was handmade. Instead, 27 samples measuring 13 x 133 mm were cut parallel to the fiber direction from samples of mitsumata, gampi, and *D. mexicana* papers using a strip cutter. Samples were not conditioned according to the TAPPI T 402 standard due to lack of equipment. Samples were loaded into the folding head and clamp of the M.I.T. tester (Fig. 10) and a 600 g weight was used to set the tension applied to the paper samples. One complete oscillation of the folding head was counted as one double-fold. The folding head oscillated until the sample fractured at the point of the double-fold. Paper thickness was measured using a dial thickness gauge at the point of fracture.

Transparency of the *D. mexicana* paper was quantified by measuring the amount of light transmitted through sheets of paper by using a spectroradiometer (PS-100, Apogee Instruments, Logan, UT) and calculated with $T=I/I_0$ (T = transmitted light, I = light measured after treatment, I_0 = light measured before treatment). The spectroradiometer was enclosed in a wooden light box and positioned directly underneath an aperture. Photosynthetic active radiation (PAR) emitted by a 14 W compact fluorescent bulb entered the aperture and was measured before and after single sheets of paper were placed between the light source and spectroradiometer.

Photosynthetic active radiation measured through a single sheet of paper was divided by PAR measured without a sheet of paper and expressed as the average percentage of four replicates.

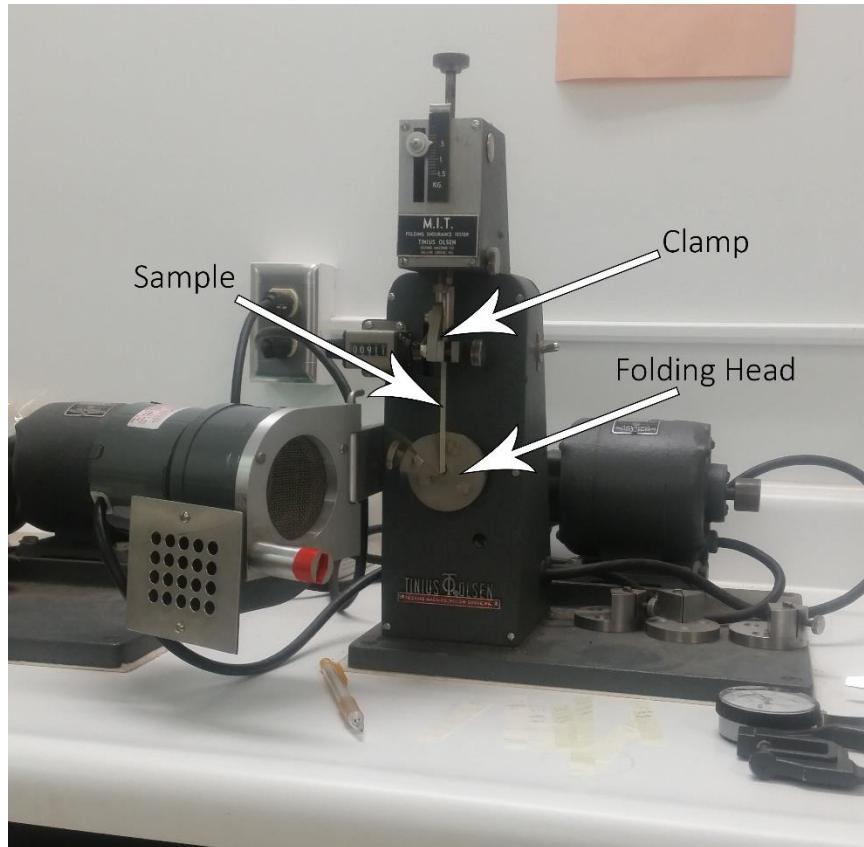


Figure 10. M.I.T. double-fold tester with a sample loaded in the folding head and clamp.

Printmaking

Participating artist were provided with sheets of *D. mexicana* and gampi papers, and given the freedom to choose the concept of their image, choose their preferred printmaking technique, and make any modifications to their printing ink. We required the artists to duplicate their print on both *D. mexicana* and gampi papers. Prints were created at the Printmaking Studio, College of Design, Iowa State University, or an artist's commercial or personal studio. The weight of the gampi and *D. mexicana* papers were 10 g m⁻².

Analysis

The fold endurance data was analyzed using an unpaired t-test with the Excel Analysis ToolPak (Office 365, Microsoft Corporation, Redmond, WA). Qualitative data such as a printmaker's opinion towards the paper, modifications to printing ink, and appealing characteristics of the paper were collected using an artist response survey. Eight evaluators used a rating-scale survey to quantify the degree of ink spread, consistency of ink, and texture by comparing identical prints on gampi and *D. mexicana* paper. Surveys were analyzed using R statistical software (R Core Team, Vienna, Austria) likert package (Bryer *et al.* 2016). Transparency was expressed as a mean percent light transmittance of four independent sheets of paper.

Results & Discussion

Papermaking

We were successful in creating handmade bark paper from the bark of *D. mexicana* using the *nagashi-zuki* sheet forming method. Fifty-three 30 x 41 cm and fifty 28 x 37 cm sheets of *D. mexicana* bark paper were made at the Center for the Book, University of Iowa. The natural

color is compared to mitsumata and gampi papers in figure 11, and identified as Pantone rutabaga 12-0806 TCX. The texture of the paper was compared to Yupo (Yupo Corporation America, Chesapeake, VA), a synthetic paper made from polypropylene, and BFK Rives (Arjowiggins, Paris, France), a moldmade cotton rag paper. 57.1 % of evaluators described the texture of *D. mexicana* bark paper as between Yupo and BFK Rives, while 50% of evaluators rated the texture of gampi as more like Yupo (Table 2). The weight of our paper is 10 g m⁻².



Figure 11. Natural color comparison between mitsumata, gampi, and *D. mexicana* papers.

Fold endurance and transparency

Dirca mexicana withstands repeated bending, folding, and creasing better than mitsumata ($p < 0.001$) and gampi ($p < 0.001$) paper with a double-fold count approximately six times greater. However, the paper thickness was different between *D. mexicana* and mitsumata ($p = 0.006$). An interaction effect of paper thickness was not investigated because no ranges of thickness were tested. All samples were taken from sheets of paper with uniform thickness. Thus, we cannot support *D. mexicana* bark paper had a double-fold count greater than mitsumata. The *D. mexicana* bark paper has a transparency of 56%.

Table 1. Double-fold count and paper thickness of *Dirca mexicana*, mitsumata, and gampi papers. Values are the mean \pm standard deviation.

Paper Type	Double-fold Count	Paper thickness (mm)
<i>Dirca mexicana</i>	$1253^a \pm 352^*$	$0.046^a \pm 0.003^*$
Mitsumata	$216^b \pm 146^*$	$0.054^b \pm 0.006^*$
Gampi	$242^b \pm 213$	$0.047^a \pm 0.004$

Values with the same superscript letter are not significantly different ($\alpha = 0.05$).

Notation with * indicates significance of double-fold count cannot be supported because the thickness of the papers was different.

Printmaking

Select prints representing each printmaking techniques used are shown in figures 12–17.

Intaglio, relief, lithography, digital, and screenprinting techniques were successfully used with our *D. mexicana* bark paper. One printmaker also used calligraphy ink, ink markers, gouache, and acrylic paint (Fig. 13).

