# PISTILLATE FLORAL AND FRUIT DEVELOPMENT IN *QUERCUS* SUBGENUS *QUERCUS* (FAGACEAE)

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Sandra Jean Borgardt

August 2013

# PISTILLATE FLORAL AND FRUIT DEVELOPMENT IN *QUERCUS* SUBGENUS *QUERCUS* (FAGACEAE)

Sandra Jean Borgardt, Ph. D.

Cornell University 2013

## ABSTRACT

A developmental series of flowers and fruits of *Quercus acutissima* Carruth. (subgenus *Quercus* section *Cerris*) in 2000 and *Q. palmeri* Engelm. (subgenus *Quercus* section *Protobalanus*) in 1995 was collected over a growing season, fixed, and sectioned using glycol methacrylate embedding and glass knife sectioning techniques for an intersectional, comparative anatomical study. For both species, pistillate flowers of the current growing season, each consisting of a pistil with three long, slightly recurved styles, six tepals, and an inconspicuous ovary subtended by a few cycles of cupule scales, emerged in early May, were pollinated by mid-May, and then were quiescent for the remainder of the growing season. Flowers from the previous growing season resumed growth in May, each forming three locules delimited by septa in the ovary, with two bitegmic, epitropous ovules developing in each locule. For *Q. acutissima*, flowers from the previous growing year resumed growth in mid-May, and mature embryo sacs were present by mid-July of the second growing season, although embryos were not observed until early August. Fruit maturation was complete by late September. For *Q. palmeri*, flowers from the previous growing year resumed growth in early May, and mature embryo sacs were

present by mid-June of the second growing year, with embryos observed thereafter. Fruit maturation was complete by early August. Features that have not been described previously for section *Cerris* (*Q. acutissima*) include early-lignifying endocarp trichomes, persistent septa, and leaf primordia buttresses on the embryo. New features for section *Protobalanus* (*Q. palmeri*) that may have phylogenetic implications include: stigmatic surfaces restricted to capitate apex of styles, a perianth flange, presence of staminodia, thick-walled style/perianth epidermis cells, and persistent septal remnants at maturity. A comparison of flower and fruit developmental features in *Q. acutissima* and *Q. palmeri* with previous subgenus *Quercus* studies in sections *Cerris*, *Lobatae*, *Protobalanus*, and *Quercus sensu stricto* reveal a mosaic of shared features among the four sections of *Quercus* subgenus *Quercus*, as well as newly described features that had been never attributed to this group of taxa previously.

## BIOGRAPHICAL SKETCH

Sandra Jean Borgardt was born November 11, 1963 in San Antonio, Texas, USA to parents John Henry Borgardt and Barbara Jean Borgardt (Higgins). She was joined by a brother, Matthew John Borgardt (born November 25, 1966), and a sister, Susanna Jean Borgardt (born January 11, 1971). Job-related moves eventually took the family to Houston, Texas.

Sandra graduated in 1981 as valedictorian from Nimitz High School. Although she had always had a deep interest in the biological sciences, an engineering expo she attended in her senior year of high school inspired a change to try electrical engineering. After attending a year at Vanderbilt University, she decided to go back to biological sciences, and concentrated on a career in pharmacy. She attended University of Houston, and obtained a B.S. in Pharmacy (R. Ph.) in 1987.

While working in Houston, Sandra became interested in horticulture and started collecting heritage or old-fashioned roses as well as learning about native Texan plants. After moving with the family in 1988 to Phoenix, Arizona, she continued collecting and growing roses (eventually maxing out at 120), and began learning about desert native plants and xeroscaping.

Sandra began taking horticulture and other botany classes at Arizona State University, Tempe, AZ in 1991, and was inspired by courses offered by Dr. Kathleen Pigg in paleobotany and anatomy. Sandra went on to study with Dr. Pigg and graduated in 1996 with a Masters of Science degree in Paleobotany, publishing her thesis “Petrified *Quercus* (Fagaceae) fruit fossils from the Middle Miocene, Yakima River Canyon, Washington”.

Wishing to continue exploring some of the questions in fruit development she encountered in her Masters work, Sandra applied and was accepted to the Ph. D. program at Cornell University, Ithaca, New York in 1997 and began her studies with Kevin Nixon and William Crepet in systematics and paleobotany.

# DEDICATION

I dedicate this dissertation to everyone that has had a positive impact on my life view, goals, and spirit.

ACKNOWLEDGMENTS

I wish to thank the advisers of my final committee, Kevin C. Nixon, William L. Crepet, Dennis Stevenson, Maria A. Gandolfo, as well as the two members that were part of my initial committee and retired before my defense: Dominick Paolillo and Karl Niklas.

This research was supported by a Mellon Fellowship Teaching Grant and the Department of Plant Biology, Cornell University, Ithaca, New York, USA.

I wish to thank Mary Hirshfeld (Gardens Curator) and the staff at Cornell Plantations for their assistance with the collections of the F.R. Newman Arboretum (Ithaca, New York, USA); Dominick Paolillo for laboratory space and assistance; H. Lloyd Mogensen (Emeritus, Northern Arizona University, Flagstaff, Arizona, USA) for the use of the slides published in his 1965 article, ‘‘A contribution to the anatomical development of the acorn in *Quercus* L.’’; and Danilo Dragoni (Cornell University, Geneva, New York, USA) for his assistance with Italian interpretations.

I also wish to acknowledge Les Landrum and Kathleen B. Pigg (Arizona State University, Tempe, Arizona, USA) for assistance in collecting the *Quercus palmeri* material.

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LIST OF ABBREVIATIONS

AO.....abortive ovules(s)
AZ.....abscission zone
C.....cupule
Ca.....caecum
CC.....central cell
CeEn.....cellular endosperm
Co.....cotyledon
CoEn.....coenocytic endosperm
CS.....cupule scale(s)
D.....druse
EA.....egg apparatus
Eg.....egg
Em.....embryo
EmAx.....embryo axis
En.....endocarp
Ep.....epidermis
ES.....embryo sac
Ex.....exocarp
FB.....floral bract(s)
FW.....fruit wall
GMA.....glycol methacrylate
H.....hypostase
IA.....inflorescence axis
II.....inner integument
L.....locule(s)
*ls*.....longitudinal section
Me.....mesocarp
N.....nucellus
O.....ovule
OI.....outer integument
OP.....ovule primordium(a)
OW.....ovary wall
P.....perianth
Pd.....peduncle
PG.....pollen grain(s)
PL.....palisade layer
S.....style
SA.....shoot apex
SC.....seed coat
Se.....septum(ae)
*s. l.* .....*sensu lato*
SPL.....secondary palisade layer
*s. s.* .....*sensu stricto*

Stam.....staminode
StS.....stigmatic surface(s)
Sy.....synergid(s)
T.....trichome(s)
TBO.....Toluidine Blue O
*ts*.....transverse section
USA.....United States of America
VB.....vascular bundle

CHAPTER 1

# INTRODUCTION

Within the traditional, wind-pollinated "Amentiferae", a group of taxa centered around the family Fagaceae was proposed to be monophyletic based on analyses of morphological characters only (Nixon, 1984, 1989; Hufford, 1992), molecular characters only (Chase et al., 1993; Manos and Steele, 1997; Chen et al., 1998; Kallersjo et al., 1998; Qiu et al., 1998; Soltis et al., 2000), as well as combined morphological and molecular characters (Nandi et al., 1998). Once termed the "higher" Hamamelidae after the subclass Hamamelidae of Cronquist (1981) and superorder of Takhtajan (1997), this clade was later demoted to an ordinal rank as "Fagales" (APG, 1998); a name that has historically been applied to only Fagaceae or Fagaceae + Betulaceae. This Fagales *s. l.* clade includes the following families: Betulaceae (birches), Casuarinaceae (she-oaks), Fagaceae (oaks, beeches), Juglandaceae (walnuts), Myricaceae (wax myrtles), and Nothofagaceae (southern beeches), as well as the monotypic families Rhoipteleaceae and Ticodendraceae (Stevens, 2001).

All species in this clade produce unisexual flowers, with the pistillate flowers (interpreted as inferior and syncarpous) maturing into fruits with only one seed. Some taxa produce only one ovule/flower, but more commonly, one or two ovules per carpel are formed in the flower and all but one abort before fruit maturation, resulting in a pseudomonomerous fruit. The aborted ovules are in an apical/distal position in the mature fruit in all the taxa found in the Fagales *s. l.* except for certain groups within

the genus *Quercus* (the oaks) of the family Fagaceae, which depending on the group may can have apical abortive ovules, basal abortive ovules, or abortive ovules with a variable position that are neither strictly basal nor apical and are described as lateral (Table 1). Even within oak groups, ovule position can be variable as is seen in subgenus *Quercus* section *Lobatae* (the red oaks) with typically apical abortive ovules that has at least 10 species in Mexico and Central America develop lateral to basal abortive ovules (Trelease, 1924; Muller, 1942a; K. C. Nixon, Cornell University, unpublished, and Flora Mesoamericana, in preparation).

A. de Candolle (1862) was the first to document the variability in abortive ovule position in *Quercus*, with subsequent classifications (Oersted, 1871; Trelease, 1924; Muller, 1942b; Nixon, 1993, 1997) also making use of this feature to distinguish among groups in the oaks, including the most complete treatment of genus *Quercus* by Camus (1934) who divided the genus into two subgenera, *Cyclobalanopsis* and *Quercus*. In the Flora of China (Huang et al., 1994), *Cyclobalanopsis* is treated at the level of genus, and although this treatment does not conflict with accepted natural groups in the oaks, the current study will follow the classification and ranks of Camus according to her monograph (Table 1), with some modifications due to priority of names (Voss, 1981). A new study published in 2008 describes the anatomy and maturation within the mostly tropical *Quercus* subgenus *Cyclobalanopsis* (Deng et al., 2008), but will not be included in the discussion of this thesis.

In *Quercus*, pistillate flowers are functionally unisexual, syncarpous, epigynous, and mature into a single-seeded indehiscent fruit subtended by a “cap” or cupule. Both

the nut and cupule together are commonly called acorns (although the nut alone is also described as an acorn), and are true nuts. Six ovules (2 ovules per carpel) are normally formed in each flower and typically all but one ovule abort during maturation into the single-seeded fruit.

Basal abortive ovule position figured prominently as a putative synapomorphy and derived character in the morphological analyses that proposed uniting the white oaks of subgenus *Quercus* section *Quercus* and the white oaks of the *Cerris* group (including *Q. ilex*) into a larger subgenus *Quercus* section *Quercus sensu lato*. (Nixon, 1984, 1993). Intersectional hybridization events between these groups, which are very rare in subgenus *Quercus*, of both natural and artificial origin (Cottam et al., 1982; Boavida et al., 2001) have also focused on the similarities between these two sections. So far, molecular analyses based on ITS sequence data have not supported a circumscription of subgenus *Quercus* section *Quercus s. l.*, with cladograms from these analyses separating the "white" oaks into two separate clades and basal abortive ovules independently derived in both groups (Manos et al., 1999; Manos and Stanford, 2001). Based on these analyses, this thesis will treat the "white" oaks *s. l.* as discrete entities and refer to them as section *Cerris* (entirely Old World, including the Ilex, Suber and Cerris groups) and section *Quercus s. s.* (both Old World and New World).

One of the most complete flower/fruit anatomical and developmental studies within *Quercus* subgenus *Quercus* was a study that described the maturation of the pistillate flowers of *Quercus alba* L. of section *Quercus s. s.* and *Q. velutina* of section *Lobatae* from anthesis to fruit maturity in some detail (Mogensen, 1965). Previous

studies that sampled species in section *Cerris* documented only morphological features (Kaul, 1985), limited the descriptions to diagnostic features, and did not describe maturation patterns (Camus, 1934; Soepadmo, 1968), or only described fruit development up to early embryogenesis (Corti, 1954, 1955, 1959; Scaramuzzi, 1960; Bianco, 1961; Boavida et al., 1999). There are no anatomical or developmental studies of the small, relictual, western North American group, section *Protobalanus*, the intermediate oaks.

The goal of this collection of studies is to conduct a complete anatomical and developmental study of a representative species from *Quercus* subgenus *Quercus*, sections *Cerris* and *Protobalanus,* with particular emphasis on understanding the processes that determine the final abortive ovule position in their respective fruits, and to discuss this new information in the context of previous work done within subgenus *Quercus*.

Herein I present the first flower/fruit anatomical and developmental study of the species *Q. acutissima*, the first intersectional comparative anatomical study for subgenus *Quercus* section *Cerris*, the first flower/fruit anatomical and developmental study of subgenus *Quercus* section *Protobalanus* (species *Q. palmeri*), and the first intersectional comparative anatomical study for all four sections in subgenus *Quercus*.

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# CHAPTER 2

# NEW HISTOLOGICAL TECHNIQUE FOR LARGE WOODY SCLERIFIED FRUITS

## INTRODUCTION

The hardness and heterogeneity of the ovary and mature pericarp in the Fagales *s. l.* (Betulaceae, Casuarinaceae, Fagaceae, Juglandaceae, Myricaceae, Nothofagaceae, Rhoipteleaceae, and Ticodendraceae), a monophyletic group of taxa (Nixon, 1989; Chase et al., 1993; Qiu et al., 1998) that form true nuts (e. g., acorns and walnuts), has been a limiting factor in studying fruit development in this both economically and ecologically important group. Of particular interest for this study is the final position of aborted ovules in *Quercus* (oaks; Fagaceae) fruits, commonly known as acorns. Most of the taxa in Fagales *s. l.* develop apical aborted ovules in the fruit (i.e., just below the styles), but fruits in *Quercus* can either have apical, lateral or basal aborted ovules and this position has often been used as a diagnostic character since it was described in oaks (Candolle, 1862). Previous studies that looked at *Quercus* flower and fruit developmental anatomy either concentrated on the maturation of the surviving ovule (and later seed) after identifying which ovules were aborting (Mogensen, 1965, 1975; Brown and Mogensen, 1972), or in most cases, only described fruit development up to early embryogenesis (Corti, 1954, 1955, 1959; Scaramuzzi, 1960; Bianco, 1961; Boavida et al., 1999). It is unknown what fruit developmental processes affect/determine the final position of the aborted ovules. A

clear understanding of these processes would be useful in deciphering shared characteristics in oak fruit development across taxa and how aborted ovule position relates to oak evolution and phylogenetic relationships.

## EMBEDDING MEDIA: PARAFFIN VERSUS GLYCOL METHACRYLATE

Paraffin has historically been the embedding medium of choice for oak anatomical studies. Heated paraffin liquifies, penetrates the specimen, is cooled to room temperature to solidify, and then sectioned. Paraffin was used to map the vasculature of the flowers and inflorescences in *Quercus* (Abbe, 1974) and to follow flower and fruit development up to early embryogenesis (Benson, 1893; Conrad, 1900; Langdon, 1939; Hjelmqvist, 1948, 1953, 1957; Rebuck, 1952; Corti, 1954, 1955, 1959; Stairs, 1964; Mogensen, 1965; Sharp and Sprague, 1967; Brown and Mogensen, 1972; Cecich, 1997). Paraffin is still valued for its economy and relatively non-toxic materials, but penetration into the maturing pericarp is most problematic during final fruit maturation stage, and techniques such as HF soaking (for 5-12 months!) and celloidin methods were used to maximize penetration (Langdon, 1920, 1939; Reece, 1938). Even in the most complete *Quercus* reproductive anatomical/developmental study, which followed pistillate flower maturation through embryogenesis and pericarp development to complete maturity, the researcher had to remove large portions of the fruit wall to facilitate paraffin penetration into the seed (Mogensen, 1965).

These problems inspired the consideration of other embedding media for this

study such as resins, plastics, and other waxes in place of paraffin. The need for rapid penetration into potentially large fruits, as well as a dense medium to support the heterogeneous tissues in the fruit walls focused attention on the various plastics: particularly glycol methacrylate (GMA), also called hydroxyethyl methacrylate (HEMA). As opposed to paraffin, plastics are liquid in their monomer state at room temperature and are catalyzed to undergo an irreversible polymerization reaction to form a solid matrix that allows sectioning.

Although GMA has never been used to embed *Quercus* flowers/fruits before, it has been used successfully for other botanical studies dealing with hard and heterogeneous materials such as conifer needles (Ruetze and Schmitt, 1986) and reconstituted herbarium specimens (Bruhl and Ashford, 1986). Animal studies that used similar plastics to infiltrate and embed rather large tissue specimens (Sims, 1974) and to minimize compression or damage during the sectioning of undecalcified bone (Franklin and Martin, 1980), also seemed to indicate that GMA would be useful in this study.

GMA has been in use for over 50 years (Rosenberg et al., 1960; Wichterle et al., 1960) and has two main components: the GMA monomer solution and the catalyzing agents that are added to the monomer to start polymerization (Cole and Sykes, 1974; Gerrits and Horobin, 1996). Other components, such as polyethylene glycol (PEG) can be added to the monomer solution to modify the final polymerized block (softening in this case) before infiltration into the specimen. The monomer solution does require special handling as it is a known carcinogen, but once GMA is polymerized, it can be

handled and disposed of in a similar manner as paraffin.

The development of commercially prepared GMA kits eliminated the need to purify and pre-polymerize raw GMA monomer, and the kit chosen for this study, Technovit 7100® (Kulzer, Germany; Energy Beam Sciences (EBS) #H7100), uses a polymerizing accelerator that is composed of a barbituric acid instead of the more commonly used, and more toxic, aromatic amines (Gerrits and Smid, 1983). This kit does not require concentrated UV exposure at room temperature to start/aid the polymerization and is not sensitive to oxygen (which can inhibit polymerization). The embedded specimens are subjected to a relatively low temperature (< 40º C) as the polymerization occurs, minimizing heat-damage artifacts. Since GMA is water-miscible, it has a high stain permeability and therefore sections can be stained without removing the GMA, which also adheres the sections to slides without pretreatment (Bennett et al., 1976; O'Brien and McCully, 1981; Ruzin, 1999).

Specimens embedded in GMA can be sectioned with metal or glass knives on a manual or automated retractable microtome. A preliminary review of articles on GMA indicated that thinner sections of <6 µm that are possible with glass knives would be more optimal in terms of seeing cell structure and staining (Bennett et al., 1976; Lindner and Richards, 1978; Butler, 1979). The Ralph or Ralph-Bennett knife (Bennett et al., 1976) was chosen for its cutting edge that spans the whole width of glass strips and would allow for the sectioning of entire flowers and fruits, thus conserving structural data.

There is a dizzying array of articles that describe GMA methods, but special

attention was given to those techniques that revealed anatomical data (versus immunological or fluorescence data), that used readily available equipment and/or materials, and could produce ribbons (Feder and O'Brien, 1968; Bennett et al., 1976; Gerrits and Smid, 1983). Since previous anatomical studies (in both paraffin-embedded and plastic-embedded specimens) that dealt with sclereid dense material required close contact with large amounts of chemicals for long periods of time, an attempt was made to streamline and/or simplify existing techniques and to minimize long-term exposure to chemicals/carcinogens. In some cases, mechanical aids were substituted for chemical-based procedures, and in others, less toxic techniques were initially tried to obtain necessary data.

It was hoped that employing alternative and non-traditional methods in this anatomical study (i.e., GMA/glass knives instead of paraffin/metal knives) would solve many of the problems previously experienced in sectioning the dense and heterogeneous flowers and fruits of *Quercus*. Successes could then be applied to future developmental studies and whole specimen sections for related taxa in this economically and ecologically valuable monophyletic group, as well as other large, woody or sclerified fruits.

## MATERIALS AND METHODS

### *Sampling*

A complete developmental series of *Quercus acutissima* Carruth (a biennial-fruiting oak from subgenus *Quercus* section *Cerris*) was collected using cultivated

material from Cornell Plantations Arboretum during the growing season of the year 2000. Because fruits from *Q. acutissima* take two growing years to mature, both flowers from the current growing year and flowers/fruits from the previous growing year were simultaneously collected and kept separate. Initial collections were unopened buds (containing immature pistillate and staminate inflorescences of the current year) and pistillate inflorescence axes of the previous year. After bud break and the current year inflorescences began to emerge, only pistillate inflorescences axes were collected. These inflorescences consisted of several pistillate flowers (each with a subtending cupule) on an inflorescence axis inserted in the axil of a leaf on a tree branch, and current year inflorescences were distal (usually on the same branch) to previous year inflorescences. Material was collected once weekly from the specimen tree (Cornell Plantations #79-119A) starting March 21 through August 1, with bimonthly collections made from August 15 through September 26. Vouchers are deposited in the Cornell University Herbarium (SB 1113).

Collections were placed in 100% cotton rag paper envelopes (13 cm high X 15 cm wide sheets that folded down to 4.4 cm high X 5 cm wide envelopes) adapted by the author from "Cornell" type envelopes commonly used on herbarium sheets. Fruits that were too large for the envelopes were placed directly in the polypropylene specimen containers for fixation.

***Fixation***

Within 2-8 hours of collecting, all specimens were placed in FAA (70% ethanol, 5% acetic acid, 5% formalin) for fixation (Johansen, 1940; O'Brien and McCully,

1981; Ruzin, 1999) and then left in the fixative for at least one week at room temperature (~25° C). Before fixation, nearly mature fruits were slashed with pruning shears to expose the embryo and the cut area was rinsed with distilled water to minimize black precipitate from chemical reactions between the steel of the cutting blade and tannins of the fruit (Li, 1954). In the cases where the FAA eventually became discolored or opaque within a few days from materials leaching from the specimens, the old solution was discarded and the containers were refilled with fresh FAA. Within a month (usually after one week of fixation), the FAA was replaced with 70% ethyl alcohol. Eventually, all specimens (most still in the paper envelopes) were transferred from the polypropylene specimen containers into wide-mouth polypropylene Nalgene® jars, with loose specimens bundled into cheesecloth and labeled. If the alcohol solution became opaque from leaching, the old solution was replaced with fresh alcohol (Figure 1).

***Specimen preparation***

After fixation and transfer into 70% alcohol, at least four specimens from each collection were dissected under a stereo dissecting microscope (Bausch & Lomb Stereozoom 4) to remove extraneous material as well as to increase the surface area for penetration of the embedding medium. Leaves and bud scales were removed from buds to expose immature inflorescences. After inflorescences emerged from the buds, each pistillate flower with its subtending cupule were removed from the inflorescence before dissection. In later stages, cupules were removed from the base of the flowers to reduce infiltration time and the load on the knives.

Figures 1-6. Materials and Methods. **1.** Collected fixed specimens in 70% alcohol: shown here in Nalgene® jars (left) and polypropylene specimen containers (right). **2.** Processing containers used in the study from top to bottom: plastic processing baskets (left unmodified, right has two stacked and tied together with top basket bottom cut out), 100% nylon manufactured lace trim folded in half and stitched, and perforated polypropylene fabric (Easy Stitch® by Pellon) folded in half and stitched. **3.** Specimens in Nalgene® jars during pre-infiltration and infiltration on an orbital shaker. **4.** Reusable molds used in this study from top to bottom: Energy Beam Sciences Molding Cup Tray, two sizes of Easy-Mold® capsule molds (left size #3, right size #00). **5.** Embedded specimens mounted on aluminum rivets and labeled: ready to be sectioned. **6.** Knife maker constructed from a small vise, with aluminum bars supporting the glass.


Quercus palmeri
ASU
4-12-95 - 8-2-95
Quercus acutissima
79-119A
Oak-Yew L46
082900 - 091500
Castanea 'seedling'
Orchards
Quercus acutissima
79-119A
Oak-Yew L46
032100 - 081500
Bulk
1
2
3
4
412.1
412.2
412.6
418.1
426.1
426.2
53.1
5
6

Undissected material was returned to corresponding envelopes and stored in the alcohol for reference and backup material.