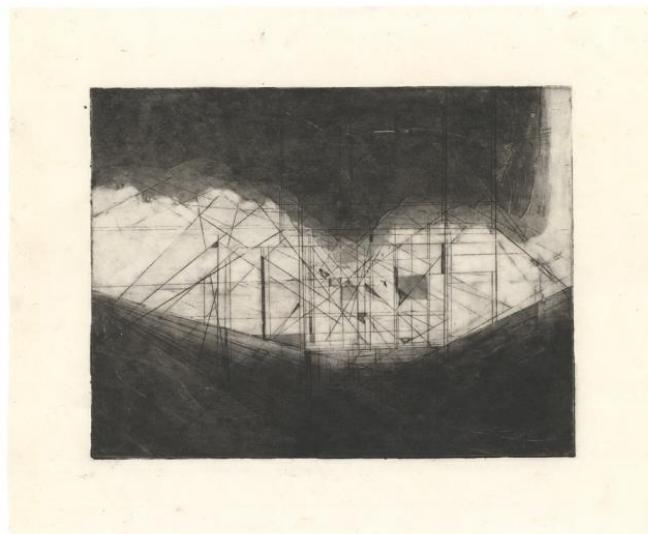


Figure 12. Hannah Becker. *Grand Ole Opry*. 2018. Intaglio. 18 x 23 cm.



Figure 13. Tibi Chelcea. *A Protocol for Packet Network Intercommunication #1*. 2018. Relief with calligraphy ink, ink markers, gouache, and acrylic paint. 27 x 18 cm.



Figure 14. Caleb Henkelman. *Doubt*. 2018. Relief. 26 x 19 cm.

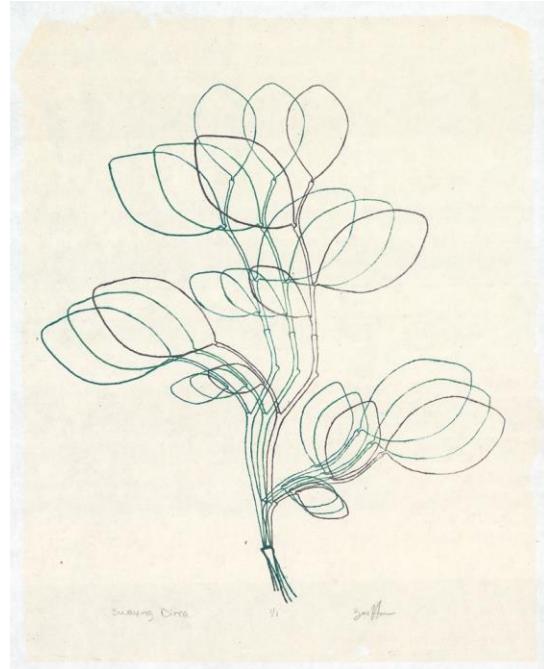


Figure 15. Zachary Hudson. *Swaying Circa*. 2018. Lithograph. 30 x 27 cm.

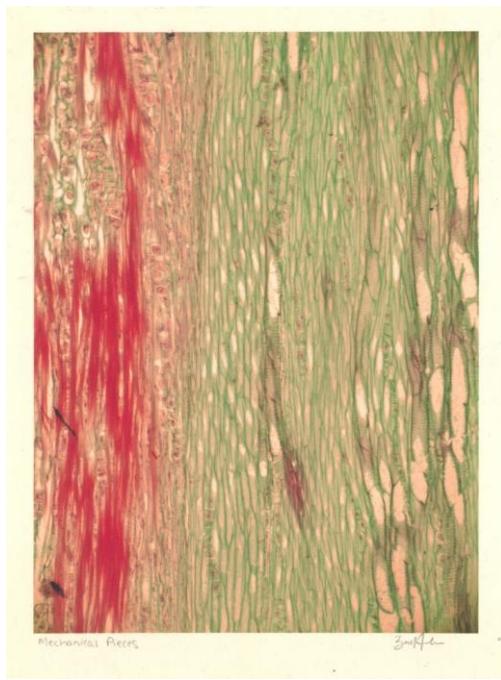


Figure 16. Zachary Hudson. *Mechanical Pieces*. 2018. Digital Inkjet. 28 x 22 cm.



Figure 17. Peter Lemken. *Luck's Got Nothing*. 2018. Screenprint. 40 x 30 cm.

Analysis

Participating printmakers valued the transparency, thinness, and texture of the paper.

Mixed experiences regarding the paper's response to ink were reported. Printmakers noted the thinness of the paper required less ink, which preserved the details of the print. Several printmakers experienced oversaturating the paper which caused the ink to bleed through the paper. Printmakers using techniques like intaglio, relief, and lithography commonly print images onto paper which has been saturated with water. Printmakers noted wet *D. mexicana* paper was not needed to successfully print an image. The paper wrinkles if wet and allowed to dry. When BFK paper is saturated with water and allowed to dry, the dimensions of the paper and image shrink. Use of *D. mexicana* paper preserved the true dimensions of the print. Another response commented on the texture of the paper. The fibers which make up the paper create a visual texture which adds depth to the final print.

Questions asked and results for rating-scale survey are presented in table 2. Comparing the crispness of edges and clarity of values of shapes, 75% of evaluators rated the digital print on gampi paper to most resemble the digital image compared to 25% of evaluators for *D. mexicana* paper. Comparing identical images on gampi and *D. mexicana* paper, 87.5% of evaluators rated the solid areas on the *D. mexicana* paper are more consistent. Comparing identical images on gampi and *D. mexicana* paper, 37.5% of evaluators rated solid lines as more consistent on *D. mexicana* paper compared to 12.5% for gampi. However, 37.5% of evaluators rated solid lines on gampi as between same consistency and more consistency. Comparing color and value of an image to its reverse side, 80% of evaluators rated color and value of the reverse side of the print to be almost the same for gampi and *D. mexicana* paper. Comparing the clarity of the reverse image of gampi to that of *D. mexicana*, 75% of evaluators rated the clarity as the same, rating gampi and *D. mexicana* papers' transparency allowing the same amount of clarity.

Table 2. Rating-scale survey used for evaluation of the technical aspects of a print. Results are expressed as percentage of evaluators to choose a response.

How would you compare the crispness of edges and clarity of values of the shapes on the *Dirca mexicana* paper printed image versus the digital image?

0	25%	75%	0	0
Most Resemblance				Least Resemblance

How would you compare the crispness of edges and clarity of values of the shapes on the gampi paper printed image versus the digital image?

0	75%	25%	0	0
Most Resemblance				Least Resemblance

How consistent are the solid areas of ink on the *Dirca mexicana* paper compared to the gampi paper?

87.5%	0	12.5%	0	0
Solid areas on the <i>Dirca mexicana</i> paper are more consistent		Same Consistency		Solid areas on the gampi paper are more consistent

Table 2 continued

How consistent are the solid lines of ink on the *Dirca mexicana* paper compared to the gampi paper?

37.5%	0	12.5%	37.5%	12.5%
Solid lines on <i>Dirca mexicana</i> print are more consistent		Same Consistency		Solid lines on gampi print are more consistent

Paying attention to the color and value of the image, how similar is the reverse side of the print on *Dirca mexicana* paper compared to its front?

0	0	0	20%	80%
Not at all the same				Almost the same

Paying attention to the color and value of the image, how similar is the reverse side of the print on gampi paper compared to its front?

0	0	0	20%	80%
Not at all the same				Almost the same

How clearly can you see the reverse side image of the print on *Dirca mexicana* paper compared to the print on gampi paper?

12.5%	75%	12.5%
Worse	Same	Better

On a scale from 1 to 5, where 1 is smooth like Yupo (synthetic paper) and 5 is coarse like BFK, rank the texture of the smoothest size of the *Dirca mexicana* paper.

0	42.9%	57.1%	0	0
Smooth like Yupo				Coarse like BFK

On a scale from 1 to 5, where 1 is smooth like Yupo (synthetic paper) and 5 is coarse like BFK, rank the texture of the smoothest size of the gampi paper.

25%	50%	25%	0	0
Smooth like Yupo				Coarse like BFK

Conclusion

Handmade *D. mexicana* had a double-fold count six times greater than gampi paper indicating it may also be used for paper art which uses bending, folding, and creasing, such as origami. The bark paper was successfully used as a paper medium to print images using intaglio, relief, lithography, digital, and screenprinting techniques. The paper is also receptive to calligraphy ink, ink markers, gouache, and acrylic paint. Transparency, thinness, and texture

were identified as the attractive characteristics of the handmade *D. mexicana* bark paper. The transparency allows background images to be seen through the paper which is an attractive characteristic for techniques such as chine-collé. The light weight of *D. mexicana* bark paper allowed less ink to be used, which preserved fine detail in the printed image.

Wetting the paper with water causes the paper to wrinkle, but intaglio, relief, and lithography prints were successfully printed without wetting the paper. Another advantage of not needing to wet the paper is the print retains its original dimensions unlike BFK Rives which shrunk in size as the paper dried. *Dirca mexicana* bark paper creates more consistent solid areas of color compared to gampi, although gampi creates more consistent solid lines of color. Transparency of *D. mexicana* bark paper was measured as 56%. Evaluators rated the transparency of gampi and *D. mexicana* equal, rating the color, value, and clarity of the reverse side of the print to be almost the same as the front.

The crispness of edges and clarity of values of shapes of a digital image printed with Inkjet ink on gampi paper was rated to resemble the digital image more than *D. mexicana* bark paper. Because of the light weight of the *D. mexicana* bark paper, taping the paper to a heavier paper backing is required. If not taped to a paper backing, the paper crumpled and was torn by the printer's feeding wheels.

We have identified *D. mexicana* as a source of fibers similar to *Wikstroemia* spp. (gampi) native to Japan, creating an American Asian-styled handmade bark paper which may be used as a paper medium for a variety of printmaking techniques, as well as paper art which involves bending, folding, and creasing.

Special Note

We ask that if you choose to create handmade bark paper from any species of *Dirca*, please be mindful of your impact when collecting bark. While the United States Fish and Wildlife Service does not list any species of *Dirca* as threatened or endangered, some state governments, such as Florida and Maryland, consider some populations of *Dirca* spp. as imperiled or endangered. We ask you do not collect from wild populations. Cultivated plants can be grown for the purpose of collecting bark.

Acknowledgements

We thank the Department of Art and Visual Culture, Iowa State University for use of the printmaking studio. Dr. Chris Currey allowed use of hydroponic tubs for sifting. Iowa State University Focus: Artist Grant Program provided funding to frame and exhibit final prints. University Print Society at Iowa State University for assisting with preparation of bark and forming of sheets of paper. Center for the Book, University of Iowa for allowing us to use their facilities to create our paper. Timothy Barrett for teaching us how to create paper using the *nagashi-zuki* sheet-forming technique.

Literature Cited

- Barrett T. 1983. Japanese papermaking: Traditions, tools, and techniques. New York: Weatherhill, Inc.
- Bell LA. 1995. Plant fibers for papermaking. McMinnville, Oregon: Liliaceae Press.
- Bryer J, Speerschneider K. 2016. likert: Analysis and visualization likert items. R package version 1.3.5.
- Dellinger SC. 1936. Baby cradles of the Ozark Bluff dwellers. American Antiquity, 1: 197--214.
- Gamble JS. 1902. A manual of Indian timbers. London: Sampson Low, Marston & Company.

- Gilmore MR. 1933. Some Chippewa uses of plants. Papers of the Michigan Academy of Science, Arts, and Letters, 7: 119--143
- Hughes S. 1982. Washi, the world of Japanese paper. Tokyo: Kodansha International.
- Koekemoer M, Steyn HM. Bester SP. 2014. Guide to plant families of southern Africa. Pretoria: Seriti Printing.
- Paul A, Arunachalam A, Khan ML, Arunachalam K. 2006. *Daphne papyraceae* Wall. ex Steud. ó [sic] a traditional source of paper making in Arunachal Pradesh. Natural Product Radiance 5: 133--138.
- Polunin O, Stainton A. 1984. Flowers of the Himalaya. Delhi: Oxford University Press.
- Pooley E. 2006. Forest plants: In the forest and in the garden. Durban: The Flora Publication Trust.
- Schmidt J, Stavisky N. 1983. Uses of *Thymelaea hirsute* (Mitnan) with emphasis on hand papermaking. Economic Botany, 37: 310--321.
- Smith HH. 1933a. Ethnobotany of the forest Potawatomi Indians. Bulletin of the Public Museum of the City of Milwaukee, 7: 1--230.
- Smith HH. 1933b. Ethnobotany of the Menomini Indians. Bulletin of the Public Museum of the City of Milwaukee, 4: 1--174.
- TAPPI Standard T 511 om-02. 2006. Folding endurance of paper (MIT tester). New York: Technical Association of the Pulp and Paper Industry.

CHAPTER 6. GENERAL CONCLUSIONS

Tensile properties underlying the use of species of *Dirca* L. and other species of the Thymelaeaceae were quantified. The tensile measurements were functional quantitative traits, which were considered by Rosell *et al.* (2014) to be highly variable within clades and therefore not reliable for characterizing groups. Because neither the ultimate tensile strength nor the modulus of elasticity of bark differed among the species of *Dirca*, I conclude that *Dirca* can be characterized by the tensile properties of its bark. However, my results show that not all species of the Thymelaeaceae have similar ultimate tensile strength or modulus of elasticity, which is consistent with the conclusion of Rosell *et al.* (2014). My findings were contextualized through comparable measures of bark from plants of other families. Species of *Dirca* had the highest mean ultimate tensile strength (82.67 ± 8.49 MPa). This, along with the intermediate modulus of elasticity, which represents resistance to deformation (2080.27 ± 261.43 MPa), explains the historical practice of binding materials together with cordage made from the bark of *Dirca palustris* L. (Moerman 1998). My findings do not support my hypothesis that species of Thymelaeaceae share similar tensile properties of bark, nor that tensile properties of species of the Thymelaeaceae are distinct from those traits of bark from other families. An additional contribution of this portion of my work was to define sampling, conditioning, and testing parameters for quantifying the tensile properties of bark samples.

Bark is a heterogeneous tissue comprising thin- and thick-walled cells, leading to difficulties when preparing sections of tissue (Barbosa *et al.* 2010; Hamann *et al.* 2011; Angyalossy *et al.* 2016). Techniques previously proposed to address the difficulty of sectioning have focused on softening lignified tissues mixed among non-lignified tissues. I found such

techniques were not adequate for heterogeneous tissues of cells with non-lignified cell walls that differ in thickness. Sections of satisfactory quality were obtained after I modified protocols reported by Barbosa *et al.* (2010). Tissue softened in ethylenediamine, embedded in polyethylene glycol 1500, and sectioned with the support of Scotch Greener packaging tape (3M, St. Paul, Minnesota, United States of America) provided anatomical sections of the highest quality. Although even these improved sections did not allow for a comprehensive description of the bark of all four species of *Dirca*, aspects of anatomy such as the diameter and length of fibers, sieve-tube elements, and axial parenchyma, and the tangential length of phellem cells were observed. The Diameter and length of fibers were shared among all species of *Dirca*. This, along with shared tensile properties suggests the bark of all species of *Dirca* could be used for cordage. Differences of diameter and length of sieve-tube elements, axial parenchyma, and phellem occurred between *D. mexicana* Nesom & Mayfield and one or two other species. However, species of *Dirca* cannot be differentiated by anatomy because no anatomical trait was different among all species. I identified the occurrence of primary xylem in *D. palustris* and *Dirca decipiens* Floden that appears to originate in the cortex and adjacent phloem. The xylem tissue could be part of an emerging axillary bud, but further investigation is needed to identify the location of origin of the tissue. The modified methods I used allowed for important contributions to our knowledge of the bark of species of *Dirca* and may be useful for other species that possess heterogeneous bark that includes stratified thick- and thin-walled cells that are free of lignin.