***Processing and embedding***

All processing and embedding was performed at room temperature (~25° C). Several trials were performed on thinly-sliced mature acorns and cupule tissue using a variety of different dehydration and infiltrating protocols described in the myriad GMA literature, with the most successful protocols described below and in Table 1. Several containers were adapted by the author to coordinate transfer of many specimens simultaneously to minimize handling of the material during processing

Table 1: Modified Technovit 7100 kit protocol according to Gerrits and Smid (1983) (Gerrits and Smid, 1983).

| | | |
|---|---|---|
| Fix tissue | | |
| Dehydrate to ethanol 95% | | |
| Pre-infiltrate with:<br>1 part ethanol 95%3<br>1 part infiltration solution (see below) | | |
| Infiltrate with:<br>Infiltration solution | | |
| | Technovit 7100 | 40 ml |
| | Hardener I | 0.5 gm |
| | PEG 400 | 4.0 ml |
| After infiltration, place tissue in molds and add embedding solution:<br>Embedding solution | | |
| | Infiltration solution | 15 ml |
| | Hardener II | 1.0 ml |
| Polymerize for 2 hours at room temperature. Further polymerization at 37º C for 2 hours is recommended. | | |

(dehydration, pre-infiltration, and infiltration) prior to embedding and to conserve the monomer solution. The most successful of these included plastic tissue processing baskets (38 mm in diameter X 8 mm high, EMS #62358-W), perforated polypropylene fabric (Easy Stitch® by Pellon), and 100% nylon lace sewing trim (Figure 3). The perforated fabric and lace trim (obtained from a local sewing supply store) allowed complete penetration of the processing solutions into the specimens and were easy to open to remove the specimens before embedding. The perforated fabric was cut into rectangles 21 cm wide and 6 cm high, and folded along the longest side and sewn down to make ten pockets 1.6 cm wide X 2.5 cm high. The lace trim, which came in several sizes was cut into 25 cm strips, and folded along the longest side and sewn down to make five pockets (Figure 2). After specimens were placed in the pockets, the excess fabric at the top was folded over the opening and sewn or basted closed.

Before pre-infiltration, the specimens were dehydrated in an alcohol series of 80%, 90%, and 95% ethanol, remaining in each step for at least one day.

Dehydrated specimens were pre-infiltrated with a solution of equal parts of 95% ethanol and the infiltrating solution (0.5 gram of Hardener I + 40 ml of Technovit 7100 + 4 ml of PEG 400) modified from the GMA Technovit 7100 kit (see Table 1) (Gerrits and Smid, 1983). The alcohol was allowed to evaporate at room temperature while being gently agitated (70 oscillations/minute) on an orbital shaker (VWR Scientific, model 57018-754) for at least one day, and in most cases, the specimens were then immediately transferred from the pre-infiltrating solution to the infiltrating solution (Figure 3). In a few cases, some specimens were maintained in the alcohol-

free pre-infiltrating solution for up to six weeks. All specimens were infiltrated for one month after preliminary trials showed that 1-3 weeks was not sufficient (Bruhl and Ashford, 1986).

Specimens were removed from the processing containers, placed in molds, and embedding solution was added according to the modified kit protocol in Table 1. No special steps were required to initiate polymerization after the embedding solution was added, and all proceeded to completion. Any tacky exposed surfaces were trimmed before mounting for sectioning (Gerrits, 1983).

The rather large size (up to 2.5 X 3.8 mm) of some of the fruits inspired the trial of several different types of reusable and non-reusable molds for embedding. Reusable molds (Figure 4) included two different Easy-Mold™ capsule molds size #3 and #00 (Electron Microscopy Sciences (EMS) item #69930-05 and EMS #69931-05) and flat molds from Energy Beam Sciences (Molding Cup Tray 12 X 16 X 5 mm; EBS #H1513). Non-reusable molds included #00 gelatin capsules (23 X 8 mm), #10 gelatin capsules (52 X 22 mm) (Bennett et al., 1976), plastic molds (Peel Away Disposable Embedding Mold, 22 X 22 mm, EMS #70182), and wide-mouth polypropylene specimen containers.

***Ralph knives***

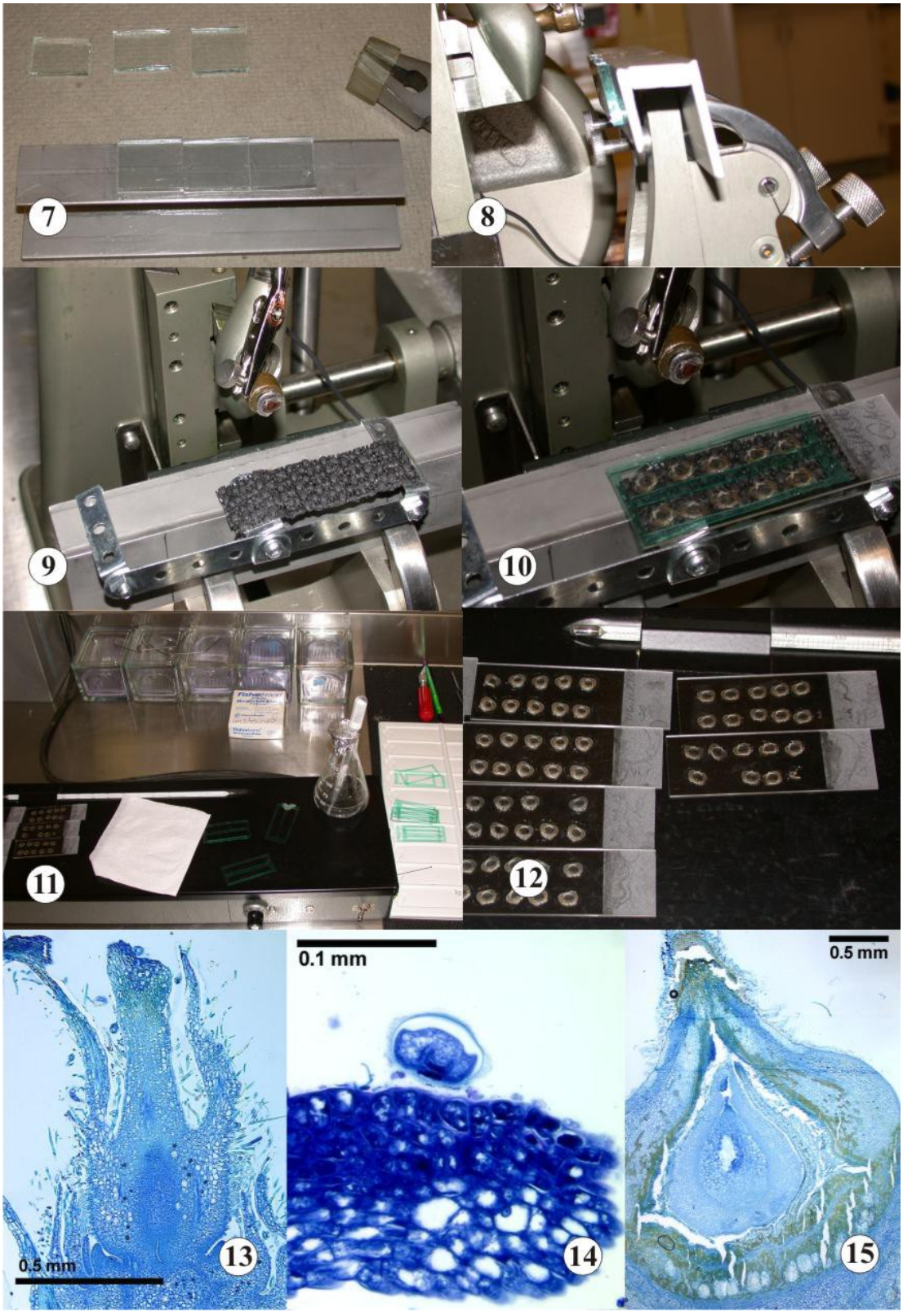
The first Ralph knives were made by hand and in a vise (Bennett et al., 1976) using 6 mm thick X 2.5 cm wide histology glass, and 2 mm and 3 mm thick window pane glass (Figure 6). After a precision knife maker, the LKB Histo-Knifemaker 2078 (Leica, Germany), was purchased midway through the sectioning process, the majority

of the sectioning was done with knives using the 3 mm thick glass (cut into 2.5 cm strips) and the LKB Histo-Knifemaker 2078. The knives obtained measured 2 cm high with a 2.5 cm wide cutting surface.

***Knife holders***

The first knife mounts were aluminum bars (3.75 cm wide X 0.3 cm thick, and cut into 15 cm lengths) that allowed one glass knife to be mounted at a time. Later, a custom-designed knife holder was constructed to the author's specifications by the Cornell Laboratory of Atomic and Solid State Physics (LASSP) shop for this study. One piece of 6 mm thick stainless steel, 1.6 cm high X 15 cm wide was machined 10 degrees on the thick edge, and then welded between two pieces of 3 mm thick stainless steel to form an inverted hook shape. One of the 3 mm pieces (2.5 cm high) held the knives, while the other 3 mm piece (3.5 cm high) clamped the holder into the microtome. Both sides were machined after welding to make them parallel (Figures 7-8). Three 2.5 cm wide Ralph knives could be mounted at a time on the custom holder using double-sided cellophane tape. A pair of vinyl-coated glass pliers (Inland) was used to tighten the tape bond between the knives and the holder (Figure 7). When all the knives became dull, they were removed (along with the tape) by soaking the holder surface with 70% ethyl alcohol and then prying the glass up with a single-edge razor blade. Tape residue was scraped off the holder with the razor blade and then scrubbed off with acetone. After the acetone dried, new knives could be immediately placed on the holder.

Figures 7-15. Materials and Methods. 7**.** Ralph knives mounted on steel custom knife holder, three 2.5 cm wide knives shown here with glass pliers on the right that was used to squeeze the knives and permanent double-sided tape onto the knife holder. **8.** Side view of knife holder with knives mounted in the microtome: note how the holder raises the knives above the clamps. Specimen on rivet in microtome clamp at top of photo. **9.** Top view of knife holder mounted in the microtome with slide support jig in place: note the grounding alligator clip on the specimen clamp. Sectioning was started on the farthest right knife on the holder, and as the knife became dull or chipped, the clamps were loosened and the holder moved to the right. The slide support jig could be shifted right or left on the holder as needed to optimize transfer of sections onto the slide. **10.** Same view as in Figure 9 with slide on the jig showing vinyl template separating the sections on a pool of water (on the slide). **11.** Work station showing slide warmer, vinyl templates, heated water, Coplin dishes, insect dissecting pins (for section placement on slide) and other tools. **12.** Examples of sections mounted on slides drying on the slide warmer: breaks in the specimen sequences show when a knife became too dull and the holder was shifted. Figures 13-16: examples of sectioned and stained *Quercus acutissima* flowers and fruits. **13**. Longitudinal section of flower showing cupule at base, ovary, perianth lobe on right, and two styles (center and left). **14.** Section showing style tissue (note extremely densely staining apex of style) and pollen grain. **15**. Longitudinal section of fruit: fruit wall surrounds the single seed in the center. Tears and bubbles occurred during placement on the slide and expansion of the section in the water.


7
8
9
10
11
12
0.5 mm
13
0.1 mm
14
0.5 mm
15

***Microtome***

A manual microtome (International Equipment Co. Minot custom microtome) with chuck advancement of 1-8 µm was set to section all the samples in 4 µm increments. Blocks with GMA embedded specimens were mounted on aluminum rivets (top surface 8 mm high X 8 mm in diameter) with cyanoacrylate adhesive (Figure 5) and allowed to set for 24 hours. Additional trimming of the block was carried out if necessary with single-edge razor blades or dulled Ralph-Bennett knives (Butler, 1980). The microtome and operator were grounded with wire to a common ground. Sectioning was performed in a small room off the main lab, and a humidifier (Bemis™ 2 in 1 air purifier and humidifier, model DP3 200) maintained ~ 60% humidity in the room.

***Section placement***

Slides (Fischer Brand frosted microscope slides; 7.5 X 2.5 X 0.1 cm) were used out of the box after a swipe with 70% alcohol. Since ribbon formation was not consistent in this study, slide templates, cut out of clear vinyl (12 gauge) and adapted by the author as an aid to place sections in sequence, were placed on each slide prior to sectioning. The vinyl templates were trimmed to fit on the non-frosted area of each slide, and had either one, two or three rectangles cut out the length of the template to form water wells or frames. Before pressing the vinyl down with a Kim wipe onto the slide, both sides of the template were swiped with 70% alcohol. Heated (~ 60º C) distilled water was placed by dropper (~ 7-8 drops total for a single slide) into the wells of the template. Each slide with vinyl template were placed on top of leveling jig

on top of the knife holder (Figure 9). Sections were picked off the knife individually with forceps and placed sequentially in the wells (Figure 10). When the glass knife became dull or was chipped, the chuck was backed off a ¼-½ turn of the wheel and the knife holder shifted to a new position. In most cases, the next sections to come off the block were usually incomplete or too thin, so a space was inserted in the sequence to indicate missing data. When filled, slides were transferred to a slide warmer set to 60º C, and the water was allowed to evaporate before removing the template (Figures 11-12). Slides were left on the warmer at least 12 hours, and allowed to cool to room temperature before staining. For each block, at least one slide was set aside as a control or for later comparative staining techniques.

***Staining***

Staining was accomplished without removing the GMA embedding medium. The Toluidine Blue O (TBO; C.I. 52040) 0.05% staining solution was modified from a standard recipe (Feder and O'Brien, 1968), with an acetate buffer (pH 4.4) substituted for the benzoate buffer. Slides were first rehydrated in distilled water for a few minutes, and then placed in the TBO stain for 1.5-2 minutes, rinsed in running tap water until the plastic is nearly free of stain (up to five minutes), rinsed a final time in distilled water, and then allowed to air-dry.

For comparative purposes with previous paraffin-embedded studies, a few slides (retained aside for this purpose) were stained with either Acid Fuchsin (C.I. 42685) (O'Brien and McCully, 1981) or a combination of Alcian Blue 8GX (C.I. 74240) and a weak aqueous Safranin O (C.I. 50240) stains (Ruzin, 1999).

No mounting media or coverslips were used, and immersion oil was placed directly on the sections for microphotography (O'Brien and McCully, 1981).

### *Microphotography*

Photographs of hand-sections were obtained with a Wild dissecting microscope using a Nikon 995 digital camera and a microscope uniadapter (ECO-1832D, Zarf Enterprises, Spokane, Washington USA). Images were obtained from an Olympus BX60 compound microscope using video capture from a CCD Sony color video camera (model DXC-9000) on a PC computer (Figures 13-15), with scale calibrated using a stage micrometer (American Optical Company, Buffalo).

## RESULTS

The developmental study of *Q. acutissima* has been published in a separate paper (Borgardt and Nixon, 2003) illustrating the sections obtained with the techniques described in this paper. Figures 13-15 show a few examples of how large structures, such as an entire flower (Figure 13) and an entire fruit (Figure 15), can be successfully sectioned in their entirety with these techniques as well as minute and delicate structures such as pollen grains (Figure 14).

### *Sampling, Fixation and Preparation*

The weekly and later bimonthly sampling schedule was sufficient to address the developmental questions in this study. FAA fixation was adequate in preserving structures prior to processing and embedding, and black precipitate (from the reaction of steel and tannins) could have been avoided with the use of ceramic blades to slash

fruits before placing them in FAA (Li, 1954). The 100% cotton rag paper envelopes adapted by the author did not fall apart in the FAA or 70% alcohol, and were useful in organizing material since the notes written in graphite remained legible even after months in the alcohol.

Initial attempts at trimming specimens sometimes introduced air bubbles that later created artifacts and problems during infiltration and/or sectioning. In some cases, the bubbles dehydrated or collapsed part of the specimen, resulting in no to little GMA infiltration in that area. These air bubbles persisted through infiltration and embedding, and these open spaces sometimes tore or split during sectioning. But when no air bubble artifacts was introduced during trimming, GMA infiltration was remarkably thorough even in areas of high sclereid density. The only structures that resisted infiltration were the waxy outer cuticle of the flower and fruit wall and the completely sclerified mature exocarp.

***Processing and Embedding***

Preliminary embedding trials by the author demonstrated that the maximum recommended amount of PEG 400 (1 part PEG 400: 10 parts GMA monomer) was required in both the pre-infiltration and the infiltrating steps or the blocks were too brittle. These trials also demonstrated that two weeks of infiltration was sufficient for mostly parenchymatous specimens, but one month of infiltration was needed for later stages (Bruhl and Ashford, 1986).

The perforated polypropylene fabric was the most successful for processing large numbers of specimens simultaneously with minimal handling. The strips remained

intact (no tearing or disintegration) throughout dehydration and infiltration, did not float or retain air bubbles, and successfully allowed circulation of the fluids around the specimens. When it was time for embedding, it was easy to tear or cut the fabric open to expose the specimen and minimize handling. The lace strips were also useful, but were not as customizable as the perforated fabric which could be cut to any dimension and sewn to form any size pockets. One unexpected benefit both the fabric and lace strips appeared when one of the infiltrating batches polymerized before the specimens could be separated out and placed into the molds. After cutting the fabric strips out of the plastic block, and the specimens inside were still salvageable, as they were polymerized in situ in the pockets. Compared to the fabric pockets, the tissue processing capsules were inefficient in processing many small specimens and were also not tall enough to enclose the later stages of the fruits.

The non-reusable molds, especially the gelatin capsules, ended up being the most useful during embedding in terms of available sizes and minimizing tacky surfaces after completion of polymerization. Most of the reusable molds eventually split or cracked when the embedded specimens were removed and eventually had to be discarded, except for the flat molds (EBS #H1513, Figure 4) which were sturdier and of a good size for the larger fruits.

***Sectioning***

Using standard glass cutting tools, the 3 mm thick window pane glass sheets were cut down to 3 cm and 2.5 cm wide strips. The 2.5 cm wide strips proved the most

useful (in conjunction with the LKB Histo-Knifemaker 2078) in terms of producing a mostly consistent cutting surface across the whole width of the glass knife.

The Ralph glass knife needs to be held securely enough to prevent tearing or "chatter" (i. e., uneven movement of the knife through the material that forms rhythmic thin and thick areas parallel to the width of the knife) during sectioning, but the holder must also allow fast and easy replacement of each knife as it chips or becomes dull. Various authors (Szczesny, 1978; Butler, 1979; Chappard and Laurent, 1983) describe holders with clamps that can be manually loosened and tightened. Other protocols describe the use of heat-softened cement (e.g., Pyseal cement, Fischer Scientific, catalog #C-228 no longer available; Bennett et al., 1976) or a cyanoacrylate adhesive (Federman and Golick, 1985) to attach the knives to metal bars or empty standard metal knife holders that were then clamped in the microtome. Butler (1979) detailed the use of permanent double-sided Scotch Brand™ tape to hold the knives for inspection before clamping, and it was decided to carry that idea further along and use the tape to hold the knives on the holder while sectioning. The cellophane double-sided tape held the knives onto the custom holder without chatter and allowed for rapid replacement of the knives, eliminating the need for clamps or custom rigs to hold the knives onto the holder.

The custom knife holder was designed to arch over the microtome clamps, which allowed three Ralph knives (each 2.5 cm wide) to mounted at a time. This significantly increased the available cutting surface compared to previous studies and also reduced downtime from switching the knives out as they became dull or chipped.

The custom holder was also propped a slide up next to the microtome chuck during sectioning (Figure 10), making it easier to pull sections as they came off the knife and place on the slide water bath (Bennett et al., 1976). One flaw that resulted from this holder design was that the angle allowed water to flow off of the slide. A prop or jig made of erector-set pieces and placed on top of the holder corrected the angle so water did not flow off the slide (Figures 9-10).

Static electricity was a serious impediment to obtaining even sections and required considerable effort to control. When static was present on the knives, sections curled up into tubes on the edge of the knives and rarely flattened out when placed on the water. Attempts to uncurl the section often resulted in tearing or further compression. When static was present between the section and the water bath, the sections would crumble or twist in various ways before landing on the surface of the bath. This problem was addressed from three main directions. First, all of the equipment and the person operating the microtome were grounded with wire to a common ground. Second, distilled water was used, every effort was made not to add impurities (such as lipids from the hands) to the water pools, slides were handled on edges only, and tweezers were used to apply and remove the vinyl templates. Finally, a humidifier was used to maintain ~ 60% humidity in the small side room in which all sectioning took place. All of these efforts in combination reduced the static problem to minimum levels, and the increase in humidity also had a side benefit of softening the blocks before they were sectioned.

Even though the infiltrating and embedding GMA protocols used for this study

formed ribbons during sectioning without the addition of external aids (Bennett et al., 1976; Gerrits and Smid, 1983), ribbon-formation was not a consistent result in this study. This was probably because the blocks in the current study were rarely even-sided polygons and were rounded or amorphous after trimming. It was decided to place individual sections on sheets of water that were contained within vinyl templates.

The vinyl templates designed in this study directed sequential placement of the sections, prevented migration of the sections across the water pool on the slide, slowed down evaporation of the water pool on the slide during sectioning, and also allowed water to be dropped onto the slide without disturbing the placed sections. When sectioning was complete, the template also kept the sections separated on the slide until evaporation completed on the slide warmer.

***Staining***

Since it was not necessary to remove the embedding media prior to staining, the GMA was able to adhere the sections to the slides.

A combination of aqueous Alcian Blue and weak aqueous Safranin O stains (Ruzin, 1999) applied to GMA embedded samples confirmed cellulosic structures that previous studies revealed with Fast Green and Safranin O stains (Mogensen, 1965), and an Acid Fuchsin stain was used to verify lignin (O'Brien and McCully, 1981). This confirmation allowed for direct comparison of the TBO stained material to previous anatomical studies.

Functioning as a metachromatic stain in this study, TBO stained

lignified/sclerified structures a light aquamarine/turquoise color, cellulosic structures a blue color, and proteins a dark purple to red-purple/violet color. Tannins in the specimens was visible as a yellow to yellow-green, depending on how many cell walls (stained blue) were between the tannin deposit and the microscope eyepiece. Although not stained, crystals could be seen in all the sections.

## CONCLUSIONS

Although not commonly used as a plant anatomical technique, GMA was able to penetrate and stabilize highly sclerified and heterogeneous material remarkably well in this study. Aside from the cuticle and the mature sclerified fruit wall, the main impediment to GMA infiltration throughout each specimen was the presence of air bubbles or air pockets, many of which were introduced during specimen preparation. Ways to address this would be either to embed large specimens and then cut them into smaller pieces using a thin-bladed saw (such as Buehler® ISOMET saw) or to cut or trim any hydrated or alcohol-preserved material while submerged to avoid introducing air bubbles/pockets. This study started with live material that was fixed and then processed, but GMA also holds great potential for looking at rehydrated material preserved on herbarium sheets (Bruhl and Ashford, 1986). The results in this study demonstrate that as long as the specimens are well hydrated and every effort is made to prevent the introduction of air or to remove all air, GMA embedding will be a powerful tool in future studies that look at highly sclerified material and/or unusually large fruits, whether they are from live or preserved material.

The Ralph-Bennett glass knife has already proven its worth in previous studies for obtaining thin-sections from large specimens (Sims, 1974; Bennett et al., 1976), and in the current study was able to successfully cut an entire mature acorn in longitudinal section! With the knife holder designed in this study, some of the limitations (knife width, slow knife switching) that have held back the full potential of this glass knife in plant anatomical studies are resolved. Holders can be designed to be wider (15 cm wide in this study) to allow more knives or wider knives to be mounted. The only limiting factor would be the microtome's capacity to clamp a wider (and heavier) holder stationary in the microtome. A minor adjustment to the angle of the sides of the holder would allow it to support a slide without the water flowing off.