Lastly, my doctoral research led to the identification of *D. mexicana* as a source of bark fibers to create an Asian-styled paper similar to gampi paper made from the bark fibers of

Wikstroemia spp. native to Japan; used as a medium for printmaking. Artists considered the transparency, thinness, and texture desirable characteristics of the Asian-styled *D. mexicana* handmade bark paper. Print detail was preserved on the *D. mexicana* bark paper because comparatively less ink was needed, and the original scale of prints were retained because it was not necessary to moisten the paper before printing. The *D. mexicana* bark paper was judged to be more receptive to solid areas of ink and less receptive to solid lines of ink than gampi paper. The *D. mexicana* bark paper withstood repeated bending, folding, and creasing six times greater than gampi paper. I have identified an Asian-style bark paper made from the bark fibers of a native North American shrub that can be used for a variety of printmaking techniques, as well as paper art which involves bending, folding, and creasing.

Future Work

I have quantified the tensile properties of bark of 14 species of Thymelaeaceae, a fraction of the ca. 900 species (Rogers 2009). The family is characterized by the traits of its stem, further investigation to quantify tensile properties of the family is needed to determine what extent the family can be defined by traits of its bark. I have proposed a method to quantify the ultimate tensile strength and modulus of elasticity of samples of bark.

My modifications of published histological techniques did not result in the quality of slides of sectioned bark tissue needed for a comprehensive description of the anatomy of bark. However, I have identified a need for development of additional techniques to section bark tissue. Future proposed techniques may be used to obtain the quality of slides of sectioned bark tissue needed for a comprehensive description of the anatomy of the bark of species of *Dirca*.

I created and demonstrated that Asian-style handmade *D. mexicana* bark paper can be used as a medium that responds to various printmaking techniques as equal or better than commercially available gampi paper. Positive feedback has been received from papermakers, printmakers, and paper artist expressing interest to use *D. mexicana* bark paper in amateur and professional contexts. Currently the only sources of bark of *D. mexicana* are its native population in Tamaulipas, Mexico, and garden plots in various locations around the United States. The feasibility and economic cost of commercial paper production needs to be investigated, especially since *D. mexicana*, or any species of *Dirca* is not considered a fast growing plant compared to other sources of commercial plant fibers.

Special Note

I ask that if you chose to create handmade bark paper from any species of *Dirca*, please be mindful of your impact when collecting bark. While the United States Fish and Wildlife Service does not list any species of *Dirca* as threatened or endangered, some state governments, such as Florida and Maryland, consider some populations of *Dirca* spp. as imperiled or endangered. I ask you do not collect from wild populations. Cultivated plants can be grown for the purpose of collecting bark.

References

- Angyalossy V, Pace MR, Evert RF, Marcati CR, Oskolski AA, Terrazas T, Kotina E, Lens F, Mazzoni-Viveiros SC, Angeles G, Machado SR, Crivellaro A, Rao KS, Junikka L, Nikolaeva N, Baas P. 2016. IAWA list of microscopic bark features. IAWA Journal 37: 517--615.
- Barbosa ACF, Pace MR, Witovisk L, Angyalossy V. 2010. A new method to obtain good anatomical slides of heterogeneous plant parts. IAWA Journal 31: 373--383.
- Hamann T, Smets E, Lens F. 2011. A comparison of paraffin and resin-based techniques used in bark anatomy. Taxon 60: 841--851.

Moerman DE. 1998. Native American ethnobotany. Portland, OR: Timber Press.

Rogers ZS. 2009. A world checklist of Thymelaeaceae. Retrieved from
<http://www.tropicos.org/projectwebportal.aspx?pagename=Introduction&projectid=26>

Rosell JA, Gleason S, Méndez-Alonso R, Chang Y, Westoby M. 2014. Bark functional ecology: Evidence for tradeoffs, functional coordination, and environment producing bark diversity. *New Phytologist* 201: 486--497.

APPENDIX A. COLLECTION PERMITS

Appendix A contains permits obtained for purpose of collection of plant material for research purposes. Permits included: Chequamegon-Nicolet National Forest research project tracking form, Wisconsin; Florida Department of Environmental Protection scientific (non-commercial) research / collection permit; and Cape Nature application to collect fauna and flora specimens for scientific research purposes, South Africa.

CHEQUAMEGON-NICOLET NATIONAL FOREST**RESEARCH PROJECT TRACKING FORM**

Today's Date: 09-24-15

PROJECT STATUS: Proposed _____ or Underway _____

Anticipated dates that activities will occur on CNNF:
Begin 09-26-15 End 09-26-15

Note: All researchers are required to 1) remove all equipment, flagging tape, etc. at the end of their study, and 2) provide the CNNF with a brief update on an annual basis, and 3) provide the Forest with a copy of final reports or publications.

PROJECT TITLE:

Anatomical and Tensile Characteristics of Bark Explain the Ethnobotanical Use of *Dirca* Species

NAME OF RESEARCHER, AFFILIATION, AND CONTACT INFORMATION:

[REDACTED] Hudson and [REDACTED]
Iowa State University, Ames IA
Email: [REDACTED] Cell: [REDACTED]

NAME OF PRINCIPLE INVESTIGATOR / MAJOR PROFESSOR / ADVISOR / GRADUATE STUDENT (if different):**OBJECTIVES:**

To characterize and compare the anatomy of bark (defined as all tissues external to the vascular cambium) of all species of *Dirca*.

Quantify the tensile properties of bark tissue of all *Dirca* species and compare these to taxonomically and ecologically allied species.

METHODS:

One branch will be pruned below the sixth year's growth scar from five individual plants of *Dirca palustris*. A four year old stem section, 5cm long, will be cut from the removed branch and placed in a plastic vial of Formalin-Acetic-Alcohol (FAA) solution for preservation. The bark on the remaining branch material will be stripped and placed into a plant press for drying. Effort will be made to collect tissue from a branch that is not a main branch and minimize damage to the plant. No plants will be entirely killed.

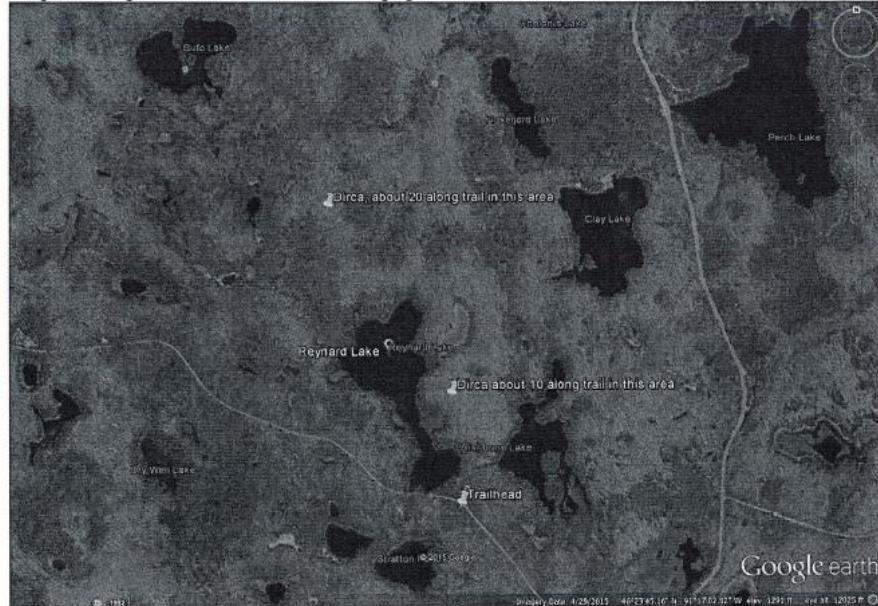
LOCATION (attach map):

Please complete and return this form to Linda Parker, or your USFS contact person

Note: Research Tracking forms are filed in

O:\NFS\ChequamegonNicolet\Program\4000Research\Research_Projects

Map shows pinned locations of *Dirca* populations that I will be visiting



IS THIS LOCATION A RESEARCH NATURAL AREA?

No

DURATION

I will arrive and leave Rainbow Lake Wilderness on Saturday, September 26, 2015.

COOPERATORS:

RESULTS: On an annual basis, please provide a brief summary reports of research status and upon completion of the project provide a copy of publications or reports.

Send copies to: Linda Parker, Forest Ecologist, 1170 4th Avenue, Park Falls WI 54555
715 762 -5169 lrparker@fs.fed.us

FOREST SERVICE CONTACT AND DUTY STATION: Matthew Bushman,
Botanist, Washburn Ranger Station, 715-373-2667 x246, mmbushman@fs.fed.us

FOREST SERVICE INVOLVEMENT: (Forest Service contribution to the study/project) None

SPECIAL USE PERMIT/AGREEMENT (if applicable):

TYPE _____ NUMBER _____

Please complete and return this form to Linda Parker, or your USFS contact person

Note: Research Tracking forms are filed in

O:\NFS\ChequamegonNicolet\Program\4000Research\Research_Projects

Page 1 of 1

Permit Number
16070512

Florida Department of Environmental Protection
Division of Recreation and Parks
Florida Park Service

SCIENTIFIC (NON-COMMERCIAL) RESEARCH / COLLECTING PERMIT

Park Visits Must Be Arranged A Minimum Of One Week In Advance. Failure To Make Required Arrangements Will Result In Denial Of Park Entry.
Permit Must Be Carried At All Times While Working In State Parks.

Permittee: [REDACTED]	Address, Phone, Email: 1137 Pearson Hall, Iowa State University Ames, IA 50011-2206 515-231-6954; [REDACTED]	Issue Date: July 5, 2016
Representing: Iowa State University		Expiration Date: December 15, 2016
Additional Authorized Researchers: Zachary Hudson	<u>Subject:</u> Characterization of tissue types for eastern leatherwood (<i>Dirca palustris</i>) <u>Permitted Activity:</u> Following up on previous work at Torreya State Park that refined the systematics of leatherwood, the researchers will be analyzing the anatomical, chemical, and physical properties of the bark and woody tissue and investigate whether the distinctive strength and flexibility of these tissues are primarily influenced by the environment or genetics,	
In the Following Park(s): Torreya State Park	<u>Permitted Collection:</u> They will collect one stem segment from each of four comparatively large individuals; each segment will measure no more than 3 cm in length. Cutting tools will be disinfected after each use to prevent the spread of any pathogens.	

** Since eastern leatherwood is Florida-listed as endangered, a separate permit from Florida Department of Agriculture and Consumer Services, Division of Plant Industry must be obtained before initiating planned field work; call (352) 395-4700 or visit this website (<http://www.freshfromflorida.com/Divisions-Offices/Plant-Industry/Business-Services/Plant-Pest-Permits/Native-Plant-Harvesting-Permit>) to obtain information and download an application for a Native Plant Harvesting Permit.

Permit Attachments:

Standard Conditions

Permit Not Valid Unless Signed By All PartiesIssuing Office

Approved By (Signature and Title)

Bureau Chief District 1 Administration
Division of Recreation and Parks
4620 State Park Lane
Panama City, FL 32408
850-233-5110 (fax: 850-233-5147)

Date

Permittee

I have read this permit and all attachments listed above. I fully understand it, and will abide by all rules and regulations.

Permittee Signature:

Date:



HEAD OFFICE

postal Private Bag X29
 physical PGWC Shared Services Center, Cnr Bosduif &
 Volstruis Streets, Bridgetown 7764
 website www.capenature.co.za
 enquiries Carlo Arendorf
 telephone +27 21 483 0122
 fax +27 86 556 7734
 email carendorf@capenature.co.za
 reference 1/2/2/1/2/O
 date 11 August 2017

[REDACTED]
 University of Johannesburg
 Department of Botany and Plant Biotechnology
 Kingsway Campus
 [REDACTED]

AUCKLAND PARK
 2006

[REDACTED]

**APPLICATION TO COLLECT FAUNA AND FLORA SPECIMENS FOR SCIENTIFIC
RESEARCH PURPOSES**

I refer to your application to collect specimens for research purposes in the Western Cape Province.

Attached is permit No. 0028-AAA008-00276 dated 11 August 2017 to collect specimens in the Western Cape Province. Please take special note of the standard conditions attached to the permits. I specifically draw your attention to permit condition (i). **It is imperative that you make contact with the Reserve Manager BEFORE you intend collecting on any nature reserve, conservation area, wilderness area and / or state forest.** No deviation is allowed from the fore-mentioned conditions without the prior written approval of the Chief Executive Officer: Western Cape Nature Conservation Board.

Please also take note of the *pro forma* (copy attached), which must please be used when submitting your collection / distribution records to CapeNature as per the conditions to your permit. Please feel free to add columns for extra data to the *pro forma* but no columns should be deleted. This *pro forma* is also available electronically from CapeNature.

Should you have any queries please do not hesitate to contact this office.

Yours faithfully,

[REDACTED]
CHIEF EXECUTIVE OFFICER

The Western Cape Nature Conservation Board trading as CapeNature
 Board Members: Prof Gavin Maneveldt (Chairperson), Mr Carl Lotter (Vice Chairperson), Mr Mervyn Burton, Mr Nico Eaton, Prof Francois Hanekom, Dr Bruce McKenzie, Ms Merle McOmbrin-Hodges, Adv Mandla Moludi, Mr Danie Nel, Prof Aubrey Redlinghuis, Mr Paul Slack

Western Cape Province

Telephone No: (027) 021 483 0000
 Email: permits.fax@capenature.co.za
 PGWC Shared Services Centre
 cnr Bosdulf and Volstruis Streets
 Bridgetown
 7764



Faxsimile No: (027)0865567734
 Internet: www.capenature.co.za
 Private Bag X29
 Gatesville
 7766

PERMIT TO

PLUCK FLORA PROTECTED AND UNPROTECTED FOR RESEARCH PURPOSES

(Issued in terms of the provisions of the Nature Cons. Ordinance 1974, (Ord 19 of 1974)Section 63(1)b & c)
Not Transferable

Holder	
Full Name	
Trade Name	University of Johannesburg
Postal Address	Department of Botany and Plant Biotechnology Kingsway Campus [REDACTED]
Suburb/Town	Auckland Park
Province/State	Gauteng
Country	South Africa
Postal/Zip Code	2006
Identity No.	
Registration No.	AAA008-01672
Physical Address	NA
Suburb/Town	NA
Province/State	
Country	
Longitude	.0000
Latitude	.0000

In terms of and to the provisions of the abovementioned Ordinance and the Regulations framed thereunder, the holder of this permit and people specified on the attached addendum is authorised to pluck the protected flora as specified below on the properties mentioned on attached addendum. See conditions on last page.