Ribbon formation, although not critical to data collection, greatly facilitates rapid and efficient sectioning. Using polygon-shaped molds, trimming embedded specimens, and/or re-embedding specimens (Greany and Rubin, 1971; Beeckman and Viane, 2000) to form parallel sides may solve the inconsistent ribbon formation in the current study. Ribbons usually require a water bath, and it would be easy to construct a miniature one using a single celled vinyl slide template such that each slide (oriented parallel to the chuck) would function as a water bath. Early developmental stages that are mostly parenchymatous would not hinder ribbon formation, but later sclerified stages would dull the knives quickly and break the ribbons at the knife. Then it would be necessary to pull sections off the knife and place them on the slide individually.

TBO, when used as a metachromatic stain, is unusually powerful in that it not only identifies structures with the same accuracy as more traditional stains, it identifies

additional features without bleaching and/or re-staining. Since GMA does not have to be removed prior to staining, features such as tannins or crystals (which are often not present after more traditional slide-preparation methods) can be found that may be useful in revealing new areas of inquiry for anatomical and phylogenetic studies.

The goal to minimize exposure to toxic materials was a trade-off. Some of the initial preparation techniques (other fixation methods, soaking in caustics) and end techniques (slide preparation for adhesion/staining) that involved toxins were eliminated, but GMA does require special handling while in monomer form. All embedding was performed in a laminar flow hood to minimize inhalation, and leftover monomer was polymerized before disposal.

In conclusion, the techniques used in this study hold great potential in furthering plant anatomical studies, especially when the material under investigation is unusually large, becomes dense or hard from sclereid formation, or positional data needs to be preserved. The improvements to GMA/Ralph knife techniques presented in this study demonstrates that even after 60 years, there is still much to explore with GMA in the near and far future.

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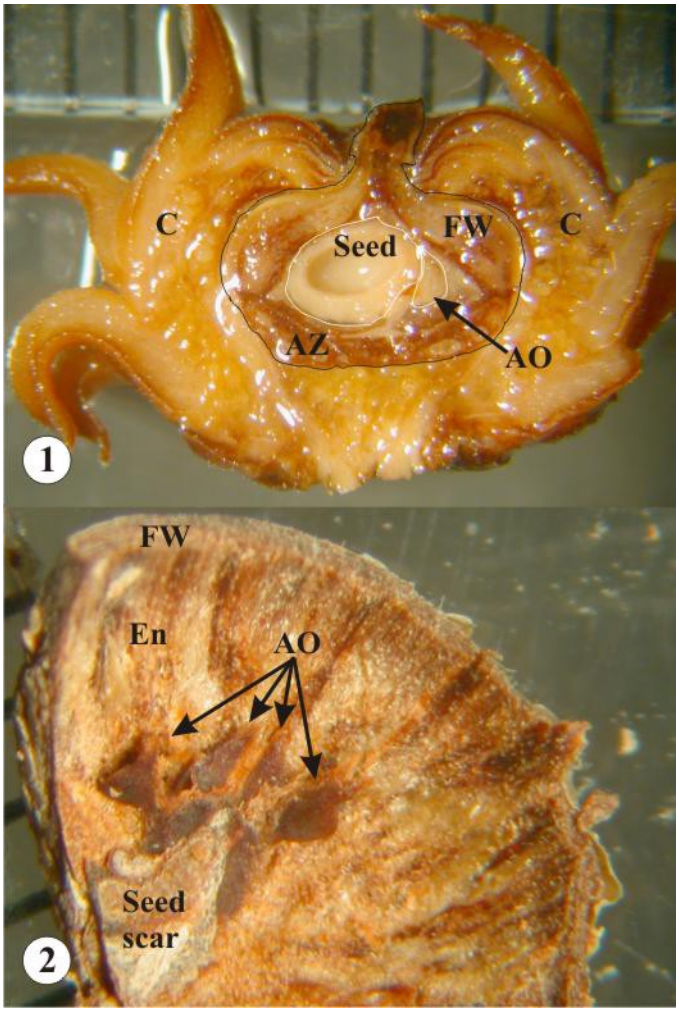
# CHAPTER 3

# A COMPARATIVE FLOWER AND FRUIT ANATOMICAL STUDY OF *QUERCUS ACUTISSIMA*, A BIENNIAL-FRUITING OAK FROM THE *CERRIS* GROUP (FAGACEAE)

## INTRODUCTION

Within the traditional, wind-pollinated "Amentiferae", a group of taxa that centered around the family Fagaceae had been proposed to be monophyletic based on analyses of morphological characters only (Nixon, 1984, 1989; Hufford, 1992), molecular characters only (Chase et al., 1993; Manos and Steele, 1997; Chen et al., 1998; Kallersjo et al., 1998; Qiu et al., 1998; Soltis et al., 2000), as well as combined morphological and molecular characters (Nandi et al., 1998). Families in this clade include Betulaceae (birches), Casuarinaceae (she-oaks), Fagaceae (oaks, beeches), Juglandaceae (walnuts), Myricaceae (wax myrtles), and Nothofagaceae (southern beeches), as well as the monotypic families Rhoipteleaceae and Ticodendraceae. Termed the "higher" Hamamelidae (henceforth referred to as the HH clade; Crane and Blackmore, 1989) after the subclass Hamamelidae of Cronquist (1981) and Takhtajan (superorder; 1997), this clade was later described as "Fagales" (APG, 1998); a name that has historically been applied to circumscriptions within the HH clade. To avoid potential taxonomic confusion, the unambiguous term HH clade will be used in this study. All flowers produced in this clade are unisexual, with pistillate flowers, interpreted as inferior and syncarpous, maturing into fruits that are

pseudomonomerous with only one seed (Figure 1).




Figures 1–2. *Quercus acutissima* fruit hand-sections in two different stages of development illustrating important morphological features. **1.** Immature (29 August) fruit longitudinal section (*ls*) showing cupule (C) enclosing a fruit (black outline) with fruit wall (FW), abscission zone (AZ), seed (white outline), and one abortive ovule (AO; white outline). The seed consists only of a seed coat with cellular endosperm in this example: the embryo is out of plane of section, and the coenocytic endosperm drained out after the fruit was cut. Scale = 1.0 mm. **2.** Mature (26 September) fruit transverse section (*ts*) in the base with the seed removed to reveal the interior of the fruit wall (FW) with its pubescent endocarp (En), a seed scar, and four (of five) abortive ovules (AO) as shown by arrows. The fifth abortive ovule is beneath the seed scar. This section was also cut longitudinally on the left of the figure. Scale = 1.0 mm.

Some taxa produce only one ovule/flower, but more commonly, multiple ovules with one or two ovules per carpel, are formed in the flower and all but one abort before fruit maturation. These abortive ovules are in an apical position (distal) in the mature fruit in all the taxa found in the HH clade except for certain groups within the genus *Quercus* (the oaks) of the family Fagaceae, which can have apical abortive ovules, basal abortive ovules, or abortive ovules with a variable position that are neither strictly basal nor apical and are described as lateral (Table 1). Even within these different oak groups, ovule position can be variable as is seen in subgenus *Quercus* section *Lobatae* (the red oaks) with typically apical abortive ovules that has at least 10 species of red oak with lateral to basal abortive ovules in Mexico and Central America (Trelease, 1924; Muller, 1942a; K. C. Nixon, Cornell University, unpublished, and Flora Mesoamericana, in preparation).

A. de Candolle (1862) was the first to document the variability in abortive ovule position in *Quercus*, with subsequent classifications (Oersted, 1871; Trelease, 1924; Muller, 1942b; Nixon, 1993, 1997) also making use of this feature to distinguish among groups in the oaks, including the most recent complete treatment of genus *Quercus* by Camus (1934–54) who divided the genus into two subgenera, *Cyclobalanopsis* and *Quercus*. In the Flora of China (Huang et al., 1994), *Cyclobalanopsis* is treated at the level of genus, and although this treatment does not conflict with accepted natural groups in the oaks, the current study will follow the classification and ranks of Camus according to her monograph (Table 1), with some modifications due to priority of names (Voss, 1981).

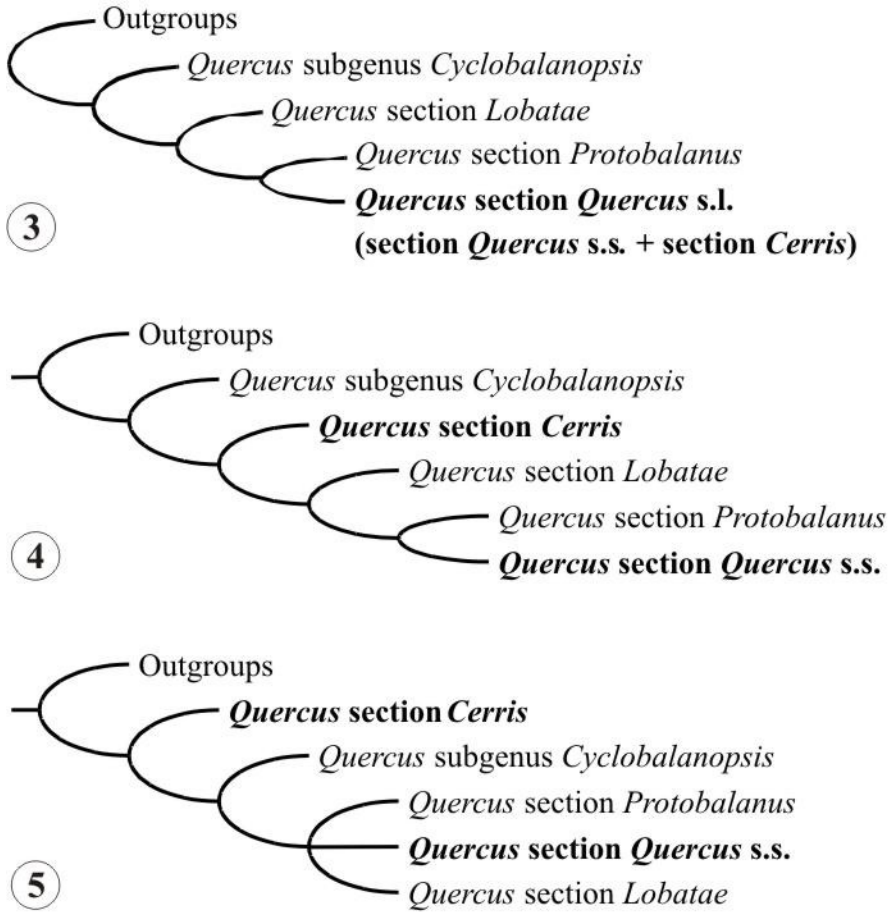
*Table 1:* Abortive ovule position in the genus *Quercus*. Old synonyms from previous classifications are below currently accepted names (Oersted, 1871; Trelease, 1924; Camus, 1934–1954; Muller, 1942a, b; Mogensen, 1965; Tucker, 1980; Kaul, 1985; Nixon, 1997).

| | Apical abortive ovules | Lateral abortive ovules | Basal abortive ovules |
|---|---|---|---|
| Subgenus *Cyclobalanopsis* (Oersted) Schneider | YES | | |
| Subgenus *Quercus* Hickel et Camus (*Euquercus)* | YES | YES | YES |
| Section *Lobatae* Loudon (*Erythrobalanus, Rubrae*) | YES | Some | Some |
| Section *Protobalanus* (Trelease) Schwarz | | YES | |
| Section *Quercus* s.s. Hickel et Camus *(Lepidobalanus, Leucobalanus, Macrobalanus, Mesobalanus)* | | Some | YES |
| Section *Cerris* Loudon | | | YES |

There is relatively little known about the anatomy and maturation of the flowers/fruits in subgenus *Cyclobalanopsis*, so the remainder of this paper will focus on subgenus *Quercus*.

Basal abortive ovule position (e.g., Figure 2) figured prominently as a putative synapomorphy and derived character in the morphological analyses that proposed uniting the white oaks of subgenus *Quercus* section *Quercus* and the white oaks of the *Cerris* group (including *Q. ilex*) into a larger subgenus *Quercus* section *Quercus sensu lato*. (Figure 3; Nixon, 1984, 1993). Intersectional hybridization events between these two groups, which are very rare in subgenus *Quercus*, of both natural and artificial origin (Cottam et al., 1982; Boavida et al., 2001) have also focused on the similarities between these two sections. So far, molecular analyses based on ITS sequence data have not supported a circumscription of subgenus *Quercus* section *Quercus s. l.*, with cladograms from these analyses separating the "white" oaks into two separate clades and basal abortive ovules independently derived in both groups (Figures 4, 5; Manos et al., 1999; Manos et al., 2001). Based on these analyses, this article will treat the "white" oaks *s. l.* as two discrete entities and refer to them as section *Cerris* (entirely Old World, including the Ilex, Suber and Cerris groups) and section *Quercus s. s.* (both Old World and New World). In order to understand the significance of basal abortive ovule position and how it relates to the sections *Cerris* and *Quercus s. s.*, additional data on the processes that underlie fruit development is needed.

One of the most complete flower/fruit anatomical and developmental studies on *Quercus* subgenus *Quercus* was a study that described the maturation of the pistillate




Figures 3–5. Cladograms of putative relationships among the subgenera and sections of the genus *Quercus*. Taxa in **bold** have predominantly basal abortive ovules. All cladograms were drawn using WinClada (Nixon, 1999–2002). **3.** Relationships based on cladistic analyses using taxonomically important diagnostic characters for the genus and outgroups. Redrawn from (Nixon, 1989, 1993). **4.** Strict consensus of two most-parsimonious cladograms based on simultaneous analysis of chloroplast DNA restriction sites and ITS sequence data. Length = 383 steps; consistency index (CI) = 0.5; retention index (RI) = 0.76. Redrawn from (Manos et al., 1999). **5.** Cladogram based on one of thousands of most parsimonious cladograms based on ITS sequence data. Length = 1038 steps; CI = 0.34; RI = 0.82. Redrawn from (Manos et al., 2001).

flowers of *Quercus alba* L. of section *Quercus* s.s. and *Q. velutina* of section *Lobatae* from anthesis to fruit maturity in some detail (Mogensen, 1965). Although there have been numerous studies of flower and fruit maturation in the oaks, the studies that sampled species in section *Cerris* only documented morphological features (Kaul, 1985), limited the descriptions to diagnostic features, and did not describe maturation patterns (Camus, 1934–1954; Soepadmo, 1968), or only described fruit development up to early embryogenesis (Corti, 1954, 1955, 1959; Scaramuzzi, 1960; Bianco, 1961; Boavida et al., 1999). To obtain a complete developmental series of fruit maturation in section *Cerris* and to allow direct comparison of features described in the Mogensen study, we sampled a species of section *Cerris*, a cultivated *Quercus acutissima* Carruth. individual, over a growing season. This species is native to temperate East Asia and is commonly planted as an ornamental in North America. Commonly called the Sawtooth Oak, this species exhibits consistent biennial fruit maturation (Figure 6), as opposed to some other species in section *Cerris* that have been reported to exhibit both annual and biennial maturation patterns (Corti, 1955; Elena-Rossello et al., 1993; Boavida et al., 1999).

The goal of this study was to collect new information that will contribute toward a greater understanding of the fruit developmental processes in subgenus *Quercus* section *Cerris*, especially those processes that influence final abortive ovule position, and to discuss these results in the context of comparable anatomical studies. This is the first flower/fruit anatomical and developmental study of the species *Q. acutissima* and

is also the first intersectional comparative anatomical study for subgenus *Quercus* section *Cerris*.

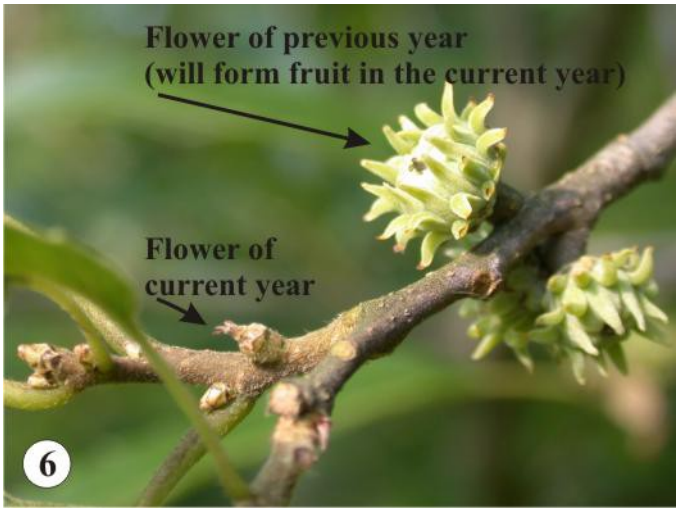



Figure 6. Biennial-fruiting habit as shown by a *Quercus acutissima* twig in late July with a flower of current year and a flower of previous year indicated with arrows. The flower of the current year was quiescent at the time this picture was taken, and the flower of the previous year was close to the time of syngamy.

## MATERIALS AND METHODS

### *Sampling*

A complete developmental series of *Quercus acutissima* Carruth. was collected using cultivated material from F.R. Newman Arboretum, Cornell Plantations, Ithaca, New York USA, during the growing season of the year 2000. To follow the biennial fruit maturation exhibited in this species, 10 flowers of the current season as well as 10 flowers/fruits of the previous season were collected once weekly from the specimen tree starting March 21 through 1 August, with bimonthly collections from 15

August through 26 September. Entire reproductive axes were collected with attached flowers and later, the maturing fruits. Once the fruit wall of the nuts became sclerified, fruits were slashed before fixation to expose the embryo and facilitate penetration of the fixative into the interior of the fruit. During specimen preparation, cupules of the later stages were removed to reduce infiltration time. Vouchers (SB 1113) of the specimen tree, Cornell Plantations #79-119A, are deposited in the Cornell University Herbarium (BH), Ithaca, New York USA.

***Fixation***

Collection materials were fixed for a minimum of seven days in a dehydrating FAA formulation (70% ethanol, 5% acetic acid, 5% formalin) to promote rapid penetration into the fruits (Johansen, 1940; O'Brien and McCully, 1981; Ruzin, 1999) and were then transferred to 70% ethanol for long-term storage.

***Pre-infiltration, infiltration and embedding***

Specimens were dehydrated, pre-infiltrated, infiltrated, and embedded using existing protocols (Gerrits and Smid, 1983; Gerrits and Horobin, 1996) for the Technovit® 7100 kit (Kulzer, Germany), which uses glycol methacrylate as the embedding medium.

***Sectioning***

Embedded specimens were sectioned on a manual microtome in increments of 4 µm using Ralph-Bennett glass knives (Bennett et al., 1976). Sections were placed in sequence on a pool of water on a microscope slide and allowed to air dry on a slide warmer set to 60ºC. No pretreatment of the slides was necessary except for a swipe

with 70% alcohol to remove dust. The next day, the slides were prepared for staining. At least one slide from each specimen was set aside as a control or for later comparative staining techniques. A more detailed account of the methods used in this study is in preparation and will be published separately.

***Staining***

A staining solution of Toluidine Blue O (TBO) 0.05% was prepared from a standard recipe (Feder and O'Brien, 1968) with an acetate buffer (pH 4.4) substituted for the benzoate buffer. Handled this way, TBO stains lignin and sclerified structures a light aquamarine or turquoise color, cellulosic structures stain a dark blue, and proteins stain a red-purple to violet. No mounting media or coverslips were used, and immersion oil was placed directly on the sections for microphotography (O'Brien and McCully, 1981). A combination of aqueous Alcian Blue and weak aqueous Safranin O (Ruzin, 1999) was used to verify that the structures stained by TBO could be compared to previous studies that used Fast Green and Safranin O stains. Acid Fuchsin stain was used to verify the staining of lignin by TBO (O'Brien and McCully, 1981).

***Photography***

Photographs of hand-sections were obtained with a Wild dissecting microscope using a Nikon 995 digital camera and a microscope uniadapter (ECO-1832D, Zarf Enterprises, Spokane, Washington USA). Photomicrographs were obtained with an Olympus BX60 compound microscope using video-capture under the control of a microcomputer, with scale calibrated using a stage micrometer.

### *Mapping characters*

The species from the current study (*Quercus acutissima*) and the Mogensen (1965) study (*Q. alba*, *Q. velutina*) were substituted for their respective sections on the appropriate terminals of a tree derived from the most recent molecular analysis that includes the major groups in *Quercus* (Manos et al., 2001). characters of fruiting habit, flowers, fruits, and seeds were scored and optimized on the tree using standard optimization procedures (Fitch, 1971) in WinClada (Nixon, 1999–2002). All 20 characters were nonadditive, with 16 binary and four multistate characters. The matrix and character coding are available from the first author.

## RESULTS

The nine developmental stages used in this study were based on definable points that were observed during the maturation of the pistillate flowers of the current year, pistillate flowers of the previous year, and the fruit in *Q. acutissima*. Within each stage, a summary of features and changes seen in the flowers/fruits is presented in order from the exterior to the interior and from the apex to the base of the flower/fruit. Because *Q. acutissima* has biennial fruit maturation, the pistillate flowers of the current year are described first, followed by the description of the flowers of the previous year and the fruits.

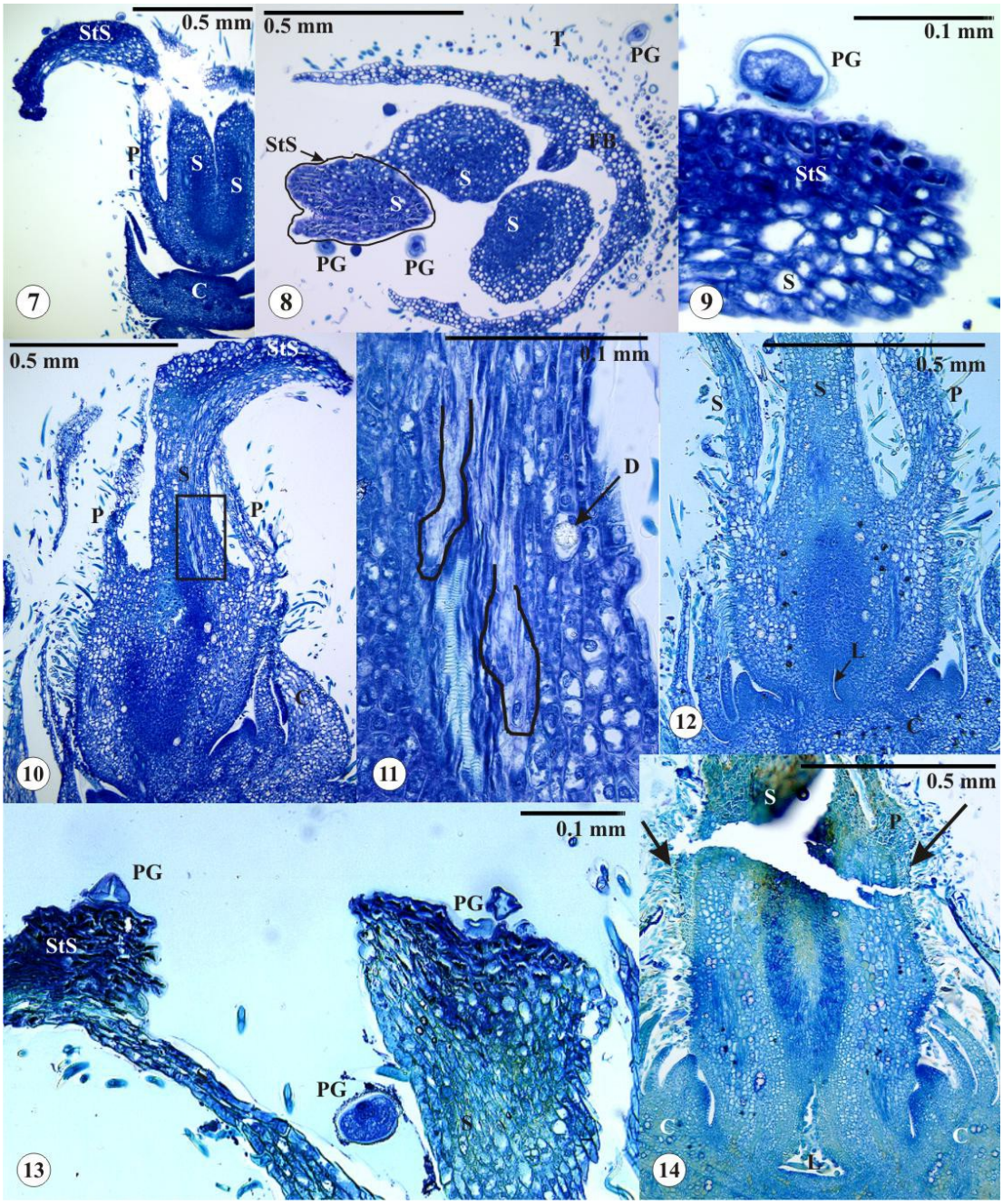
### *Flowers of current year*

*Stage 0, Winter dormancy: 21 March–25 April*–Pistillate flowers of the current year were either not yet differentiated on the inflorescence axis, or were differentiating on the inflorescence axis within closed terminal buds.

*Stage 1, Bud break: 2–9 May (Figures 7–9)*–At bud break, there were two subopposite flowers on each inflorescence axis in most cases, although some collections had up to five flowers arranged as decussate, subopposite pairs. Each pistillate flower was in the axil of one large bract that was usually flanked by two ephemeral secondary bracts on the inflorescence axis. The cupule could be seen subtending the flower with one to two scale cycles (Figure 7). The most prominent features of the flowers were the (usually) three styles which were slightly recurved, tapering to slightly flared apices with scattered druses visible in section. Stigmatic surfaces on the styles stained an intense violet, and were present from the style apices down adaxial central furrows to the point where the styles fused together (Figures 7–9). Ungerminated pollen grains, 24–32 μm in diameter, were present on the stigmatic surfaces other as well as other surfaces of the flowers (Figures 7–9). Unicellular-lignified and multicellular-glandular trichomes and a blue-staining surface distinguished the perianth lobes from the styles in section. At the base of the pistil, the ovary was not yet differentiated or distinguishable from the style bases (Figure 7).

*Stage 2, Anthesis and pollination: 16 May–23 May (Figures 10–13)*–The cupule was considerably larger than the previous stage, with four to six cycles of scales. Some sclerification of the outermost cycle of scales was obvious by the end of this stage (Figures 10, 12). The styles remained the prominent feature of the flowers, with some cells highly vacuolated in the style apices in both the stigmatic and nonstigmatic areas (Figure 10). Some senescence in the stigmatic areas was visible at the end of this stage as compressed cells with densely staining, coagulated contents (Figure 13).

Figures. 7–14. *Quercus acutissima* flowers of current year. **7.** Stage 1; 9 May. Flower *ls* including cupule (C), perianth (P), and stigmatic surface (StS) of styles (S). **8.** Stage 1; 9 May. Flower *ts* with a floral bract (FB) pubescent with glandular and lignified trichomes (T) enveloping three styles (S). One style is sectioned obliquely through its stigmatic area (StS; arrow). Several pollen grains (PG) are present, with a pollen grain outside of floral bract. **9.** Stage 1; 9 May. High magnification of style apex *ls* showing densely staining cells in stigmatic surface (StS) relative to lighter-staining cells in nonreceptive style tissue (S), with a pollen grain (PG). **10.** Stage 2; 16 May. Flower *ls* with cupule (C), stigmatic surface (StS) of one style (S), and perianth lobes (P). Box enlarged in next figure. **11.** Stage 2; 16 May. Higher magnification of style tissue from Figure 10 showing pollen tubes (outlined) as tip growth. Scattered cells with druses (D) also present. **12.** Flower *ls* showing cupule (C), two style (S) bases, one perianth lobe (P), and the appressed walls of locule (L) at arrow. **13.** Stage 2; 23 May. Flower apex *ls* of same specimen as Figure 12 in another section showing stigmatic surface (StS) on styles (S) with pollen grains (PG). Pollen grains in upper left and upper right have discharged their contents; the pollen grain in the center still has a gametophyte. Separation of center pollen grain from style occurred during mounting. **14.** Stage 8; 26 September. Flower *ls* at end of current growing season showing cupule (C), senesced styles (S) with several layers of suberized cells (arrows) apical to where the cupule scales are appressed to the pistil, one perianth lobe (P), and locule (L) containing trichomes from the endocarp epidermis. Torn area in upper part of specimen occurred during sectioning.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Germinated and ungerminated pollen grains were present (Figure 13), with pollen tube growth visible within the style in one specimen (Figure 11). The ovary was not yet enlarged at the base of styles, but locules were visible in sections as thin slits (Figure 12).

*Stages 3–7, Quiescence: 23 May–15 August*–The styles and perianth senesced completely in stage 3, with cells variously losing cohesion, becoming compressed, suberized, or filled with tannins. For the rest of the current growing season, the flowers remained relatively unchanged except for some enlargement of the ovary at the base of the styles.

*Stage 8, Quiescent: 29 August–26 September (Figure 14)*–Before the onset of winter dormancy, each pistillate flower appeared enveloped within a cupule with only the styles and perianth exserted. Cupules at this stage had at least six cycles of awl-shaped, imbricate scales. The outermost scales were highly sclerified with the scale tips appressed to the pistil below the perianth (Figure 14). The styles and perianth were often broken so that only the bases of the styles remained. In cases where the styles or perianth were still present at the apex of the flower, the outermost cells were either filled with tannins or suberized (Figure 14; at arrows). Within the styles, cells internal to the tannin-filled or suberized cell rows were highly vacuolated and some had druses. At the base of the styles, some cells remained parenchymatous and stained intensely (Figure 14). The ovary was still not well-differentiated at the base of the styles, but locular spaces delimited by intruded septa were more obvious. Unicellular trichomes were now present on the walls of the locules (Figure 14). No ovule

primordia were visible.

***Flowers of previous year***

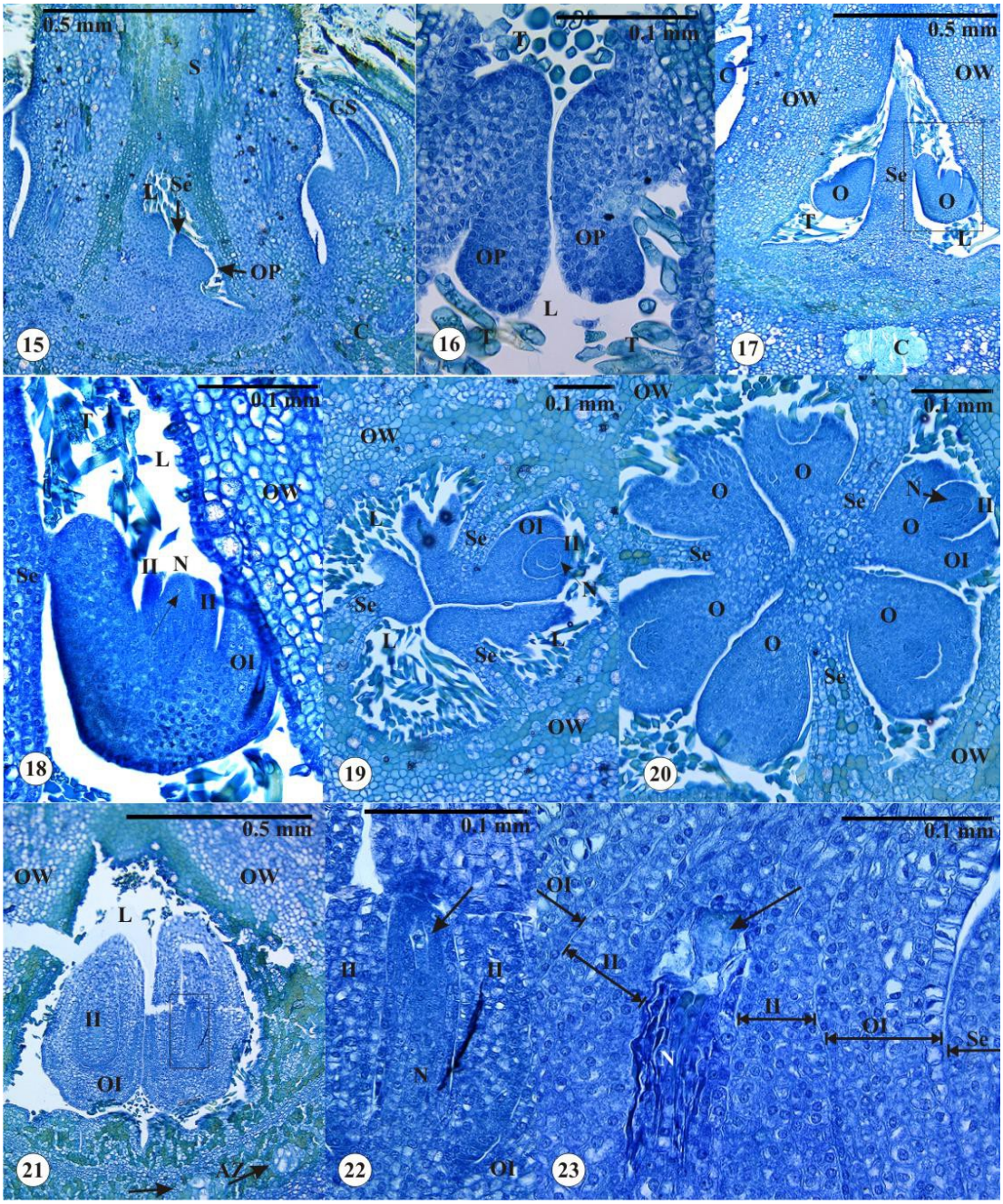
*Stage 0, Winter dormancy: 21 March–25 April (e.g., see Figure 14)*–At the conclusion of winter dormancy, the pistillate flowers formed in the previous year were very similar to those of stage 8 of the current year (see earlier).

*Stages 1–2, Quiescent: 2–16 May*–No change from stage 0.

*Stage 3, Resume development: 23 May (Figures 15–16)*–In the cupule, new scale primordia were seen as well as vascular tissue and sclereid clusters in the cupule base (Figure 15). Styles and perianth lobes were most often broken, with portions that remained sclerified or suberized. The ovary, with its locules and developing ovules, could now be distinguished from the style bases (Figure 15). Some endocarp differentiation in the ovary could be seen as a uniseriate epidermis. Unicellular, lignified and unlignified trichomes filled the locules (Figures 15, 16). Two collateral ovule primordia were present on each septum, oriented perpendicular to the axis of the ovary (Figure 15; at arrow). At later stages, these became pendulous with the apices of the primordia oriented toward the base of the ovary (Figure 16). Six ovule primordia were normally present, although one specimen only had four ovules in two locules. Each funiculus appeared sessile.

*Stage 4, Ovule maturation: 30 May–20 June (Figures 17–20)*–The cupule was no longer tightly appressed to the ovary. Cupule scales were considerably larger and longer than scales in earlier cycles (Figure 17). The ovary was larger in diameter than the style bases (Figure 17), with some tannin accumulation visible in the endocarp and

Figures 15–23. *Quercus acutissima* flowers of previous year. **15.** Stage 3; 23 May. Flower *ls* showing cupule (C), cupule scales (CS), base of styles (S), and ovule primordium (OP) on a septum (Se) in locule (L) filled with trichomes. **16.** Stage 3; 23 May. Flower *ls* showing pendulous ovule primordia (OP) in locule (L) with trichomes (T). **17.** Stage 4; 20 June. Flower *ls* with cupule (C), ovary wall (OW), one septum (Se) with two ovules (O), locule (L) and trichomes (T). The light staining mass at the base of the figure is a cluster of sclereids in the cupule. Box enlarged in next figure. **18.** High magnification of flower *ls* in Figure 17 showing ovary wall (OW), septum (Se), locule (L) with lignified and unlignified trichomes (T). Ovule with outer integument (OI), inner integument (II), and nucellus (N) distinguishable, with possible megaspore mother cell indicated at arrow. Lignified trichomes stain more homogeneously than unlignified trichomes, which have a dark-staining exterior around a light-staining interior. **19.** Stage 4; 20 June. Flower transverse section (*ts*) in apical portion of ovary with ovary wall (OW) and three septa (Se) intruding into the ovary to form three locules (L). The outer integument (OI), inner integument (II), and nucellus (N) in the most apical ovule can be distinguished, with a portion of three of the remaining five ovules visible in this section. **20.** Stage 4; 20 June. Flower *ts* of same specimen as Figure 19 in a more basal section of the ovary with ovary wall (OW), three septa (Se) and all six ovules (O) visible in this section. The outer integument (OI), inner integument (II) and nucellus (N) are indicated in one ovule. **21.** Stage 5; 27 June. Flower *ls* showing ovary wall (OW), locule (L), and two ovules with the outer integument (OI) and inner integument (II) distinguishable in the left ovule. Expanded cells that will form the sclereid clusters in the abscission zone (AZ) are now visible at base of ovary (arrows). Tear in ovary wall and ovule occurred during specimen preparation. Box magnified in next figure. **22.** Stage 5; 27 June. High magnification of ovule in Figure 21 with outer integument (OI), inner integument (II) and nucellus (N). Megaspore mother cell (at arrow) distinguishable in nucellus as a light-staining cell that is expanded relative to adjacent cells. **23.** Stage 6; 11 July. High magnification of flower *ls* including young embryo sac (at arrow) with septum (Se), outer integument (OI), inner integument (II), and the senescing nucellus (N).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septa (Figures 19, 20). The septa were separate and tightly appressed near the apex of the three locules (Figure 19), were fused into a single structure in the middle of in the ovary (Figure 20), and were separate and formed a unilocular space at the base of the ovary. The ovules were attached in a shallow helical pattern within the ovary (Figures 19, 20). The ovules were oriented with the micropyle toward the pistil apex with the raphe on the ventral side of the ovule. In most ovules, the outer and inner integuments and the nucellus were completely differentiated (Figures 17–20), with some cells in the nucellus expanded relative to adjacent cells (Figure 18). The ovules did not fill the locules, with all open spaces not occupied by septa or ovules in the locules filled with trichomes from the locule walls.

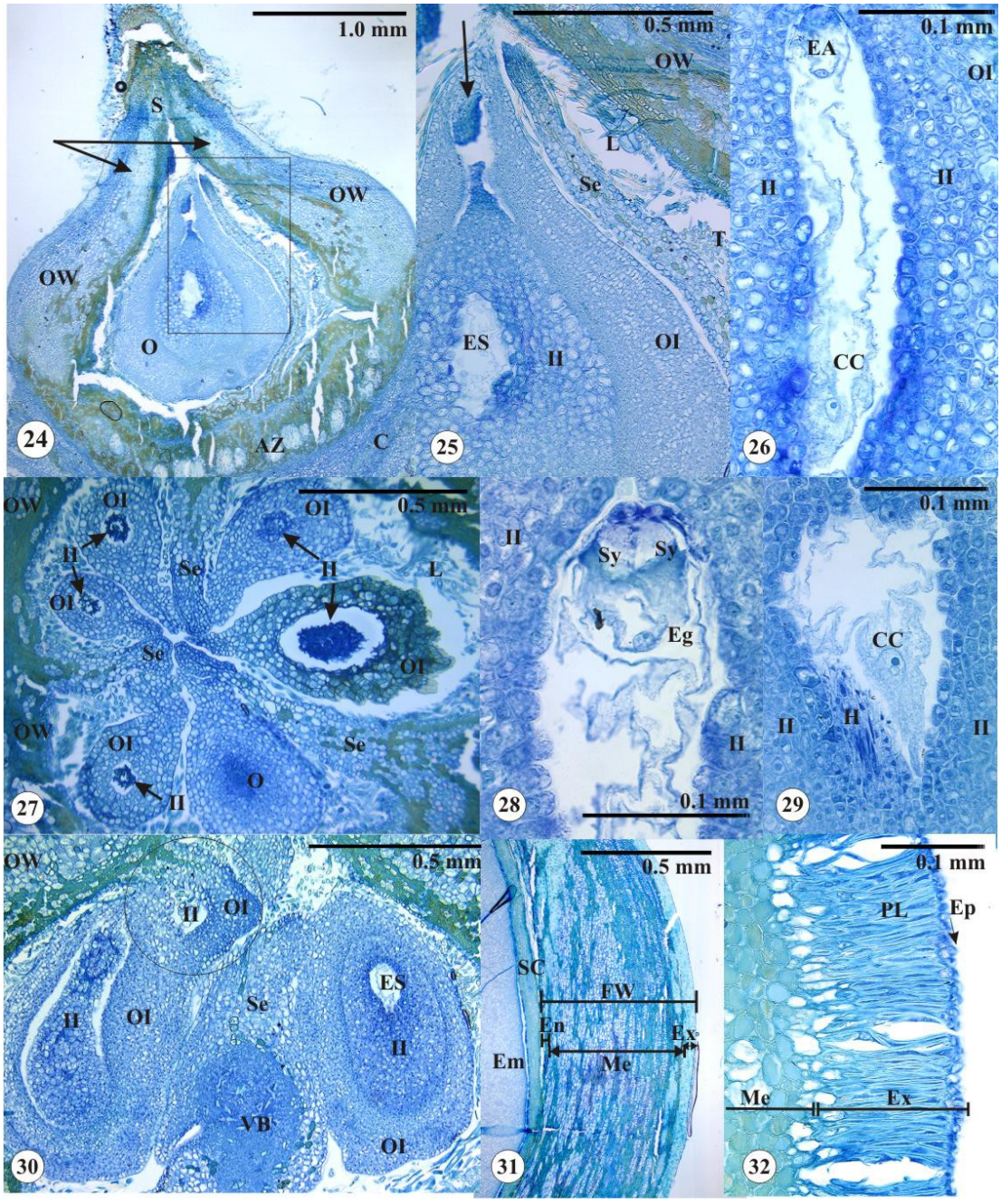
*Stage 5, Megasporogenesis: 27 June (Figures 21, 22)*–Cupule and scales remained unchanged relative to Stage 4. The ovary remained unchanged relative to Stage 4 except for the differentiation of isolated sclereid clusters in the abscission zone at the base of the ovary (Figure 21). The ovules were expanded to fill the locules by the end of this stage, and most of the ovules examined had one megaspore mother cell that was visible as a lighter-staining cell 2–3 cells below the apex of the nucellus (Figure 22). Ovules were crassinucellar with the inner and outer integuments 5–6 cells wide at the level of the megaspore mother cell.

*Stage 6, Megagametogenesis: 4–11 July (Figure 23)*–Cupule and scales remained unchanged relative to Stage 4. Differentiation of the exocarp was visible with an external uniseriate epidermis and 3–4 rows of small and densely staining cells oriented perpendicularly to the epidermis, forming a palisade layer. The numerous cells (50–

100) in the mesocarp were not as densely staining as the exocarp, with some cells highly vacuolated, some with druses, and some vascular tissue present. The 5–10 cells that formed the endocarp were parenchymatous and contained tannins. Lignified unicellular trichomes filled in the spaces in the locules around the ovules. Vascular tissue could be seen in the base of each septum. Embryo sacs of various developmental stages were visible in many of the ovules as lighter-staining tissue with free nuclei (Figure 23). As the embryo sacs developed, each nucellus deteriorated in a basipetal direction, beginning with cells that were immediately basal to the embryo sac, to form a caecum (Figure 23). By the end of this stage, the ovules had expanded to fill the locules.

*Stage 7, Mature embryo sacs: 18–July–15 August (Figures 24–30)*–By the end of this stage, the cupule base was more sclerenchymatous, with more numerous and larger sclereid clusters and more cells showing tannin accumulation. The outermost scale cycles became reflexed away from the ovary due to adaxial surface expansion (25 July; e.g., see Figure 6). The exocarp showed basipetal sclerification of the palisade layer beginning from the base of the styles (25 July) to about 50% of the ovary. The mesocarp was mostly parenchymatous, with some highly vacuolated cells, some scattered druses, and some vascular tissue present. Below the style bases, an apical portion of the mesocarp was sclerified into a cone-shaped structure (1 August; Figure 24, at arrows). The sclerification of the abscission zone was mostly complete, forming a disklike structure at the base of the ovary composed of clusters of sclereids that were interdigitated with parenchymatous cells (Figure 24).