Details	
Permit/Licence No	0028-AAA008-00276
Expiry Date	31/08/2018
Date Issued	11/08/2017
Amount Paid	R 0.00
Reference	None
File Code	1/2/2/1/2/O
Stamp:	
Description	Property
Organization	University of Johannesburg
Person	[REDACTED]
ID	
Properties	Within the Western Cape only
Physical Address	Within the Western Cape only
District	N/A
Province/State	Western Cape
Country	South Africa
Longitude	.0000
Latitude	.0000

Species(Scientific Name)	Qty	Note
A) Note(NA)	0	see special conditions; special conditions apply
Acmadenia spp.(Acmadenia spp.)	0	None
Adenandra spp.(Adenandra spp.)	0	None
Agathosma spp.(Agathosma spp.)	0	None
Aspalathus spp.(Aspalathus spp.)	0	None
Aulax spp.(Aulax spp.)	0	None
Brabejum spp.(Brabejum spp.)	0	None
Calodendrum spp.(Calodendrum spp.)	0	None
Capnophyllum spp.(Capnophyllum spp.)	0	None
Clausenia spp.(Clausenia spp.)	0	None
Coleonema spp.(Coleonema spp.)	0	None
Cyclopia spp.(Cyclopia spp.)	0	None
Dasispermum spp.(Dasispermum spp.)	0	None
Diastella spp.(Diastella spp.)	0	None
Diosma spp.(Diosma spp.)	0	None
Empleurum spp.(Empleurum spp.)	0	None
Euchaetes spp.(Euchaetes spp.)	0	None
Euphorbia spp.(Euphorbia spp.)	0	None
Helinus spp.(Helinus spp.)	0	None
Hermas spp.(Hermas spp.)	0	None
Lachnaea spp.(Lachnaea spp.)	0	None
Leucadendron spp.(Leucadendron spp.)	0	None
Leucospermum spp.(Leucospermum spp.)	0	None
Lichtensteinia spp.(Lichtensteinia spp.)	0	None
Liparia spp.(Liparia spp.)	0	None
Macrostylis spp.(Macrostylis spp.)	0	None
Mimetes spp.(Mimetes spp.)	0	None
Nanobubon spp.(Nanobubon spp.)	0	None
Noltea spp.(Noltea spp.)	0	None
Notobubon spp.(Notobubon spp.)	0	None
Orothamnus spp.(Orothamnus spp.)	0	None
Paranomus spp.(Paranomus spp.)	0	None
Passerina spp.(Passerina spp.)	0	None
Phyllica spp.(Phyllica spp.)	0	None
Phylloasma spp.(Phylloasma spp.)	0	None
Protea spp.(Protea spp.)	0	None
Rhamnus spp.(Rhamnus spp.)	0	None
Scutia spp.(Scutia spp.)	0	None
Serruria spp.(Serruria spp.)	0	None
Sorocephalus sp.(Sorocephalus spp.)	0	None
Spatalla spp.(Spatalla spp.)	0	None
Stirtonanthus spp.(Stirtonanthus spp.)	0	None
Struthiola spp.(Struthiola spp.)	0	None
Trichocephalus spp.(Trichocephalus spp.)	0	None
Vepris spp.(Vepris spp.)	0	None
Vexatorella spp.(Vexatorella spp.)	0	None
Xiphotheca spp.(Xiphotheca spp.)	0	None
Zanthoxylum spp.(Zanthoxylum spp.)	0	None

11/08/2017

Issued by:
Carlo ArendorfApproved on Behalf CEO
Western Cape Nature Conservation Board

Effective Date

Signature of Holder
I acknowledge, accept and understand fully the
permit conditions as described

Standard Conditions

1. The holder of this permit shall return it together with a return of the species flora and the number of each species which he plucked thereunder, to the Chief Executive Officer: Western Cape Nature Conservation Board, Private Bag X29, Gatesville, 7766, within fourteen days from the date of expiry thereof.

2. THIS PERMIT IS SUBJECT TO THE SPECIAL CONDITIONS:

Special Conditions

TITLE OF PROJECT:

Stem anatomy of selected plant families of the South African flora.

This permit is subject to the following special conditions:

1. A maximum of 3 specimens per species per population or locality may be collected.
2. Data of all specimens collected must be captured on the SOB datasheet and submitted electronically to CapeNature for incorporation into the SOB database.
3. No threatened species will be collected (as per the application).
4. The permit holder must contact the relevant conservation manager at least a week in advance to make arrangements for the visit, as they may require that a field ranger accompanies the applicant on the field visit.
5. No vehicle access is allowed in the Cederberg Wilderness Area during weekends.
6. The permit holder must note that most of the CapeNature protected areas have Permanent Protea monitoring plots in the field and therefore needs to ask the relevant conservation managers where these plots are in order to avoid collection of material at these sites.
7. Copies of all reports or publications emanating from this research must be forwarded to CapeNature for internal dissemination to relevant staff.

CONDITIONS APPLICABLE TO RESEARCHERS UNDERTAKING RESEARCH OR OTHER COLLECTING WORKS ON PROVINCIAL CONSERVATION AREAS AND / OR PRIVATELY OWNED LAND IN THE PROVINCE OF WESTERN CAPE:

1. THE MANAGER OF THE RELEVANT CONSERVATION AREA(S) (IF ANY) MUST BE INFORMED TIMEOUSLY BEFORE ANY CONSERVATION AREA IS ENTERED FOR COLLECTING OR RESEARCH PURPOSES AND THE MANAGER'S WRITTEN PERMISSION TO ENTER SUCH RESERVE MUST BE ACQUIRED BEFOREHAND. THIS PERMIT DOES NOT GRANT THE PERMIT HOLDER AUTOMATIC ACCESS TO ANY NATURE RESERVE, CONSERVATION AREA, WILDERNESS AREA AND / OR STATE FOREST. ANY OTHER / FURTHER CONDITIONS OR RESTRICTIONS THAT THE MANAGER MAY STIPULATE AT HIS / HER DISCRETION MUST ALSO BE ADHERED TO. THIS PERMIT MUST BE AVAILABLE TO BE SHOWN ON DEMAND.
2. The owner of any other land concerned (be it privately or publicly owned land) must give WRITTEN consent allowing the permit holder to enter said property to collect flora / fauna. This written permission must reflect the full name and address of the property owner (or of the person authorised to grant such permission), the full name and address of the person to whom the permission is granted and the number and species of the flora / fauna, the date or dates on which such flora / fauna may be picked / collected and the land in respect of which permission is granted. Copies of this written permission must be made available to The Western Cape Nature Conservation Board upon request.
3. Type-specimens of any newly described / discovered species or other taxon collected must be lodged with a recognised South African scientific institution / museum / herbarium (preferably within the Province of Western Cape) where such material will be available to other researchers. For every flora specimen collected on a Western Cape Nature Conservation Board nature reserve, one additional (extra) herbarium specimen must be forwarded to the Western Cape Nature Conservation Board Herbarium at Jonkershoek (c/o MJ Simpson, Private Bag X5014, Stellenbosch 7599).
4. A list of all collected specimens / material including the; species name, the number collected, the collection date and the precise locality of the collection must be submitted within 14 days from the date of expiry of your permit to The Chief Executive Officer: CapeNature, Private Bag X29, Gatesville, 7766
5. The maximum number of specimens per species specified in the permit (if at all) may not be exceeded without the prior permission of The Chief Executive Officer: Western Cape Nature Conservation Board.
6. For projects of more than one year's duration a progress report must be submitted to The Chief Executive Officer: Western Cape Nature Conservation Board before 31 December of each year.
7. One copy of all completed reports, publications, or articles (including books, videos, CDs, DVDs etc.) resulting from the project/collection must be submitted to The Chief Executive Officer: Western Cape Nature Conservation Board free of charge.
8. Should a report, publication, article or thesis arise from this project/collection, an acknowledgement to Western Cape Nature Conservation Board must be included.
9. The Forest Act 1984 (Act 122 of 1984) and regulations, the Nature Conservation Ordinance, 1974 (Ordinance 19 of 1974) and all regulations in terms of the Ordinance must be adhered to.
10. Should it be envisaged to export any material / specimens across the boundaries of the Western Cape Province, an export permit will be required in respect of certain species and a further application form will have to be completed. The permit holder must confirm with the Western Cape Nature Conservation Board whether an export permit is required BEFORE exporting any material / specimens from the Western Cape Province.
11. No species that appear on the Red Data List or species listed as endangered in terms of the Nature Conservation Ordinance, 1974 (Ordinance 19 of 1974) may be collected, except for those mentioned on the permit.
12. Unless otherwise specifically indicated in writing, no material or specimens collected with this permit or material or specimens bred or propagated, from material or specimens collected with this permit, may be donated, sold or used for any commercial purpose by any party.