Figures 24–32. *Quercus acutissima* flowers of previous year. **24.** Stage 7; 1 August. Flower *ls* showing the base of the cupule (C), the base of the styles (S), ovary wall (OW), the abscission zone (AZ), a septum (Se), and surviving ovule (O). Sclerified mesocarp apex indicated by arrows. Cupule scales were trimmed in this specimen. Box is enlarged in next figure. **25.** Stage 7; 1 August. High magnification of flower *ls* in Figure 24 ovary wall (OW), locule (L) with lignified trichomes (T), septum (Se), outer integument (OI), inner integument (II), and embryo sac (ES). Fragment of inner integument that remains persistent in micropyle indicated by arrow. The highly vacuolated appearance of the inner integument cells precedes breakdown of the inner integument. **26.** Stage 7; 25 July. Flower *ls* showing outer integument (OI), inner integument (II), with the egg apparatus (EA), and central cell (CC) of one embryo sac in the same section. **27.** Stage 7; 15 August. Flower *ts* median in the ovary showing the ovary wall (OW), three septa (Se), and six ovules with five of the six ovules abortive as shown by their shrunken and dark-staining inner integuments (II; at arrows). The ovule (O) on the bottom of this figure has a viable embryo sac visible in other sections. Tannin infiltration is visible as opaque cells that form mottled areas in the ovary wall, the septa, and the right abortive ovule outer integument. **28.** Stage 7; 18 July. Flower *ls* of egg apparatus in mature embryo sac with two synergids (Sy) and the egg (Eg) enclosed within the inner integument (II). **29.** Flower *ls* of same specimen in Figure 28 but different embryo sac showing the central cell (CC), inner integuments (II) and the hypostase (H). **30.** Stage 7; 1 August. Flower *ls* showing ovary wall (OW), two ovules with outer integument (OI), inner integument (II), and one embryo sac (ES) visible in one ovule, and a septum (Se) with a well-developed vascular bundle (VB). In the left ovule, the base of the integument lobes that form the micropyle is visible at the top of the figure within the circle, and the separation of the inner integument from the outer integument in this ovule is typical of the beginning of ovule abortion. **31.** Stage 8; 29 August. Fruit *ls* showing fruit wall layers with exocarp (Ex), mesocarp (Me), and endocarp (En) delimited, seed coat (SC), and embryo (Em). Most of the fruit wall is still parenchymatous with the exception of the exocarp and the apex of the fruit. Dark lines in this section are folds that occurred during mounting. **32.** Stage 8; 29 August. High magnification of the exocarp (Ex), external epidermis (Ep), and sclereids in the palisade layer (PL) and underlying mesocarp (Me). The tears in the palisade layer occurred during sectioning.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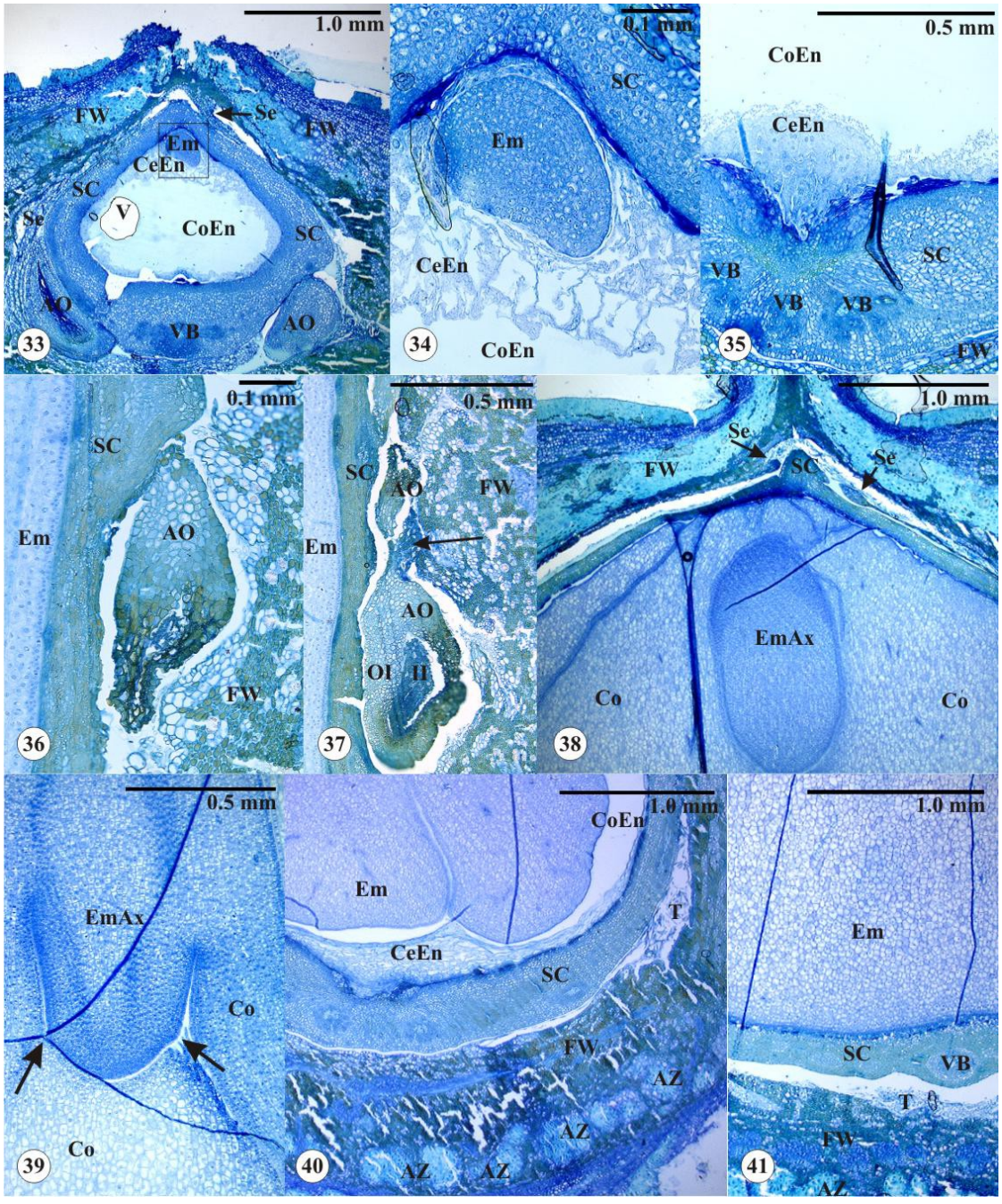
Lignified unicellular trichomes from the endocarp filled spaces in the locules around the ovules (Figures 24, 25, 27, 30), and the parenchymatous septa that were present from the apex to the base of the ovary had significant vascular tissue differentiation in their bases (Figure 30). In each ovule with an embryo sac, the caecum was completely filled by the embryo sac (Figures 24–29) except for a remnant of the nucellus, the basal lateral densely staining hypostase (Figure 29). The egg apparatus with two synergids and an egg cell was present at the apex of the ovule (Figures 26, 28), with the central cell usually midway down the caecum (Figures 26, 29). The antipodals were not located in any of the samples. Most of the flowers had at least one ovule with a viable embryo sac with two syngergids, an egg, and a central cell. Ovules that did not have a viable embryo sac were either underdeveloped or were aborted, and were smaller than the ovules with viable embryo sacs. Toward the end of this stage, endosperm was usually visible in at least one ovule, even if zygotes or embryos could not be located in the embryo sac. When endosperm was present in an ovule, its inner integument was either highly vacuolated or broken down around the embryo sac so that only the outer integument remained, except for a small plug of the inner integument in the micropyle (Figures 24, 25). By the end of this stage, one ovule with endosperm was usually much larger than the others. The rest of the ovules showed signs of abortion, as inner integuments and internal structures coagulated to form amorphous, dark-staining structures contained within outer integuments (Figure 27, at arrows). A zygote was tentatively identified in one sample (1 August, not illustrated).

Figures 33–41. *Quercus acutissima* fruits. **33**. Stage 8; 29 August. Fruit ls showing the fruit wall (FW), two septa (Se), two basal abortive ovules (AO), and seed with seed coat (SC), vascular bundles (VB), cellular endosperm (CeEn), coenocytic endosperm (CoEn), and embryo (Em). A vacuole (V outlined) can be present in the endosperm; this may be a preparation artifact. Note persistent septum (Se) at apex of fruit (at arrow) and another septum on the left side of the fruit between seed coat and fruit wall. Tears in fruit wall, seed coat and embryo occurred during sectioning. Box enlarged in next figure. **34**. Stage 8; 29 August. Fruit ls of same specimen in Figure 33 showing apex of seed with seed coat (SC), coenocytic endosperm (CoEn), and cellular endosperm (CeEn) localized around the embryo (Em). Object to left of embryo is a fold in the embedding media that occurred during mounting. **35**. Stage 8; 29 August. Fruit ls of same specimen as Figure 33 in another section showing the base of the seed with the fruit wall (FW), seed coat (SC) with its vascular bundles (VB), and endosperm. The coenocytic endosperm (CoEn) occupies most of the embryo sac, with the cellular endosperm (CeEn) localized to the base of the seed in this section as a haustorium-like structure. Angular object to the right of this structure is a fold in the embedding media that occurred during mounting. **36**. Stage 8; 29 August. Fruit ls of an abortive ovule (AO) at the base of the fruit between the fruit wall (FW) and seed coat (SC). This abortive ovule is mostly outer integument. Note attachment of abortive ovule to the seed as a result of sharing the same septum. **37**. Stage 8; 29 August. Fruit ls in same specimen as Figure 36 in another section showing two other abortive ovules (AO) between the fruit wall (FW) and seed coat (SC) with embryo (Em). In the lower abortive ovule, the coagulated mass (II) that is the remnant of the inner integument, nucellus, and aborted embryo sac is clearly distinguishable as a separate structure from the outer integument (OI). Note attachment of both ovules to a senesced septum (arrow). **38**. Stage 8; 29 August. Fruit ls with fruit wall (FW), two septa (Se; arrows), seed coat (SC), cotyledons (Co), and embryo axis (EmAx). Compare with specimen in Figure 33 that was collected at the same time. Dark lines in embryo are folds in embedding media that occurred during mounting. **39**. Stage 8; 29 August. Fruit ls of same specimen as Figure 38 in another section showing the cotyledons (Co) around the shoot apex of the embryo axis (EmAx) with leaf primordia *buttresses* visible (arrows). Dark lines are folds in embedding media that occurred during mounting. **40**. Stage 8; 16 September. Fruit ls showing the base of the fruit with the fruit wall (FW), trichomes (T) between fruit wall and seed coat, abscission zone (AZ), and seed coat (SC) with its vascular bundles (VB), and both coenocytic endosperm (CoEn) and cellular endosperm (CeEn) present in the embryo sac. Tears in fruit wall occurred during sectioning, and dark lines in embryo are folds in embedding media that occurred during mounting. **41**. Stage 8; 26 September. Fruit ls of base of fruit showing fruit wall (FW), trichomes (T) between fruit wall and seed coat, with abscission zone (AZ), and seed coat (SC) with one vascular bundle (VB) indicated, and embryo (Em). Dark lines in embryo are folds in embedding media that occurred during mounting.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*Stage 8, Embryo, seed, and fruit: 29 August–26 September (Figures 31–41)–* Throughout this stage, the cupule and scales became increasingly woody. Sclerified cells were present in all wall layers of the fruit except the endocarp (Figure 31). The palisade layer of the exocarp was sclerified from the apex to over 75% of the fruit (29 August; Figures 31, 32, 38) to completely sclerified (26 September), with a small unsclerified zone just apical and lateral to the abscission zone. In the mesocarp, additional clusters of cells became highly vacuolated (Figure 31) and differentiated into sclereids at fruit maturity (26 September), adding to the sclerified apex of the mesocarp. The remaining parenchymatous cells in the mesocarp became compressed between the sclereid clusters. The cells in the endocarp were parenchymatous, filled with tannins, and compressed between the mesocarp and the seed coat (Figures 31, 38, 40, 41). Lignified unicellular trichomes were present between inner fruit epidermis and seed coat. The remnants of all three septa were present from apex to base of the fruit, but often were senesced and hard to detect in section (indicated by arrows in Figures 33, 38). The septal remnant that was attached to the seed was sometimes distinguishable at the base of the nut with tannin-filled cells and vascular tissue. The expanding seed compressed the septal remnants against the endocarp so that only one locule remained. Fruits from the 29 August collection showed a wide range of stages of maturity, with both a heart-shaped embryo (Figures 33–35) and a fully differentiated embryo (Figures 38, 39). The seed coat, which had significant vascular tissue differentiation in the base (Figures 33, 35, 40, 41), was exotestal, except for a small plug of inner integument tissue in the micropyle (e.g., Figure 25, at arrow). The

free-nuclear endosperm, or coenocytic endosperm, with nuclei at its periphery, completely filled the embryo sac (Figure 33–35, 40), although in one specimen a single vacuole (29 August; Figure 33) that may have been a preparation artifact appeared in section. Cellular endosperm was restricted to the area around the maturing embryo and the periphery of the embryo sac (29 August; Figures 33–35) until almost all the endosperm was totally assimilated (26 September; Figure 41). In the early stages of embryo maturation, the seed was shaped by both the cellular and coenocytic endosperm. After all the endosperm was assimilated, the embryo enlarged to fill the locules. A concentration of cellular endosperm was observed at the chalazal end of the embryo sac. In early embryogenesis, this structure was isolated at the base (Figure 35), but later became continuous with the rest of the cellular endosperm as the seed matured (Figure 40). The base of a septal remnant connected the seed to the nut. This septal remnant was attached to the seed coat basal to the chalazal concentration of the endosperm and was attached to the nut at the base, and was often adjacent to the aborted ovules (seed scar in Figure 2). Leaf or scale-leaf buttresses were present in a embryo shoot apex (29 August; Figure 39). Aborted ovules were basal to the seed throughout maturation of the fruit (Figures 33, 36, 37). The outer integuments with tannin-filled cells were the only recognizable structures in the aborted ovules, as the inner integuments and contents were detached from the outer integuments and shrunken into densely staining masses (Figures 36, 37).

***Distribution of morphological characters on molecular tree***

The results from mapping 20 characters of fruiting patterns, cupules, flowers,

fruits, and seeds of this study and the Mogensen (1965) study onto a tree derived from molecular data are presented in Figure 42. Tree length for the morphological characters alone was 31 steps, with a consistency index (CI) of 0.74, and a retention index (RI) of 1.0. Total ambiguities (characters coded as either missing values or as full polymorphisms) in the matrix was 21%, and 12 characters were uninformative (either pleisiomorphic or autapomorphic) as mapped on this tree. Of the remaining characters that were informative, two characters appear as parallelisms between *Q. acutissima* (section *Cerris*) and *Q. alba* (section *Quercus* s. s.): character 13 (locules are filled with ovules for much of development; CI = 0.50) and character 16 (basal abortive ovules; CI = 0.66).

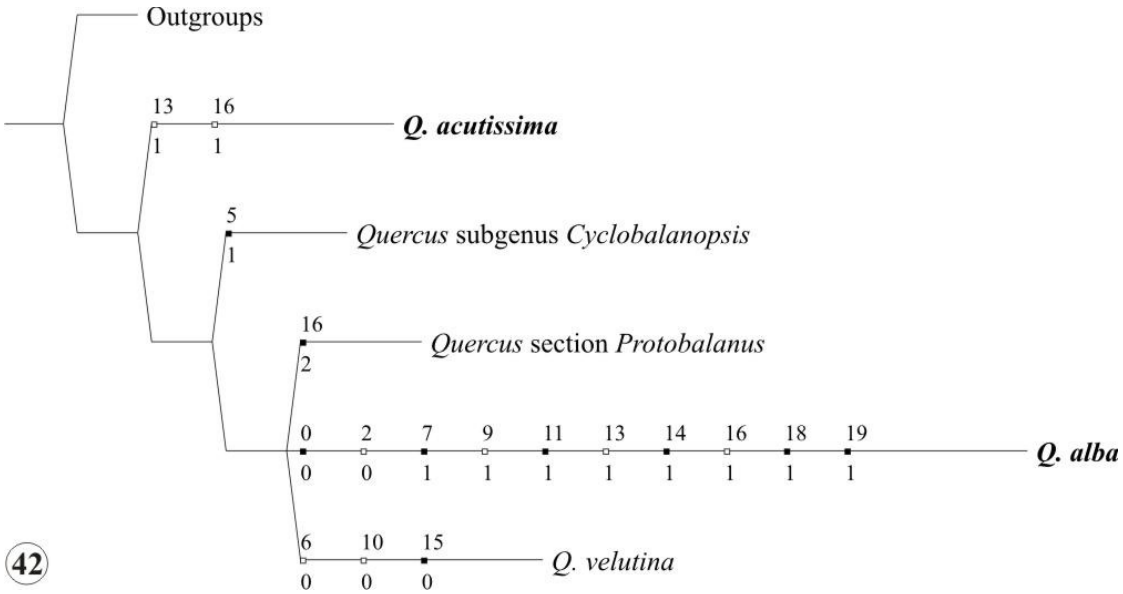


Figure 42. New and historical fruiting, cupule, flower, fruit, and seed characters of the current study and Mogensen (1965) mapped onto a tree based on the latest published molecular tree Manos *et al.* (2001) using WinClada (Nixon, 1999–2002). Example species were substituted for their respective sections of subgenus *Quercus*: *Q. acutissima* for section *Cerris*; *Q. alba* for section *Quercus* s.s.; *Q. velutina* for section *Lobatae*. Length = 31 steps; consistency index = 0.74; retention index = 1.0.

## DISCUSSION

For the purposes of discussion, *Q. acutissima* will function as an example species for section *Cerris*, with previous works on the maturation of other species in section *Cerris* supplementing the anatomy and development seen in the current study. *Quercus alba* of section *Quercus s. s.* and *Q. velutina* of section *Lobatae* will also function as example species for their respective sections because there are numerous anatomical and comparative studies that exist for these two species, including the Mogensen (1965) work that provided a framework for the current study.

### *Shared features among sections Cerris, Quercus s.s., and Lobatae*

For all three species, *Q. acutissima*, *Q. alba*, and *Q. velutina*, the meristems of the pistillate inflorescence axes axes form at the end of the growing season and remain quiescent in terminal buds until the following spring. These reproductive meristems are usually indistinguishable from vegetative meristems until a few weeks before the buds break. The pistillate inflorescences, ranging in length from almost sessile up to several centimeters long, are formed on the main branch in the axils of leaves distally to the staminate inflorescences. The pistillate inflorescence axis bears 1–several flowers (as observed in a wider sample of species) that are mostly sessile and in decussate subopposite pairs (Trelease, 1924; Langdon, 1939; Muller, 1942b; Turkel, 1950; Corti, 1954, 1955, 1959; Bianco, 1961; Sharp and Sprague, 1967; Soepadmo, 1968; Fey, 1981; Kaul, 1985; Boavida et al., 1999).

At anthesis of the pistillate flowers, the ovary is an insignificant part of the pistil,

and each flower is essentially three styles with a perianth (Figure 7), with the cupule present as a toruslike structure at the base of the pistil before the first few cupule scales are formed (Turkel et al., 1955; Scaramuzzi, 1960; Bianco, 1961; Fey, 1981; Cecich, 1997; Boavida et al., 1999; Boavida et al., 2001). Stigmatic surfaces are restricted in area to the style apices and along adaxial grooves on the styles and consist of the nonpapillate, "dry" type with a pellicle (Heslop-Harrison, 1971). The styles senesce within a month after pollination. Although pollen tube penetration into the styles (Turkel et al., 1955; Stairs, 1964; Cecich, 1997) was not directly observed in the current study, the adherence of *Quercus*-type pollen to the stigmatic surfaces, attached empty pollen grains that have presumably discharged their microgametophytes, and a specimen with pollen tube growth within the style (Figure 11) inferred that pollination was successful.

Species with annual fruit maturation (e.g., *Q. alba*) have a delay of approximately one month between pollination and syngamy, while species with biennial fruit maturation (e.g., *Q. acutissima* and *Q. velutina*) are quiescent ~ 13 month between pollination and syngamy. Despite the difference in quiescence time, ovule initiation and maturation in the flowers are similar in all three species. Typically, there are three locules in the pistillate flowers, with each locule containing two collateral ovules that at maturity are oriented with the micropyle toward the apex of the flower (epitropous) with the raphe on the ventral side of the ovule (Figures 17, 18, 21). This final ovule orientation is best described as semianatropous (Corti, 1954). Soon after the nucellus is distinguishable within the developing ovule, one or more megaspore mother cells,

or megasporocytes, can be seen within the apex of the nucellus (Benson, 1893; Langdon, 1939; Turkel, 1950; Rebuck, 1952; Hjelmqvist, 1953; Corti, 1954, 1955; Turkel et al., 1955; Corti, 1959; Scaramuzzi, 1960; Bianco, 1961; Stairs, 1964; Mogensen, 1965; Fey, 1981; Boavida et al., 1999). Only one megaspore mother cell was found in each ovule in *Q. acutissima* (Figures 18, 21, 22). Although not directly observed in the current study, megagametogenesis is of the monosporic, Polygonum-type with a 7-celled, 8-nucleate embryo sac (Hjelmqvist, 1953; Corti, 1959; Bianco, 1961; Stairs, 1964), although several authors have mistakenly described the process as tetrasporic, Adoxa-type (Conrad, 1900; Bagda, 1952; Turkel et al., 1955). The ephemeral antipodals were not detected in the current study, although some authors illustrated the position of these three nuclei as apical to the senescing nucellus (Benson, 1893; Langdon, 1939; Turkel, 1950; Rebuck, 1952; Hjelmqvist, 1953; Corti, 1954, 1955; Turkel et al., 1955; Corti, 1959; Scaramuzzi, 1960; Bianco, 1961; Stairs, 1964; Mogensen, 1965; Fey, 1981; Boavida et al., 1999). A few studies that examined *Q. alba* (section *Quercus s. s.*), *Q. rubra* from section *Lobatae*, and other species from section *Cerris* reported tracheids or procambium-like tissue in the hypostase or surrounding tissue (Benson, 1893; Langdon, 1939; Corti, 1954, 1955; Turkel et al., 1955), but this was not observed in the current study.

During the maturation of the ovules and embryo sacs, the pollen tubes (Figures 10, 11), are quiescent in a basal area of the styles that remains parenchymatous. After megasporogenesis and during megagametogenesis, the pollen tubes resume growth and enter the ovules through the micropyle (porogamy; Benson, 1893; Kerner Von

Marilaun, 1895; Conrad, 1900; Klebelsberg, 1910; Hjelmqvist, 1953; Corti, 1954; Mogensen, 1972; Cecich, 1997; Boavida et al., 1999). Pollen tube growth into the ovules as well as the resulting syngamy was not directly observed in the current study, but there was no evidence of ovular disruption that would indicate that the pollen tubes entered the embryo sac other than through the micropyle. All of the ovules in the ovary usually have embryo sacs of various developmental stages at the time of syngamy, but only one ovule matures into the seed. The abortive ovules can be distinguished in the early stages of abortion from the surviving ovule by the loss of cohesion of the embryo sac and the coagulation of the inner integument and contents into a densely staining structure as seen in *Q. acutissima* (Figures 27, 30, 36, 37). The abortive ovules remain attached to their respective septal remnants (Conrad, 1900; Langdon, 1939; Corti, 1954, 1955; Turkel et al., 1955; Corti, 1959; Scaramuzzi, 1960; Bianco, 1961; Stairs, 1964; Mogensen, 1965; Brown, 1971; Brown and Mogensen, 1972; Mogensen, 1972, 1973, 1975a, b; Fey, 1981; Boavida et al., 1999).

For the remainder of the discussion, any ovule with a multicellular embryo will be referred to as a seed, with its respective ovary now a fruit. Multicellular embryos were common in this study, and assumed to be the result of syngamy, and not apomixis or parthenogenesis (Johri, 1984). In the embryo sac, the free-nuclear or coenocytic endosperm proliferates before the first division of the zygote and can have vacuoles of various sizes (Hjelmqvist, 1957), although only one specimen showed possible evidence of a vacuole in *Q. acutissima* (see Figure 33, outline). In one collection (29 August) of *Q. acutissima*, fruits with almost mature seeds (e.g., Figure

38) were collected at the same time as a fruit that had a heart-shaped embryo (Figures 33, 34), supporting the observation made by other authors that the level of maturity in fruits from an individual tree can vary widely at any given time (Conrad, 1900; Langdon, 1939; Corti, 1954, 1955; Turkel et al., 1955; Corti, 1959; Scaramuzzi, 1960; Bianco, 1961; Stairs, 1964; Mogensen, 1965; Fey, 1981).

In the current study, a concentration of cellular endosperm was observed at the chalazal end of the embryo sac in the more mature fruits and may be haustorial (Figures 35, 40). Adjacent to this chalazal concentration of cellular endosperm is the hypostase. Several authors have discussed the possibility that the hypostase functions as a haustorium for the growing embryo (Benson, 1893; Langdon, 1939; Hjelmqvist, 1953; Corti, 1954; Mogensen, 1973). Whether the chalazal cellular endosperm or the hypostase function separately or together as a haustorium is not known at the present time.

***Differences between section Cerris and sections Quercus s. s. and Lobatae***

A comparison of the historically important features of section *Cerris* to both the sections *Quercus s. s.* and *Lobatae* is presented here with features unique to section *Cerris* described first, followed by those features that group section *Cerris* with either section *Quercus s. s.* or section *Lobatae* (Table 3).

*Autapomorphic features for section Cerris*–In *Q. acutissima* and related species within section *Cerris*, the scales emerge from the cupule meristem in an open imbricate series as three-dimensional, awl-shaped, parenchymatous structures that are separate and distinct from adjacent scales. At one point in maturation (around late July

in the current study), the outermost cycles of scales become reflexed away from the cupule as a result of increased growth on the adaxial portion of the scales, forming a three-dimensional burlike structure (see Figure 6 for a slightly reflexed stage of an immature cupule). In the sections *Quercus s. s.* and *Lobatae*, the scales emerge in a tight imbricate series in which the bases of the scales are tightly appressed to adjacent scales and appear to be fused together. Each scale is flattened, with the distal portion sclerified into a papery, two-dimensional structure, and the scales usually remain appressed to the cupule throughout maturation (Oersted, 1871; Camus, 1934–1954; Muller, 1942b; Soepadmo, 1968; Maleev, 1970; Soepadmo, 1972; Kaul, 1985; Nixon, 1997).

It is possible to compare the time delay between pollination and syngamy in both annual- and biennial-fruiting species in subgenus *Quercus* if the time of quiescence of the biennial species (1 yr) is subtracted from the time between pollination and syngamy. The approximate one month time delay that remains is comparable across both annual- and biennial-fruiting taxa (Conrad, 1900; Corti, 1954; Turkel et al., 1955; Stairs, 1964; Mogensen, 1965; Sharp and Sprague, 1967; Cecich, 1997). Syngamy usually occurs one month after pollination in both the annual-fruiting (e.g., *Q. alba*) and biennial-fruiting species (e.g., *Q. velutina*), but in the current study, no zygotes or embryos could be located in the fruits of *Q. acutissima* for at least two months after pollination occurred. A 3–5 month delay in syngamy for section *Cerris* has been reported previously for an annual-fruiting form of *Q. aegilops* (Scaramuzzi, 1960). It is possible that the delay in syngamy observed in the current study may be

environmental and is not a consistent phylogenetic characteristic. Samples of other *Quercus* species that were collected for anatomical studies at the same time and from the same study area as *Q. acutissima* may provide some answers to this particular question.

One feature that was noticeable early in the development of the pistillate flower in *Q. acutissima* was the lignification of the trichomes in the locules. A large number of trichomes were lignified before megasporogenesis and fractured during sectioning to form artifact spaces in the ovary (e.g., Figures 17, 18, 21). Because lignification patterns of endocarp trichomes have not been followed in previous studies, this feature needs to be examined more closely in the future. In a preliminary examination of the original slides provided by Mogensen (1965), endocarp trichomes do not appear to lignify until after syngamy, with trichomes in *Q. alba* lignifying prior to *Q. velutina*.

In the current study, one of the most mature fruits collected from *Q. acutissima* contained a seed that had an embryo shoot apex with leaf or scale primordia buttresses (Figure 39). In section *Quercus s. s.*, the shoot apex forms several leaf or scale primordia before the acorn falls from the tree, and in studied species of section *Lobatae*, the shoot apex remains naked (Mogensen, 1965; Sutton, 1969; Sutton and Mogensen, 1970). A larger sampling of mature acorns and the examination of seedling germination morphology in *Q. acutissima* and other species from section *Cerris* may reveal whether these buttresses are a consistent state that is intermediate between section *Lobatae* and section *Quercus s. s.*, or if the embryo shoot apex in section *Cerris* later forms leaf or scale primordia before falling the tree.

***Potential synapomorphies between sections Cerris/Lobatae and sections Cerris/Quercus s. s.***

Style morphology of the pistillate flower in subgenus *Quercus* has historically been an important character in distinguishing among the major groups (Oersted, 1871; Trelease, 1924; Camus, 1934–1954; Reece, 1938; Muller, 1942b; Tillson and Muller, 1942; Soepadmo, 1968; Maleev, 1970; Tucker, 1980; Nixon, 1984; Kaul, 1985; Nixon, 1993). The styles in *Q. acutissima*, with slender and thin apices that remain straight or may become slightly curved, have closest affinities to the styles seen in section Lobatae (e.g., *Q. velutina*). The styles do not lengthen appreciably in *Q. alba* with the style apices spreading out laterally to become rounded and blunt.

The pistillate flowers in both *Q. acutissima* and *Q. velutina* have perianth lobes that are longer than they are wide and can extend up the style column and be mistaken for styles in longitudinal section (e.g., Figures 10, 12). The perianth in *Q. alba* is considerably shorter, with tepals as long as they are wide.

Although the perianth tepals of *Q. acutissima* and *Q. alba* are not morphologically similar in height/width proportions, they are morphologically similar in that the perianth lobes do not invaginate at the base to form a skirt or flange. This flange can be seen in biennial-fruiting taxa in the section *Lobatae*, such as *Q. velutina*, which seals the parenchymatous flower during quiescence by interlocking the perianth with the outermost cupule scales (Oersted, 1871; Trelease, 1924; Camus, 1934-1954; Muller, 1942b; Soepadmo, 1968; Tucker, 1980; Nixon, 1984; Kaul, 1985; Nixon, 1993).

Distinguishing between the different layers in the fruit wall can be arbitrary, particularly in the early stages of ovary development, but for discussion purposes, the mature fruit layers described by Harz (1885) and Soepadmo (1968) for the genus *Quercus* were used. The exocarp and mesocarp of *Q. acutissima* can be compared to the exocarp and mesocarp of *Q. alba* and *Q. velutina* as separate layers, but when the two layers combine at fruit maturity to form the "shell" of the nut, the structure seen in *Q. acutissima* is best interpreted as a different character state when compared to the other two species. The exocarp palisade layer in *Q. acutissima* and *Q. alba* is thin with short sclereids and is a minor component of the fruit wall when compared to the robust and dense palisade layer of *Q. velutina.* The mesocarp in *Q. acutissima* and *Q. velutina* has some scattered groups of sclereids at maturity, but is relatively parenchymatous at maturity compared to the mesocarp in *Q. alba*. The total composition of the "shell" of the nut at maturity results in three different fruit wall compositions across the three species. At maturity, the exocarp in *Q. velutina*, the mesocarp in *Q. alba* (Mogensen, 1965), or the combination of both layers in *Q. acutissima* makes up the mature "shell". Because this is one of the few studies to compare the patterns of fruit wall differentiation across several sections, this observation needs further verification with more sampling.

The absence or presence of a pubescent or tomentose endocarp, historically cited as a useful character in differentiation among the sections of subgenus *Quercus*, can be problematic unless applied consistently. For example, when the fruit of section *Quercus s. s*. is opened, the seed coat often detaches from the seed, adhering to the

fruit wall, and the inside of the seed coat is then mistaken for the endocarp (Nixon, 1984, 1997). The true endocarp in section *Quercus s*. *s*. is mostly glabrous at maturity, although sometimes patches of short and sparse pubescence can be found. The trichome type, size, and form seen in *Q. acutissima* are similar to trichomes in *Q. alba*, but in *Q. acutissima* the endocarp remains tomentose at maturity. In contrast, the endocarp in *Q. velutina* is very tomentose and is densely covered by trichomes of considerable number and length (Oersted, 1871; Trelease, 1924; Camus, 1934–1954; Muller, 1942b; Soepadmo, 1968; Tucker, 1980; Nixon, 1984; Kaul, 1985; Nixon, 1993).

One developmental pattern found in the present study that may be taxonomically useful among the sections of subgenus *Quercus* is the locule volume occupied by the ovules as they develop. In *Q. acutissima*, the ovules occupy most of the volume in the locules (Figures 20, 24, 30) except near the time of megasporogenesis when the ovary expands faster than the ovules. The ovules in *Q. alba* fill the locules in a similar manner, although it is unknown as to whether the ovary in *Q. alba* also expands at a greater rate during megasporogenesis. In contrast, the ovules in *Q. velutina* occupy only half of the volume of the locules until late in maturity when the seed finally fills the fruit (Conrad, 1900; Corti, 1954; Turkel et al., 1955; Mogensen, 1965).

As previously mentioned in the introduction, the abortive ovules adhere to the base of the seed or fruit wall in the mature fruit in both sections *Cerris* and *Quercus s*. *s*. All mature eggs are fertilized at the time the pollen tubes descend into the locules, and competition among the ovules with fertilized eggs determines which

of these ovules will go on to form the seed (Stairs, 1964; Mogensen, 1975b). In the current study, it was noted in *Q. acutissima* fruits that the base of each septum became heavily vascularized during megagametogenesis. We believe that it is this vascular differentiation in the base of the septa before syngamy that anchors the aborted ovules and the seed to the base of the ovary. It is unknown at this time whether the basal abortive ovules that occur in all of section *Quercus s. s.* and a few species in section *Lobatae* are the result of similar developmental processes.

The septa, which originally delimited the locules in the ovary, begin to senesce as the embryo expands to form a unilocular space in the nut. These septal remnants can be displaced between the seed coat and the endocarp as the embryo expands, or they can remain intruded into the interior of the fruit. The septal remnants are almost indistinguishable at the base of the fruit with the basal aborted ovules in *Q. alba*, but they are a significant feature in *Q. velutina* in which the septal remnants sclerify into ridges extending from the apex to the base of the fruit. In *Q. acutissima*, the septal remnants can be found from the apex to the base of the fruit as in *Q. velutina*, but they are thin papery structures (Figures 24, 25, 33, 38).

The surviving ovule that matures into the seed is attached to the fruit wall by a compound structure that is composed of the ovule placenta, the funiculus, and part of a septal remnant [the "umbilical complex" of Borgardt and Pigg (1999); the "columella" of Nixon (1984, 1993, 1997)]. In *Q. alba*, the aborted ovules, the septal remnants, and the compound structure that attaches the seed to the nut are all basal in the fruit. In *Q. velutina*, the aborted ovules are apical, the septal remnants are present from the apex

to the base of the fruit, and at this time the location of the structure that attaches the seed to the nut is unknown (Mogensen, 1965; Borgardt, 1996, 1997). In the current study, a new combination of these features is found in *Q. acutissima*, with basal aborted ovules and a basal compound structure connecting the seed to the fruit, but the septal remnants remain present from the apex to the base of the fruit.

As mentioned previously, in *Q. alba* the seed coat can adhere to the endocarp and be left behind when the embryo is removed. In *Q. acutissima* and *Q. velutina*, the seed coat is adherent to embryo (Oersted, 1871; Trelease, 1924; Camus, 1934–1954; Muller, 1942b; Soepadmo, 1968; Tucker, 1980; Nixon, 1984; Kaul, 1985; Nixon, 1993).

***Distribution of morphological characters on molecular tree***

Although the new and existing features presented here are not sufficient for a complete, independent morphological analysis, characters described in this study and the Mogensen (1965) study are mapped on the latest tree derived from molecular data (Manos et al., 2001) to evaluate potential synapomorphies or inconsistencies based on that tree topology (Figure 42). With a total CI of 0.74, the morphological characters as a whole show less conflict mapped on this tree than the molecular characters (CI = 0.34) used to derive the tree. Some characters are coded as missing for taxa that have not yet been examined using the protocol in Mogensen (1965), and some of the outgroup characters are coded as full polymorphisms. As a result, the total ambiguity in the matrix is 21%, with 12 characters uninformative. Of the remaining eight characters that do provide grouping information, two characters appear to be

parallelisms on this tree. These are: character 13 (locules are filled with ovules for much of development; CI = 0.50) and character 16 (basal abortive ovules; CI = 0.66).

These two characters neatly summarize the conflict that exists between the relationships that are derived from morphological data versus relationships derived from molecular data for subgenus *Quercus*. When these characters are mapped on trees obtained from molecular data, they appear as parallelisms, but in the absence of molecular data, they contribute to uniting sections *Cerris* and *Quercus s*. *s*. into a larger "white" oak clade.

## CONCLUSIONS

Examination of the historically important taxonomic features, as well as new features that are described for the first time in this study, reveals a mosaic of morphological and anatomical characteristics among the three sections of *Quercus* subgenus *Quercus* (Table 2).

Some of the features, such as basal abortive ovules and exocarp anatomy, unite the section *Cerris* with section *Quercus s*. *s.* in agreement with previously published morphological analyses (Nixon, 1984, 1993). Other features, such as stigma morphology and mesocarp anatomy, are shared between section *Cerris* and section *Lobatae*, and contradict a "white" oak clade *s*. *l*., although not directly supporting the collateral position of section *Cerris* as a sister taxon to the remainder of *Quercus* subgenus *Quercus* (excluding *Cyclobalanopsis*) as found in the latest molecular analyses (Manos et al., 1993; Manos et al., 2001). There are also features, such as the

embryo shoot apex morphology and pericarp anatomy, that appear to be autapomorphic for section *Cerris* and do not provide any grouping information among the three sections.

Further comparative flower and fruit anatomical and developmental work on other groups within the genus *Quercus* (subgenus *Quercus* section *Protobalanus* and subgenus *Cyclobalanopsis*) as well as related genera in the family Fagaceae is in progress and should provide more resolution on the significance of features that have been used taxonomically as well as the phylogenetic relationships among these taxa.

*Table 2:* Morphological and anatomical differences between three sections of subgenus *Quercus* based on features of three species used as examples of their respective sections (Mogensen, 1965).

| | ***Q. acutissima*** **Section *Cerris*** | ***Q. alba*** **Section *Quercus*** s.s. | ***Q. velutina*** **Section *Lobatae*** |
|---|---|---|---|
| **Syngamy** | Delayed by 14 mo | Delayed by 1 mo | Delayed by 13 mo |
| **Cupule scales** | Thickened in apex and base of scale | Apex of scale papery, base of scale thickened | Both apex and base thin, papery |
| | Reflexed | Appressed to cupule | Appressed to cupule |
| **Styles** | Long and thin | Short and broad | Long and thin |
| **Perianth** | Long tepals | Short tepals | Long tepals |
| | Does not form a flange or interlock with cupule during quiescence | Does not form a flange | Forms a flange basally that interlocks with cupule during quiescence |
| **Exocarp** | Palisade layer thin | Palisade layer thin | Palisade layer thick |
| **Mesocarp** | Few, scattered clusters of sclereids | Concentrated and dense clusters of sclereids | Few, scattered clusters of sclereids |
| **Endocarp** | Pubescent at maturity, not conspicuously pubescent | Glabrous at maturity | Pubescent at maturity, very conspicuously pubescent |
| **Pericarp** | Both exocarp and mesocarp combine to form mature fruit wall | Mesocarp primary component of mature fruit wall | Exocarp primary component of mature fruit wall |

| | ***Q. acutissima*** **Section *Cerris*** | ***Q. alba*** **Section *Quercus*** s.s. | ***Q. velutina*** **Section *Lobatae*** |
|---|---|---|---|
| **Locules** | Filled with ovules/seed during most of development | Filled with ovules/seed during most of development | Not filled with ovules/seed until late in maturity |
| **Septal remnants** | Present from base to apex of seed at maturity | Present only at base of seed at maturity | Present from base to apex of seed at maturity |
| | Do not invaginate seed | Do not invaginate seed | Invaginate seed |
| **Abortive ovules** | Basal | Basal | Apical |
| **Connection of seed to fruit** | Base of fruit | Base of fruit | Unknown |
| **Seed coat** | Adherent to embryo at maturity | Adherent to fruit wall at maturity | Adherent to embryo at maturity |
| **Embryo** | Buttresses on shoot apex of embryo | Leaf primordia on shoot apex of embryo | Naked shoot apex |

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# CHAPTER 4

# AN ANATOMICAL AND DEVELOPMENTAL STUDY OF PISTILLATE FLOWERS AND FRUITS FROM *QUERCUS PALMERI*, AN INTERMEDIATE OAK FROM SECTION *PROTOBALANUS* (SUBGENUS *QUERCUS*, FAGACEAE)

## INTRODUCTION

In contrast to closely related taxa in Fagales *s*. *l*., which only develop apical abortive ovule(s), abortive ovule position is variable among historically recognized taxonomic groups within *Quercus* with the occurrence of apical abortive ovules, basal abortive ovules, and lateral abortive ovules (Table 1). A. de Candolle (1862) was the first to document this variability of abortive ovule position in *Quercus*, with subsequent classifications (Oersted, 1871; Engelmann, 1880; Trelease, 1924; Camus, 1934; Muller, 1942a; b; Nixon, 1993, 1997) recognizing this feature and its utility in differentiating among oak groups, particularly in *Quercus* subgenus *Quercus*.

Complete anatomical and developmental studies of flower/fruit maturation have previously been published for taxa in three of the four sections within *Quercus* subgenus *Quercus*: *Q. alba* L. (section *Quercus sensu stricto*) and *Q. velutina* Lamb. (section *Lobatae*) were studied by Mogensen in 1965, with *Q. acutissima* Carruth. (section *Cerris*) by Borgardt and Nixon in 2003. Features described from these studies (and the current study) are summarized in Table 2.

*Table 1*. Abortive ovule (AO) position in the genus *Quercus*: definitions of these states in the text. Old synonyms from previous classifications follow currently accepted names and are enclosed in parentheses (Loudon, 1830; Oersted, 1871; Trelease, 1924; Camus, 1934; Muller, 1942a; b; Mogensen, 1965; Tucker, 1980; Kaul, 1985; Nixon, 1997).

| | Apical AO | Lateral AO | Basal AO |
|---|---|---|---|
| Subgenus *Cyclobalanopsis* (Oersted) Schneider | YES | ? | ? |
| Subgenus *Quercus* Hickel et Camus (*Euquercus)* | YES | YES | YES |
| Section *Lobatae* Loudon (*Erythrobalanus, Rubrae*) | YES | Few | Few |
| Section *Protobalanus* (Trelease) Schwarz | | YES | |
| Section *Cerris* Loudon | | | YES |
| Section *Quercus s. s.* Hickel et Camus *(Lepidobalanus, Leucobalanus, Macrobalanus, Mesobalanus)* | | | YES |

*Table 2:* Morphological and anatomical differences between all four sections of subgenus *Quercus* based on features of four species used as examples of their respective sections. Additional relevant sectional variability follows within parentheses (Mogensen, 1965; Borgardt and Nixon, 2003).

| | ***Q. palmeri*** | ***Q. acutissima*** | ***Q. velutina*** | ***Q. alba*** |
|---|---|---|---|---|
| | **Section *Protobalanus*** | **Section *Cerris*** | **Section *Lobatae*** | **Section *Quercus s. s.*** |
| **Fruit Maturation** | Biennial only | Biennial<br>(Biennial, or annual, or both) | Biennial<br>(Biennial or annual) | Annual only |
| **Syngamy** | Delayed by 13 mo | Delayed by 14 mo | Delayed by 13 mo | Delayed by 1 mo |
| **Primary floral bract** | Persistent | Ephemeral | ? | ? |
| **Cupule** | Hemispherical/flared<br>Scales not prominent, mostly ground tissue<br>(Composed mostly of scales) | Hemispherical<br>Composed mostly of scales<br>(Combination of ground tissue and scales) | Hemispherical<br>Composed mostly of scales<br>(Combination of ground tissue and scales) | Hemispherical<br>Composed mostly of scales<br>(Combination of ground tissue and scales) |
| **Cupule scales** | Apex of scale papery, base of scale thickened<br>Appressed to cupule | Thickened in apex and base of scale<br>Reflexed | Both apex and base thin, papery<br>Appressed to cupule | Apex of scale papery, base of scale thickened<br>Appressed to cupule |
| **Styles** | Short and broad | Long and thin | Long and thin | Short and broad |

| | ***Q. palmeri*** | ***Q. acutissima*** | ***Q. velutina*** | ***Q. alba*** |
|---|---|---|---|---|
| | **Section *Protobalanus*** | **Section *Cerris*** | **Section *Lobatae*** | **Section *Quercus s. s.*** |
| **Stigmatic surface** | Capitate, restricted to style apex | Broad, extending down style adaxial surface | Broad, extending down style adaxial surface | Broad, extending down style adaxial surface |
| **Perianth** | Short tepals | Long tepals | Long tepals | Short tepals |
| | Forms a flange that interlocks with cupule during quiescence | Does not form a flange | Forms a flange that interlocks with cupule during quiescence | Does not form a flange |
| **Exocarp** | Palisade layer thin | Palisade layer thin | Palisade layer thick | Palisade layer thin |
| **Mesocarp** | "Secondary" palisade layer with few scattered clusters of sclereids | Few, scattered clusters of sclereids | Few, scattered clusters of sclereids | Concentrated and dense clusters of sclereids |
| **Endocarp** | Pubescent at maturity, can be conspicuously pubescent | Pubescent at maturity, not conspicuously pubescent | Pubescent at maturity, very conspicuously pubescent | Mostly glabrous at maturity, some scattered or patchy pubescence |
| **Pericarp** | Both exocarp and mesocarp combine to form mature fruit wall | Both exocarp and mesocarp combine to form mature fruit wall | Exocarp primary component of mature fruit wall | Mesocarp primary component of mature fruit wall |
| **Locules** | Not filled with ovules/seed until late in maturity | Filled with ovules/seed during most of development | Not filled with ovules/seed until late in maturity | Filled with ovules/seed during most of development |

| | *Q. palmeri* | *Q. acutissima* | *Q. velutina* | *Q. alba* |
|---|---|---|---|---|
| | **Section *Protobalanus*** | **Section *Cerris*** | **Section *Lobatae*** | **Section *Quercus s. s.*** |
| **Septal remnants** | Present from base to apex of seed at maturity | Present from base to apex of seed at maturity | Present from base to apex of seed at maturity | Present only at base of seed at maturity |
| | Do not invaginate seed | Do not invaginate seed | Invaginates seed | Do not invaginate seed |
| **Abortive ovules** | Lateral to basal, never apical | Basal | Apical | Basal |
| | | | (other spp. basal) | |
| **Connection of seed to fruit** | Base of fruit | Base of fruit | Unknown, presumed apical | Base of fruit |
| **Seed coat** | Adherent to embryo at maturity | Adherent to embryo at maturity | Adherent to embryo at maturity | Adherent to fruit wall at maturity |
| **Embryo** | Naked shoot apex | Buttresses on shoot apex of embryo | Naked shoot apex | Leaf primordia on shoot apex of embryo |

This study is the first developmental and intersectional comparative anatomical study of flowers/fruits for subgenus *Quercus* section *Protobalanus*. This section contains only five species which are restricted to southwestern USA and northwestern Mexico, including islands off both California and Baja California. Originally placed within section *Lobatae* or the red oaks (Michaux, 1817), these "intermediate" oaks exhibit a mosaic of flower and fruit features that are found in both sections *Lobatae* and *Quercus s. s.* (Table 2), prompting Trelease (1916; 1922) to place these five species, *Q. cedrocensis* C. H. Muller, *Q. chrysolepis* Liebm., *Q. palmeri* Engelm., *Q. tomentella* Engelm., and *Q. vaccinifolia* Kellogg, in their own section based on this character distribution as well as unique features such as mucronate leaf tips and lateral abortive ovules (Manos, 1997; Nixon, 1997).

To allow direct comparison of characters described in previous flower/fruit studies of subgenus *Quercus*, sections *Cerris*, *Lobatae*, and *Quercus s. s.* (Mogensen, 1965; Borgardt and Nixon, 2003), two individuals of *Q. palmeri* were sampled from a wild population over a single growing year. In previously published literature (e. g., (Tucker, 1980), this species may be referred to by the illegitimate name "*Q. dunnii* Kell.". Native to the southwestern US (California and Arizona) and northwestern Mexico, *Q. palmeri* (Figures 1-4) is found in canyons, mountain washes, dry thickets and chaparral as small, disjunct populations (Manos, 1997; Nixon, 1997). Commonly called the Palmer Oak (or Dunn Oak), this species exhibits biennial fruit maturation, in which flowers of the current year and the flowers/fruits of the previous year are normally present simultaneously on the same individual, and often on the same

branches (with current year flowers distal to older flowers). Distinguishing between current and previous year growth is critical in collecting relevant material because biennial-fruiting oak species found in dry habitats can abort current year's growth if drought conditions occur early in the growing season. This results in flowers/fruits of the previous year appearing terminal and borne alone on the twig: a characteristic of annual-fruiting oaks. This “pseudo-annual” fruit maturation pattern was first described by Engelmann (1876) for *Q. chrysolepis* and *Q. tomentella*, and has been observed for *Q. palmeri* (K. Nixon, personal observation, Cornell University, Ithaca, NY, USA).