APPENDIX B. ANATOMICAL DATA COLLECTED FOR ALL SPECIES OF *DIRCA*

Species	Population	Specimen Number	Cell Type	Observation	Diameter	Length	Shape	Arrangement	Number of Cells Width
DM	ISU	1	fiber	1	9.151	3104.79			
DM	ISU	1	fiber	2	8.309	1810.718			
DM	ISU	1	fiber	3	8.309	2361.265			
DM	ISU	2	fiber	1	14.02	3424.615			
DM	ISU	2	fiber	2	8.189	3122.101			
DM	ISU	2	fiber	3	10.59				106
DM	ISU	3	fiber	1	14.466	3172.261			
DM	ISU	3	fiber	2	13.473	2288.743			
DM	ISU	3	fiber	3	8.398				
DM	ISU	4	fiber	1	15.707	3004.505			
DM	ISU	4	fiber	2	11.309	2612.864			
DM	ISU	4	fiber	3	11.894	3198.634			
DM	ISU	4	sieve	1	34.928	208.087		3	

Table continued

DM	ISU	4	sieve	2	29.53	249.303		3
DM	ISU	4	sieve	3	39.395	214.448		3
DM	ISU	4	phellem	1		62.29	flat_crescent	5
DM	ISU	4	phellem	2		150.864	flat_crescent	4
DM	ISU	4	phellem	3		92.347	flat_crescent	5
DM	ISU	5	fiber	1	6.203	2534.487		
DM	ISU	5	fiber	2	6.471	3091.337		
DM	ISU	5	fiber	3	9.151	2931.419		
DM	ISU	5	sieve	1	11.479	44.739		3
DM	ISU	5	sieve	2	9.689	66.404		3
DM	ISU	5	sieve	3	6.056	50.317		3
DM	ISU	5	phellem	1		13.974	flat_crescent	4
DM	ISU	5	phellem	2		17.227	flat_crescent	4
DM	ISU	5	phellem	3		13.158	flat_crescent	4

Table continued

DM	RI	1	fiber	1	8.576	3381.359	
DM	RI	1	fiber	2	9.026	2491.544	
DM	RI	1	fiber	3	8.101	3068.893	
DM	RI	1	sieve	1	8.272	35.434	3
DM	RI	1	sieve	2	6.149	39.326	3
DM	RI	1	sieve	3	9.13	59.572	3
DM	RI	1	phellem	1			
DM	RI	1	phellem	2			108
DM	RI	1	phellem	3			
DM	RI	3	fiber	1	12.26	2739.326	
DM	RI	3	fiber	2	10.645	3658.676	
DM	RI	3	fiber	3	11.828		
DM	RI	4	fiber	1	8.309	3107.331	
DM	RI	4	fiber	2	11.507	2489.428	

Table continued

DM	RI	4	fiber	3	8.511	2929.213	
DO	Japser	1	fiber	1	10.953	3373.119	
DO	Japser	1	fiber	2	6.812	2270.397	
DO	Japser	1	fiber	3	6.203	2698.33	
DO	Jasper	1	sieve	1	7.109	31.082	3
DO	Jasper	1	sieve	2	5.751	20.074	3
DO	Jasper	1	sieve	3	7.528	27.22	3
DO	Jasper	1	phellem	1	20.141	flat_crescent	6
DO	Jasper	1	phellem	2	20.169	flat_crescent	6
DO	Jasper	1	phellem	3	22.779	flat_crescent	5
DO	Japser	2	fiber	1	10.59	1516.032	
DO	Japser	2	fiber	2	10.965	2257.168	
DO	Japser	2	fiber	3	11.827	2568.233	
DO	Japser	4	fiber	1	11.07	3250.508	

Table continued

DO	Japser	4	fiber	2	10.806	1948.261
DO	Japser	4	fiber	3	7.754	2617.476
DO	Japser	5	fiber	1	13.159	2318.244
DO	Japser	5	fiber	2	7.671	3407.654
DO	Japser	5	fiber	3	7.522	3106.698
DD	Eureka	1	fiber	1	10.526	2450.238
DD	Eureka	1	fiber	2	9.055	1643.709
DD	Eureka	1	fiber	3	6.138	1478.867
DD	Eureka	2	fiber	1	11.309	3447.164
DD	Eureka	2	fiber	2	9.027	3740.377
DD	Eureka	2	fiber	3	8.309	3137.238
DD	Eureka	2	sieve	1	6.536	42.925
DD	Eureka	2	sieve	2	8.261	41.963
DD	Eureka	2	sieve	3	9.13	52.477

Table continued

DD	Eureka	2	phellem	1	14.859	flat_crescent	5
DD	Eureka	2	phellem	2	15.017	flat_crescent	5
DD	Eureka	2	phellem	3	17.084	flat_crescent	5
DD	Eureka	3	fiber	1	12.276	3677.285	
DD	Eureka	3	fiber	2	12.002	2368.641	
DD	Eureka	3	fiber	3	9.474	1885.074	
DD	Eureka	4	fiber	1	20.219	3206.195	
DD	Eureka	4	fiber	2	15.563	3587.699	111
DD	Eureka	4	fiber	3	14.172	2556.59	
DD	Overland	1	fiber	1	12.854	3360.041	
DD	Overland	1	fiber	2	8.511	2987.725	
DD	Overland	1	fiber	3	11.458		
DD	Overland	1	sieve	1	8.235	68.882	1
DD	Overland	1	sieve	2	11.314	43.449	1

Table continued

DD	Overland	1	sieve	3	9.571	52.777	1	
DD	Overland	1	phellem	1		30.606	flat_crescent	6
DD	Overland	1	phellem	2		29.754	flat_crescent	7
DD	Overland	1	phellem	3		25.725	flat_crescent	6
DD	Overland	2	fiber	1	10.092	3011.894		
DD	Overland	2	fiber	2	9.808	1996.009		
DD	Overland	2	fiber	3	7.447			
DD	Overland	3	fiber	1	7.522	3370.172		112
DD	Overland	3	fiber	2	7.671	2902.893		
DD	Overland	3	fiber	3	10.531	3341.116		
DD	Overland	4	fiber	1	12.165	3916.203		
DD	Overland	4	fiber	2	12.992	2340.928		
DD	Overland	4	fiber	3	9.187	3193.784		
DP	ME	1	fiber	1	6.203	2934.106		

Table continued

DP	ME	1	fiber	2	10.849	2231.284	
DP	ME	1	fiber	3	7.671	2400.083	
DP	ME	2	fiber	1	6.541	2492.275	
DP	ME	2	fiber	2	8.669	2427.211	
DP	ME	2	fiber	3	8.189	2184.904	
DP	ME	3	fiber	1	7.671	2790.747	
DP	ME	3	fiber	2	11.457	1728.985	
DP	ME	3	fiber	3	12.451	525.508	
DP	ME	3	sieve	1	6.522	70.135	1
DP	ME	3	sieve	2	6.536	73.767	1
DP	ME	3	sieve	3	6.102	35.11	1
DP	ME	3	phellem	1	26.177	flat_crescent	4
DP	ME	3	phellem	2	24.161	flat_crescent	4
DP	ME	3	phellem	3	21.477	flat_crescent	5