## MATERIALS AND METHODS

### *Sampling*

A complete developmental series of *Q. palmeri* was collected from two wild individuals in the Seven Springs Campground (Cochise County) north of Carefree, Arizona, USA, during the growing year of the year 1995. To follow the biennial fruit maturation exhibited in this species, 10 pistillate flowers that emerged in the current year (1995) as well as 10 pistillate flowers/fruits that emerged the previous year (1994) were collected once weekly from 12 April through 19 June, followed by bimonthly collections from 30 June through 2 August. Current year flowers were collected attached to their respective inflorescence axis, while previous year flowers and maturing fruits were collected with only the subtending cupule. Cupules from the last stages of fruit maturation were removed to reduce infiltration time, conserve preserving fluid, and to fit the fruits in the available fixing containers.


Figures 1-4. *Quercus palmeri* morphology: all photos taken by S. Borgardt except for Figure 4 which was provided by Kevin Nixon (Cornell University, Ithaca, NY, USA). **1**. Canopy view of the two trees sampled growing below an embankment next a road in the Seven Springs Campground (Cochise County) north of Carefree, Arizona, USA. S. Doan (Arizona State University, AZ) included for scale. **2**. Leaves with mucronate tips of section *Protobalanus*: flowers of previous year in the axil of the lowest leaf, flowers of current year still enclosed in buds. **3**. Close-up of flowers from previous year showing characteristic arrangement of flowers on the inflorescence. **4**. Mature acorns showing flaring, undulate rimmed cupule common to this species.

Vouchers (SB1400, SB1401) of the specimen trees, are deposited in the Cornell University Herbarium (BH), Ithaca, New York USA.

***Fixation***

Collection materials were fixed in Farmer's or Carnoy's Fluid (Johansen, 1940). To facilitate penetration of the fixative, fruits with sclerified exocarp were slashed with a pruning shear blade at the time of collection. Specimens were transferred a week later to 70% ethanol for storage.

***Pre-infiltration, infiltration and embedding***

Fixed specimens were dehydrated, pre-infiltrated, infiltrated, and embedded using existing protocols (Gerrits and Smid, 1983; Gerrits and Horobin, 1996) for the Technovit® 7100 kit (Kulzer, Germany) which uses glycol methacrylate as the embedding medium.

***Sectioning***

Embedded specimens were sectioned on a manual microtome in increments of 4 µm using Ralph-Bennett glass knives (Bennett et al., 1976) which allowed for unusually wide anatomical sections even in sclerified material. Sections were placed in sequence on a pool of water on a microscope slide and allowed to air dry on a slide warmer set to 60ºC overnight, and prepared for staining the next day.

***Staining***

A staining solution of Toluidine Blue O (TBO) 0.05% was prepared from a standard recipe with an acetate buffer (pH 4.4) substituted for the benzoate buffer. Handled this way, TBO stains lignin and sclerified structures a light aquamarine or

turquoise color, cellulosic structures stain dark blue, and proteins stain red-purple to violet. No mounting media or cover slips were used, and immersion oil was placed directly on the sections for microphotography (O'Brien and McCully, 1981).

***Photography***

Photographs of hand-sections were obtained with a Wild dissecting microscope using a Nikon 995 digital camera and a microscope uniadapter (ECO-1832D, Zarf Enterprises, Spokane, Washington, USA). Photomicrographs were obtained with an Olympus BX60 compound microscope using video-capture under the control of a PC microcomputer, with scale calibrated using a stage micrometer.

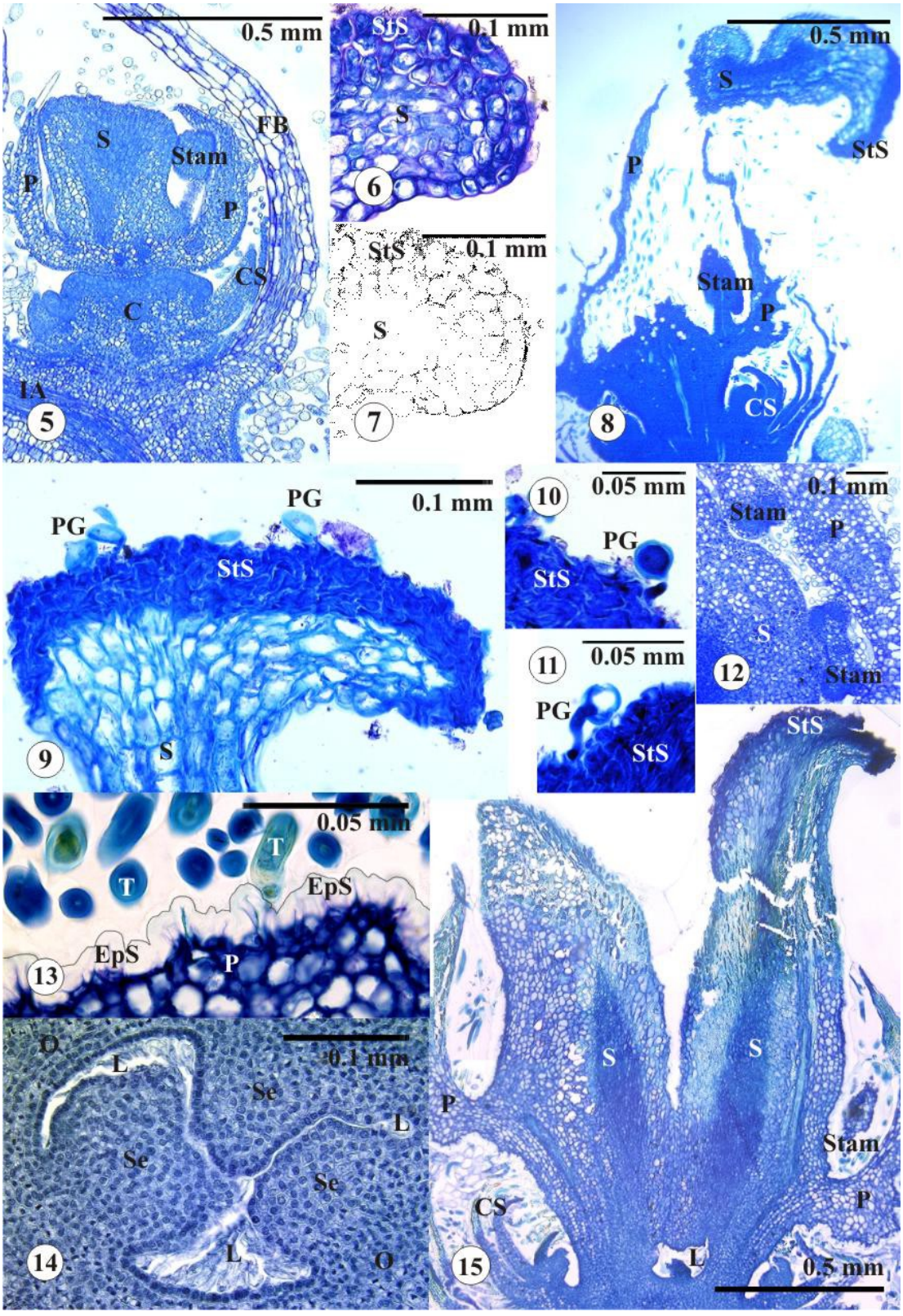
**RESULTS**

Biennial fruit maturation, as seen in *Q. palmeri*, creates a special challenge for the researcher to describe in a clear and concise way the sometimes simultaneous maturation of reproductive structures from different years on the same individual. For this study, the development of the pistillate flowers of the current year (1995) will be described first, followed by the description of the flowers of the previous year (1994) and subsequent fruit maturation. Seven developmental stages are defined for this study based on observable anatomical changes in *Q. palmeri* during the maturation of the pistillate flowers of both the current year (1995), the previous year (1994), as well as the fruits. **Stage 0** is winter dormancy with flowers of the current year either undifferentiated or differentiating on the inflorescence axis that were still enclosed within bud scales/bracts. Flowers of the previous year are quiescent. **Stage 1** describes

bud break and emergence of the flowers of the current year. Flowers of the previous year are quiescent. **Stage 2** begins when the stigmatic surfaces of the styles on flowers of the current year show adherent pollen grains. Flowers of the previous year show differentiation of ovules and ovary wall layer changes. **Stage 3** describes quiescent flowers of the current year. Flowers of the previous year have two integuments and a nucellus in most ovules. **Stage 4** describes quiescent flowers of the current year. Flowers of the previous year have an immature embryo sac in most ovules. **Stage 5** describes quiescent flowers of the current year. Flowers of the previous year have at least one ovule with a mature embryo sac, or endosperm visible in a developing seed (ovary now a fruit). **Stage 6** describes quiescent flowers of the current year. The embryo, seed, and fruit continue to mature until fruit drop.

Stages are presented in order from youngest to mature in the anatomical figures (Figures 5-38), with the flowers from the current year presented first (Figures 5-15) and the flowers/fruits from the previous year presented last (Figures 16-38). Within each stage, a summary of features and changes seen in the flowers/fruits is presented in order from the exterior to the interior and from the apex to the base of the flower/fruit. To allow comparison between different developmental stages and to save space, terms used to refer to mature or end stages will be applied to applicable immature or earlier stages. For example, fruit wall layer terms such as “exocarp” will be used to indicate parts of the ovary that will eventually differentiate into that part of the fruit. The term “staminode” has historically been used in floras to describe

Figures 5-15. *Q. palmeri* flowers of current year. **5.** Stage 1; 3 May. Flower *ls* including inflorescence axis (IA), floral bract (FB), cupule (C), perianth lobes (P), a staminode (Sta) and one style (S). **6.** Style apex *ls* showing purplish staining cells in stigmatic surface (StS) with lighter-staining cells in non-receptive style tissue (S). 7**.** Same as Figure 2 with purplish staining cells in stigmatic surface (StS) emphasized using bitmap editing software. **8.** Stage 2; 17 May. Flower *ls* (dead) with cupule scales (CS) lodged under perianth lobes (P), one staminode (Stam), and ovary (O), with styles out of section. **9.** Style apex *ls* showing the non-receptive style tissue (S), stigmatic surface (StS), and germinated pollen grains (PG). **10.** Style apex *ls* showing stigmatic surface (StS) with a germinating pollen grain (PG). **11.** Style apex *ls* showing stigmatic surface (StS) with a germinating pollen grain (PG). **12.** Stage 2; 9 May. Flower *ts* with perianth (P), two staminodia (Stam) and style (S). **13**. Stage 6; 16 July. Perianth lobe *ls* showing sclerified epidermis (EpS; line added to figure showing outer edge of cell wall) and lignified trichomes (T). **14.** Stage 5; 19 June. Ovary *ts* showing three locules (L) and three irregular septa (S). **15**. Stage 6; 2 August. Composite image merged from two images of flower *ls* at end of growing year showing cupule (C), cupule scales (CS) lodged under perianth flange, perianth lobes (P), one staminode (Sta), two styles (S) with the stigmatic surface (StS) of one style visible. In the ovary, one locule (L) with the base of a septum (Se) is visible. Separation in style occurred during sectioning.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the vestigial organs/ non-functioning stamens in pistillate flowers of *Quercus* subgenus *Cyclobalanopsis* (Camus, 1934; Soepadmo, 1972) and will be used in this study in a similar way.

Some collections made on and after 22 May (from both current and previous years' growth) were later discovered to contain flowers that were dead or senesced at the time of collection. In many cases, the fact they were dead was not discovered until they were cut open. These dead flowers were very similar in size to the living flowers, but on closer inspection, the ovaries of these completely senesced flowers were darker and appeared shrunken. The tissues in these dead flowers had more druses and stained more intensely (often without visible cell walls) relative to living flowers, and also had druses in tissues that were crystal-free in living flowers (e. g., Figures 8, 18). Dead flowers also had large air spaces in the ovary wall resulting from the separation of prematurely sclerified ovary wall layers. Despite these differences, it is assumed that floral/ovule development in the dead flowers is comparable to living flowers until the time of death. Even though some flowers of the current year died, “pseudo-annual” fruit maturation (total drop of the current year pistillate flowers) was not observed in this study. Interestingly, for a few years following this study, flowers emerged on current year's growth and were persistent, but the flowers/fruits of the previous year were dropped resulting in no acorns for that year (S. Doan, personal communication, Arizona State University, Tempe, AZ, USA).

Measurements of the flowers and fruits were obtained from sections of the flowers/fruits and are provided only for comparative purposes within this study. Since

the same methods were used on all the samples, any shrinkage (fixation, dehydration, and embedding), compression (glass knife cutting), or expansion (placement and hydration on the slide) are assumed to be similar across all samples. Some of the samples had very few specimens, so sample size also has to be considered.

***Flowers of current year (1995)***

***Stage 0****, Winter dormancy: 12-26 April*–Within closed terminal buds, the pistillate inflorescences were differentiated enough to be recognizable in the axils of leaves on the immature branch. The pistillate flowers were most often arranged on the inflorescence axis as a sub-opposite pair (with a flower slightly lower on the axis), although some collections had up to four flowers arranged as pairs, with each pair perpendicular on the axis relative to the lower pair (decussate-like). In the earliest samples, the flower primordia (~ 0.3 mm high X 0.3-0.4 mm wide) were bowl-like with perianth lobes around the rim and a few cupule scale primordia emerging in the axil between the flower and the enclosing bract. Just before bud break, some flowers had recognizable styles and tepals with cupule scale primordia emerging from a cupule that was now a mass of cells elevating the base of the flower from the enclosing bract (Figure 5). Some of the largest flowers (with cupules) in bud measured (~1.1-1.3 mm high X 1 mm wide). Many flowers had at least one staminode inserted in an axil of a tepal, and a few flowers had more than one staminode (each inserted in the axil of a tepal). Figure 5 is a good representation of flower morphology just before bud break, but it is actually the most apical flower still enveloped by its floral bracts on an inflorescence collected on 3 May that had more basal flowers exposed on the

inflorescence.

***Stage 1****, Bud break/anthesis: 3 May* – At the time of bud break, each pistillate flower (~0.7 mm high X 0.9-1.1 mm wide), subtended by a cupule (~0.3-0.4 mm high X 1.1 mm wide) with 3-4 cupule scale cycles, was in the axil of a primary bract flanked by two smaller, ephemeral secondary bracts on the inflorescence axis. The perianth was comprised of two alternate cycles of three tepals with the inner cycle opposite the styles and inserted onto the pistil just below where the styles became appressed or fused together in the apex of the pistil. The tepals (~0.7 mm high) enveloped the bases of the styles up to the stigmatic surfaces with the styles barely exserted from the perianth. Lignified trichomes and multicellular glandular trichomes were present on the surfaces of cupule scales and flower tepals. One to two staminodia were inserted in the axil of at least one tepal in most flowers but were not visible outside the perianth. At this stage, the most prominent feature of the pistillate flowers were (usually) three styles which comprised half the flower mass (styles spread out to ~0.7 mm across at top and were ~0.7 mm high). Stigmatic surfaces were restricted to the style apices and stained darker (with violet hues), but had no pollen present at this stage (Figures 6-7). Features unique to the ovary (such as locules) were not yet distinguishable at the base of the flower.

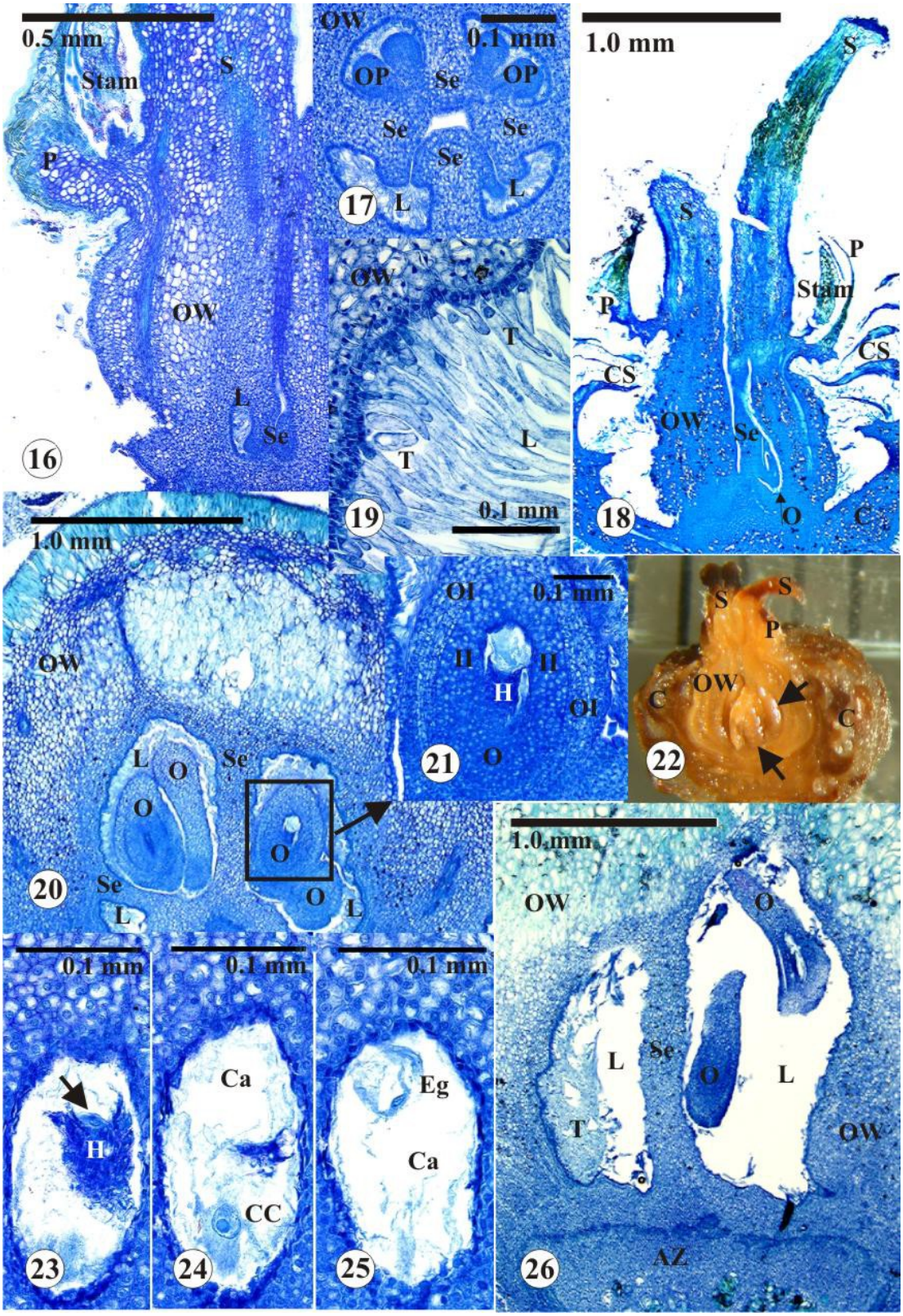
***Stage 2****, Pollination: 9 May–17 May* –The subtending primary floral bract was still present below each pistillate flower, secondary floral bracts were no longer visible in sections. Pistillate flowers (~1.3-1.4 mm high X 1.1 mm wide) and cupules (~0.5 mm high X 1-1.2 mm wide), with four to five cycles of cupule scales, were

slightly larger that those at bud break. Sclerification of the outermost cycle of cupule scales had occurred by the end of this stage, with the innermost scales still lodged under the abaxial surface of the perianth lobes (Figure 8). Tepals (~1 mm high) still enveloped the bases of the styles, but the styles were now exerted out of the perianth by ~0.5-0.7 mm, and had pollen grains (20–25 μm in diameter) adhered to the stigmatic surfaces. These grains appeared viable with contents either visible inside the grain or germination and/or pollen tube growth evident (Figures 9-11). Most staminodia were club-shaped undifferentiated masses at their apex and attached by slender bases to the axils of tepals (Figure 8), but a few showed remarkable stamen-like differentiation (Figure 12). The styles were still the most prominent feature of the flowers, with some cells now highly vacuolated in the nonstigmatic areas of the style apices (Figure 9). Senescence in the stigmatic areas was visible at the end of this stage as compressed cells with densely staining, coagulated contents. The ovary was not yet distinguishable at the base of styles, but locules were visible in the sections as thin slits (Figure 8, at arrows).

***Stages 3–6****, Quiescence: 22 May–2 August* –These four stages encompass the end of pollination (no viable pollen visible on stigmatic surfaces in these sections), the senescence of the stigmatic surfaces, and gradual changes of the pistillate flowers prior to the end of the growing year. Cupules grew moderately through this period, and by the end, each cupule (~0.6-0.7 mm high X 1.7-2 mm wide) had five to six cycles of imbricate flattened scales. The outermost scale cycle was highly sclerified and each scale curved in to touch the outside of the perianth before spreading out in such a way

as to appear convex in section. Innermost scales were still mostly parenchymatous and lodged beneath a flange formed by the undulate abaxial surface of the perianth lobes. Sclerification of the epidermal cells on all style and perianth surfaces occurs by the end of these stages, and in some cases form very thick cell walls (outside edge indicated by line added to Figure 13). Senescence of the styles (~1.7 mm wide across all the styles at the top X 1.4-1.6 mm high) and perianth (~0.7-1 mm high) progresses through this period. A few rows of cells just basal to the epidermis became compressed, or suberized, or filled with tannins, but most interior cells of the styles and perianth remain parenchymatous, with the cells in the style bases staining densely relative to cells in other tissues. Staminodia in most flowers senesce in the same manner as the perianth and styles. The ovary changed the most during these stages: going from indistinguishable at the bases of the styles to being clearly visible (0.2-0.4 mm high) between the perianth insertion on the pistil and the cupule. The ovary (0.4-0.6 mm wide at insertion into cupule) was smaller in diameter than the style bases (~1 mm wide at perianth insertion) and had locular spaces filled with unicellular parenchymatous trichomes that were delimited by intruded septa (Figure 14). No ovule primordia were found at the end of these stages, although the irregular margins of the septa appeared to be ovule primordia in section (Figure 15). One extraordinary inflorescence in Stage 6 had two flowers with staminodia that contained pollen grains and evidence of pollen tube growth inside the “anthers” (Figures 39-40). It is unknown if developing viable pollen in pistillate flowers was characteristic of this individual tree, of *Q. palmeri* in general, or occurs in other species of section *Protobalanus*.

Figures 16-26. *Quercus palmeri* flowers of previous year. **16.** Stage 2; 9 May. Flower *ls* showing ovary wall (OW), septa (Se), locules (L), perianth (P), staminode (Stam) and styles (S). Example of how the septa can appear to be ovules in section. Cupule removed before embedding, tear on left ovary wall occurred during preparation. **17.** Stage 2; 9 May. Flower *ts* showing ovary wall (OW), ovule primordium (OP) on septa (Se) in locules (L) filled with trichomes. This specimen had four styles, locules, septa and eight ovule primordia rather than the normal three styles, locules, septa, and six ovule primordia. Example of the shallow helical placement of the ovules on the septa. **18.** Stage 3; 4 June. Dead flower *ls* showing cupule (C), with inner cupule scales (CS) still lodged under the perianth (P). Two styles (S) in section with two perianth lobes (P) and one staminode (Stam) visible. Ovary wall (OW) with numerous druses, one septum (Se), and one ovule (O) visible at arrow in this section. **19.** Flower *ls* showing ovary wall (OW), with locule (L) filled with unlignified trichomes (T). **20.** Stage 5; 19 June. Flower *ls* with ovary wall (OW), two septa (Se), three locules (L) with four ovules (O) and trichomes. The light staining area at the apex of the ovary wall below the palisade layer is the ring of sclerified cells in the mesocarp (described in text). All of these ovules in section have developing embryo sacs. High magnification of ovule in box presented in Figure 21. **21.** Stage 5; 19 June. Flower *ls* high magnification of ovule in Figure 19 (in box). Ovule (O) with outer integument (OI), inner integument (II) and hypostase (H) in caecum. **22.** Stage 5; 19 June. Hand cut flower *ls* showing cupule (C), styles (S), perianth (P), ovary wall (OW), and ovules (at arrows). **23.** Stage 5; 19 June. Flower *ls* showing ovule with inner integument (II), antipodal (at arrow) in the hypostase (H). **24.** Flower *ls* of same specimen as Figure 23 in another section showing central cell (CC) within the caecum (Ca). **25.** Flower *ls* of same specimen in Figure 23 in another section showing egg (Eg) in the caecum. **26.** Flower *ls* showing ovary wall (OW) with abscission zone (AZ), one septum (Se), two locules (L) trichomes (T), with two ovules (O) visible in section in only one of the locules. Ovules shifted to a more apical position in locule after being placed in the water bath.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***Flowers of previous year (1994)***

***Stage 0-1****, Winter dormancy: 12 April- 3 May (e. g., see Figure 15)*–At the conclusion of winter dormancy, the pistillate flowers (1.8-2.4 mm high X 1.3-1.5 mm wide) of the previous year were similar (although at a larger scale) to those of Stage 6 of the current year, with the exception of the wider ovary (0.8-1 mm high X 1-1.3 mm wide; insertion of ovary into cupule 0.6-0.8 mm wide).