Table continued

DP	FL	1	fiber	1	9.913	2238.392	
DP	FL	1	fiber	2	9.13	2654.069	
DP	FL	1	fiber	3	6.541	1939.027	
DP	FL	1	sieve	1	4.783	40.907	1
DP	FL	1	sieve	2	5.669	58.89	2
DP	FL	1	sieve	3	6.957	71.316	2
DP	FL	1	phellem	1	18.588	flat_crescent	5
DP	FL	1	phellem	2	20.982	flat_crescent	5
DP	FL	1	phellem	3	20.042	flat_crescent	4
DP	FL	2	fiber	1	10.036	2165.671	
DP	FL	2	fiber	2	9.089	2183.493	
DP	FL	2	fiber	3	9.515	1535.862	
DP	FL	3	fiber	1	10.478	3088.737	
DP	FL	3	fiber	2	7.671		

Table continued

DP	FL	3	fiber	3	7.522			
DP	FL	3	sieve	1	4.348	37.889		1
DP	FL	3	sieve	2	8.793	33.599		2
DP	FL	3	sieve	3	5.669	49.148		1
DP	FL	3	phellem	1		23.142	flat_crescent	7
DP	FL	3	phellem	2		26.047	flat_crescent	7
DP	FL	3	phellem	3		21.238	flat_crescent	5
DP	ND	1	fiber	1	10.036	2747.482		
DP	ND	1	fiber	2	8.773	3043.599		
DP	ND	1	fiber	3	10.478			
DP	ND	2	fiber	1	12.036	3073.273		
DP	ND	2	fiber	2	10.531	2476.981		
DP	ND	2	fiber	3	9.027	2457.779		
DP	ND	3	fiber	1	10.953	3007.802		

Table continued

DP	ND	3	fiber	2	9.151	1641.941	
DP	ND	3	fiber	3	10.036	1011.544	
DP	ND	3	sieve	1	10.145	66.389	1
DP	ND	3	sieve	2	5.452	59.1	1
DP	ND	3	sieve	3			
DP	ND	3	phellem	1			
DP	ND	3	phellem	2			
DP	ND	3	phellem	3			
DP	ND	4	fiber	1	11.581	3356.303	
DP	ND	4	fiber	2	8.398	3685.313	
DP	ND	4	fiber	3	9.25	2780.307	
DP	ND	4	sieve	1	5.19	77.585	1
DP	ND	4	sieve	2	6.465	80.244	1
DP	ND	4	sieve	3			

Table continued

DP	ND	4	phellem	1	24.572	flat_crescent	6
DP	ND	4	phellem	2	30.549	flat_crescent	5
DP	ND	4	phellem	3	19.948	flat_crescent	7
DP	ND	5	fiber	1	9.677	3837.243	
DP	ND	5	fiber	2	10.581	2637.309	
DP	ND	5	fiber	3	9.187	3177.091	
DP	Rainbow	1	fiber	1	12.947	1763.881	
DP	Rainbow	1	fiber	2	12.021		117
DP	Rainbow	1	fiber	3	8.866		
DP	Rainbow	2	fiber	1	10.526	1829.176	
DP	Rainbow	2	fiber	2	12.675	2019.225	
DP	Rainbow	2	fiber	3	13.398	2699.099	
DP	Rainbow	3	fiber	1	10.478	2825.172	
DP	Rainbow	3	fiber	2	10.092	1857.346	

Table continued

DP	Rainbow	3	fiber	3	9.515	2684.281	
DP	Rainbow	4	fiber	1	9.617	2999.932	
DP	Rainbow	4	fiber	2	7.213	2328.51	
DP	Rainbow	4	fiber	3	8.398	2499.474	
DP	Jersey	1	fiber	1	21.197	2839.147	
DP	Jersey	1	fiber	2	10.531	7151.235	
DP	Jersey	1	fiber	3	8.309	2945.682	
DP	Jersey	2	fiber	1	6.812	2720.367	118
DP	Jersey	2	fiber	2	6.018	2634.325	
DP	Jersey	2	fiber	3	12.036	2148.422	
DP	Jersey	2	sieve	1			
DP	Jersey	2	sieve	2			
DP	Jersey	2	sieve	3			
DP	Jersey	2	phellem	1	24.295	flat_crescent	5

Table continued

DP	Jersey	2	phellem	2	18.97	flat_crescent	5
DP	Jersey	2	phellem	3	23.312	flat_crescent	5
DP	Jersey	3	fiber	1	11.507		
DP	Jersey	3	fiber	2	12.036		
DP	Jersey	3	fiber	3	9.574		
DP	Jersey	3	sieve	1	8.696	62.476	3
DP	Jersey	3	sieve	2	7.778	42.326	3
DP	Jersey	3	sieve	3	7.48	58.967	1
DP	Jersey	3	phellem	1	20.626	flat_crescent	5
DP	Jersey	3	phellem	2	25.298	flat_crescent	4
DP	Jersey	3	phellem	3	20.262	flat_crescent	5
DP	Jersey	4	fiber	1	11.071	2165.02	
DP	Jersey	4	fiber	2	5.79	2038.58	
DP	Jersey	4	fiber	3	7.754	1323.939	

APPENDIX C. *DIRCA MEXICANA* BARK PAPER: ORIGAMI EVALUATION

We determined *Dirca mexicana* bark paper has a fold endurance six times greater than commercially available gampi, suggesting the paper can be used for paper arts and crafts which involve repeated bending, folding, and creasing. Appendix C includes an evaluation of handmade *Dirca mexicana* bark paper for use as an origami paper. The evaluation was performed by artist James Lucas.

When Lucas received the paper, he was initially impressed with the paper's strength, crispness, and thinness, and he set forth to fold a design which would test these qualities. Lucas decided to fold a Jacana (fig. 1) (genus of bird) from a 30 cm square sheet of paper. A Jacana requires pleats the entire length of the sheet of paper to form spindly legs and toes. He noted that only the thinnest, strongest sheets of paper would yield a good result. If the paper is too thick, the bird's feet will not look delicate, and if the paper is too weak, the paper will rip or shed fibers, potentially preventing completion of the design.

Before folding, the paper was treated a solution of methylcellulose and water, a technique which aids in shaping and molding origami. Brown acrylic ink was also added to the methylcellulose solution to change the paper's color. The paper responded well to this treatment, it did not become wrinkled as it dried, which was observed during printmaking.

Lucas commended on the "memory" of the paper, "With many papers, like thin abaca or thin kozo, the paper can be folded bidirectional easily (e.g., a valley fold can be easily reversed along the same crease into a mountain fold, and vice versa). The *Dirca* paper was less amenable to changing crease polarity than other fibers I have folded with." Another observation noted that while the paper is extremely strong for its thinness, it tore more often compared to other

origami papers. Despite the tearing, minute splits and tears that formed over the course of folding did not appear to compromise the structural integrity of the model, nor did they prevent completion of the design.

In Lucas' artist opinion he said, "I highly recommend it for folding complex insect designs, with their numerous, thin appendages. Its crispness, which significantly exceeds every other folding medium I can think of except maybe Glassine, also lent well to shaping, as once creased, the paper more easily retained its folded conformation than many other papers like chiri unryu. Based on my experience folding with it, I believe that your *Dirca* paper has truly remarkable characteristics that enable it to rival the Origamido and O-Gami abaca papers in their suitability for the most complex of origami designs, even despite its greater brittleness. I think your paper shows great promise as a folding medium among the origami community, and would likely be adopted by many talented folders if it could be made available at comparable sizes, colors, thicknesses, and prices as the abaca papers currently on the market (currently they are sold roughly between \$15-\$20 per 25" x 37" sheet, at 10-20 gsm weight)."



Figure 1. James Lucas. *Jacana*. 2019. 6 x 5 cm.