***Stage 2****, Ovule initiation: 9-17 May (Figure 16-17)*–The cupule (1.7-1.9 mm high X 2.6-2.8 mm wide) with six to seven scale cycles was still mostly parenchymatous, with only the outermost scales sclerified (not illustrated, see Figure 18 for an example). Tips of the inner scale cycles were lodged under the perianth flange and showed some sclerification. The exserted portions (relative to the cupule) of the pistil, perianth, staminodia and styles were mostly sclerified or cells were filled with tannins or coagulated, with epidermal cells uniseriate and sclerified. The fused style bases (0.8-1 mm wide) at the level of the perianth were densely stained and parenchymatous, with the individual styles measuring 1-1.6 mm high X 0.3-0.5 mm wide. The ovary (0.8-1 mm high X 1-1.3 mm wide) was still mostly undifferentiated, with an uniseriate inner epidermis and unicellular unlignified trichomes filling the locules around the ovule primordia (Figure 17). Early in this stage, two collateral ovule primordia were present on each septum, initially oriented perpendicular to the axis of the ovary, and each occupied ca. 10-20% of the locule volume in section. Later in this stage, the ovules (~0.1 mm high X 0.1 mm wide) with integumentary and nucellus primordia were oriented mostly parallel with the axis of the ovary. Six ovule primordia in three

locules were normally present, although two specimens had eight ovules in four locules (Figure 17).

***Stage 3****, Ovule maturation: 22 May–4 June (Figure 18; all flowers collected were dead)*–Cupule (1.6-2.3 mm high X 2.7-4 mm wide) was wider in diameter than the perianth (0.6-1 mm high X 1.3-1.5 mm wide), no longer tightly appressed to the ovary, and contained small sclereid clusters. Outermost cupule scales were free of perianth flange and basally thickened. Innermost cupule scales sometimes still lodged under perianth flange (Figure 18). No cupule scale primordia were present. In the apex of the ovary, a sclerified ring of cells was present just basal to the densely stained style bases in the mesocarp and extended down to the apex of the locules. The ovary wall was differentiated enough to distinguish the areas that would mature into the exocarp, mesocarp and endocarp of the mature fruit. The exocarp consisted of an epidermis that appeared to be two-layered and a palisade layer of three to four rectangular elongated cells oriented perpendicular to the epidermis. One or two palisade cells just below the epidermis usually contained crystals. The palisade layer was mostly sclerified from the apex of the ovary (from the base of the styles) down 75% of the ovary. The mesocarp was mostly parenchymatous with scattered druses and vascular tissue. The endocarp consisted of an uniseriate inner epidermis and unicellular unlignified trichomes. The septa were parenchymatous, present from the apex to the base of the ovary, and delimited the locules. Ovules on the septa formed a shallow helical pattern within the ovary, occupied ca. 75% of the locule volume in section, and each ovule had completely differentiated outer and inner integuments (both 6-10 cells thick) and a

nucellus.

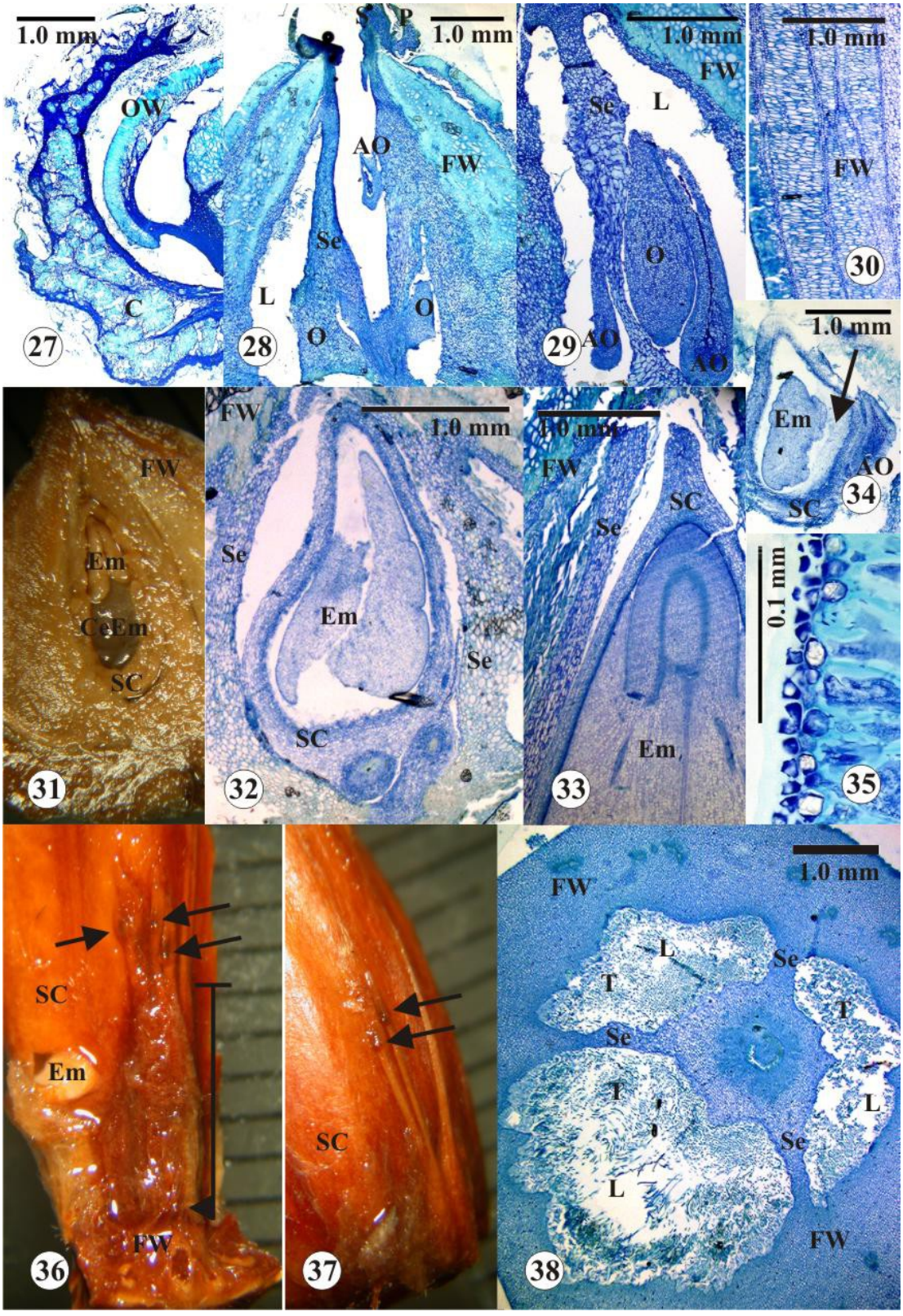
***Stage 4****, Megasporogenesis, megagametogenesis: 12 June (Not figured; all flowers collected were dead)*–Cupule and cupule scales were still mostly parenchymatous with sclereid clusters. Outermost cupule scales were free of perianth flange and basally thickened. Innermost cupule scales sometimes still lodged under perianth flange. No cupule scale primordia were present. Exocarp palisade layer was sclerified over entire ovary except for a small zone just apical and lateral to abscission zone. A ring of cells in the apical portion of the mesocarp was sclerified from the style bases down to ca. 3 mm from apex of ovary enclosing densely staining parenchymatous cells in the center of the flower just apical to the locules. Sclereid clusters were present in abscission zone in base of ovary. Septa with vascular differentiation at their bases were present from apex to base of ovary, and ovules occupy ca. 50% of the locule volume in section. The ovules at this stage had either one megaspore mother cell or an immature embryo sac. Both inner and outer integuments were each 6-10 cells in thickness.

***Stage 5****, Mature embryo sacs: 19 June (Figures 19-26)*–Cupule and scales contain more sclereid clusters than previous stages, but were still mostly parenchymatous. Outermost cupule scales were free of perianth flange and basally thickened. Innermost cupule scales sometimes still lodged under perianth flange (Figure 22). There were no cupule scale primordia present. Cells in the center of the style bases were now highly vacuolated (instead of densely staining). The ovary wall had both basipetal and centripetal differentiation and sclerification compared to earlier

stages, with some cells in the mesocarp in stacked cell tiers parallel to the exocarp palisade layer. The apical portion of the mesocarp was now completely sclerified down to about 3 mm from the apex of the ovary: no darkly staining parenchymatous cells present just apical to the locules as in former stages (Figure 19, 26). Unlignified unicellular trichomes present in the spaces in the locules around the ovules (Figures 19-20, 26). Sclereid clusters and druses were present in abscission zone in base of ovary. Each ovule contained a differentiated embryo sac with a densely stained hypostase (Figure 21) at the base. An egg apparatus with two synergids and an egg cell was present at the apex of the ovule (Figure 23), with the central cell toward the base of the caecum (Figure 24). The antipodals were sometimes visible at the apex of the hypostase (Figure 25). Toward the end of this stage, endosperm was usually visible in at least one ovule, even if zygotes or embryos could not be located in the embryo sac. When endosperm was present in an ovule, its inner integument was either highly vacuolated or broken down around the embryo sac so that only the outer integument remained (except for a small plug of the inner integument in the micropyle). In specimens with endosperm visible in section, there was also an accumulation of vascular tissue visible at bases of septa, below ovule attachment.

***Stage 6****, Fruit, seed and embryo: 19 June–2 August (Figures 27-38) Fruits from 30 June dead)*–Throughout this stage, the cupule became increasingly woody (Figure 27). In specimens where the cupule had not been removed prior to embedding, the cupule was completely free of the perianth flange. Sclerified cells were present in all wall layers of the fruit. The exocarp palisade layer (0.2-0.3 mm wide) of the exocarp

Figures 27-38. *Q. palmeri* flowers and fruits of previous year. **27.** Stage 6; 30 June. Dead flower *ls* showing cupule (C) and ovary wall (OW). Good example of ovary wall separation between the exocarp palisade layer and mesocarp that is seen in the dead flowers. Note cupule sclerification and basal expansion, no cupule scale primordia present at apex of cupule. **28.** Fruit *ls* with fruit wall (FW), two septa (Se), three locules (L) with three ovules (O; one aborted AO). Left lower ovule has embryo and endosperm in other sections, right lower ovule still has intact embryo sac in other sections. Senesced perianth (P) and styles (S) at apex. **29.** Stage 6; 16 July. Fruit *ls* showing fruit wall (FW), one septum (Se), two locules (L) with three ovules (O; two aborted OA) visible. Central ovule has embryo and endosperm in other sections. Note vacuolated cells near apex of septum. **30.** Stage 6; 2 August. Fruit *ls* showing fruit wall (FW), exocarp and mesocarp. Exocarp palisade layer visible as a dark line at far left: separation occurred during sectioning. Mesocarp "palisade" layer is visible as elongated cells organized into 2-3 tiers high adjacent to exocarp. Lines of non-elongated parenchyma cells that separate the mesocarp "palisade" layer also visible in this section. **31.** Stage 6; 16 July. Hand cut fruit *ls* showing fruit wall (FW), seed with seed coat (SC), and embryo (Em). Notice shiny, translucent cellular endosperm (CeEn) basal to embryo in seed. The coenocytic endosperm dribbled out immediately after sectioning. **32.** Stage 6: 16 July. Fruit *ls* showing fruit wall (FW), two septa (Se), and one seed with seed coat (SC) and embryo (Em). Bundle of vascular tissue at base of seed is attached in other sections to the bundle in the septal remnant immediately next to it on the right. **33.** Stage 6; 2 August. Fruit *ls* showing fruit wall (FW), one septum (SE), trichomes (T) and one seed with with seed coat (SC) and embryo (Em). **34.** Stage 6; 16 July. Fruit *ls* with seed and seed coat (SC), cellular endosperm (at arrow), embryo (Em), and aborted ovule (AO). **35.** Stage 6; 2 August. Fruit *ls* of exocarp only. Exocarp epidermis on left with two-layered appearance, with rhomboidal crystals in exocarp palisade layer immediately below the epidermis. **36.** Stage 6; 2 August. Hand cut fruit *ls* of base of fruit and seed showing fruit wall (FW), small seed with seed coat (SC) and embryo (Em). Seed coat was cut during preparation. Not figured is the large seed that was removed during dissection and was attached on the right. The structure in front of seed and in center of figure indicated by the bracket is an example of the woody pillar-like structure (Nixon's "columella") that can form in the base and side of fruit. Aborted ovules (at arrows) are visible at the apex of this structure, with a persistent septal remnant visible on the far right and continuing up out of the figure. **37.** Stage 6; 2 August. Hand cut seed *ls* removed from fruit showing seeed coat (SC) and aborted ovules (at arrows) on separate septal remnants. The pillar-like structure was not present at the base of this fruit. **38.** Stage 6; 2 August. Fruit *ts* showing base of fruit wall (FW) and three septal remnants (Se) delimiting three locules (L) filled with trichomes (T).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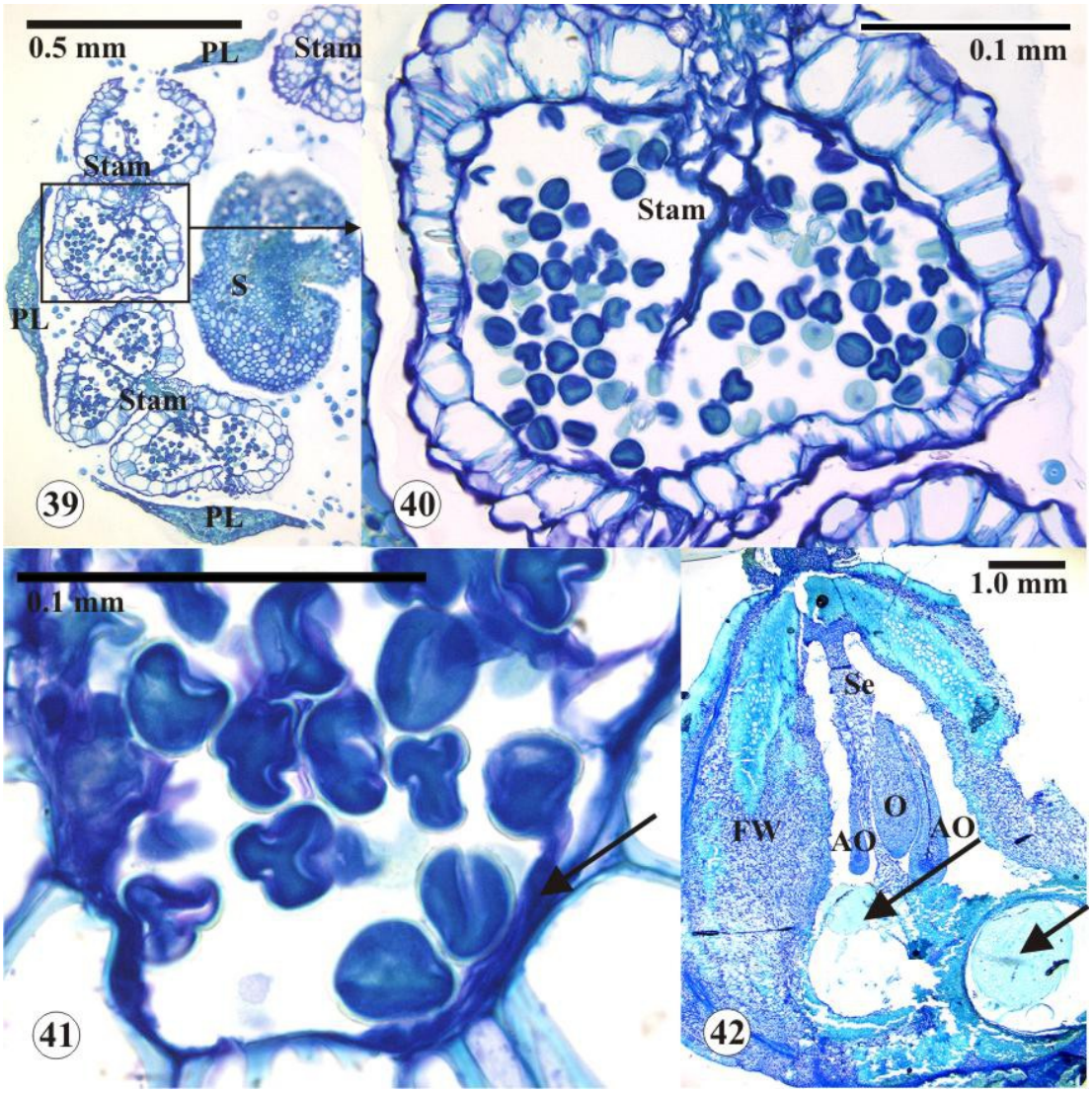
was completely sclerified (Figures 27-28, 35), with a small unsclerified zone just apical and lateral to the abscission zone (Figure 27). The outer mesocarp contained tiers of elongated cells that were parallel and just below the exocarp palisade layer and appeared to be a "secondary" palisade layer (Figure 30) with varying degrees of sclerification. Each "secondary" palisade cell tier (~1 mm wide) was separated by several layers of parenchymatous cells, with the total mesocarp layer measuring ~4 mm at the widest part of the fruit and decreasing to ~2 mm wide on the top and bottom of the fruit. The endocarp epidermis was parenchymatous with proximal unicellular trichomes unlignified, but some trichomes in the interior of the fruit were lignified. The delimitation of the abscission zone (1 mm high X 3 mm wide) at the base of the fruit was mostly complete, forming a disk-like structure composed of loose sclereid clusters interdigitated with parenchymatous cells around its periphery. At the beginning of this stage, the remnants of all three septa were present from apex to base of the fruit (Figures 28-29) and the maturing seed(s) occupied ca. 50-75% of the fruit cavity in section. More than one fruit had a large seed with a well developed embryo and a much smaller seed next to it. The seed coat, which had significant vascular tissue differentiation in the base, was exotestal. In the early stages of seed maturation, the seed was shaped by both the cellular and coenocytic endosperm surrounding a small embryo that was at the micropylar end of the seed coat (Figures 31). Cellular endosperm was restricted to the area around the maturing embryo and the periphery of the seed, while coenocytic endosperm filled the remainder of the space in the seed until the embryo assimilated all the endosperm (Figures 31-34). Simultaneous with




Figures 39-42 *Q. palmeri* flower of current year and fruit of previous year. **39.** Stage 6; 2 August. Pistillate flower of current year *ts* is above level of perianth insertion into styles. One style (S), perianth lobes (PL), and three staminodia (Stam) are visible in this section. Note differentiated tissues in staminodia. **40.** Higher magnification of one pollen sac in Figure 39. **41.** Higher magnification of pollen sac of same specimen, different section. Pollen tube growth visible at arrow. **42.** Fruit *ls* , same as Figure 29, not cropped. Fruit with Fruit wall (FW), one septum (Se), two aborted ovules (AO), one ovule (O). Good illustration at apex of fruit of the sclerified mesocarp ring. Exocarp on right was cut off prior to embedding. Evidence of herbivory or galls at base of fruit (at arrows).

endosperm assimilation, the seed enlarged to fill the locular spaces, and during the last stages of maturation, compressed the septal remnants against the endocarp so that only one locule remained (Figures 32-33). At the base of a septal remnant, the seed was connected to the fruit (Figures 36-37). Aborted ovules were lateral to basal to the seed throughout maturation of the fruit, and were usually found together in a group in the bottom half of the fruit; attached to a compound structure consisting of the ovule placenta, the funiculus, and at least one septal remnant (Figure 36). The aborted ovules were attached either in a tight spiral around the compound structure (Figure 36), or were spread out a short distance next to the seed coat attached to septal remnants (Figure 37): aborted ovules were never found beneath the seed (Figure 38). The outer integuments were the only recognizable structures in the aborted ovules, as the inner integuments and contents were detached from the outer integuments and/or shrunken into densely staining masses (Figures 28: aborted ovule at apex, 29: aborted ovule on right, 34: aborted ovule on right). No leaf primordia or leaf buttresses in the embryo axis were found (Figure 33).

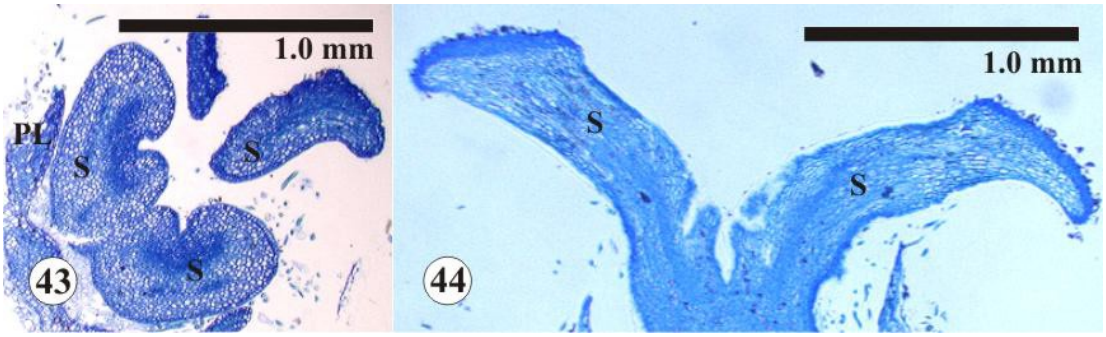
## DISCUSSION

### New features for section *Protobalanus*

*Cupule scale meristem inactive in second year* – In the *Q. palmeri* (P) study, the cupule scale meristem was active in the first year of growth, but did not produce new scale primordia in the second growth year when flower development resumed in the spring (Figures 18, 27). As the cupule matured in the second year, the scales became

evenly spaced and further apart (although still imbricate/helically arranged on the surface of the cupule) while the scale bases simultaneously expanded and thickened. Mature *Q. palmeri* (P) cupules can vary from hemispherical with an undulate rim, to a flat disk with flared edges (Figure 4), to an uneven, warty surface with no scales visible at maturity (Nixon, 1997). Since there is no cupule scale meristem active in the second year, growth in the ground tissue concentrated around the cupule rim may account for this unique morphology. The canyon live oak, *Q. chrysolepis*, also in section *Protobalanus* and known to hybridize with *Q. palmeri* (Tucker and Haskell, 1960; Manos, 1993, 1997; Nixon, 1997), develops cupules that are unusually thickened with blunt rims and may be the result of similar growth processes.

*Capitate stigmas* – Stigmatic surface morphology has historically been described as different between the two *Quercus* subgenera: with *Quercus* subgenus *Cyclobalanopsis* having capitate stigmas that are restricted to the apex of the style and *Quercus* subgenus *Quercus* having broadly spreading stigmatic surfaces that cover the apex of each style and continue down an open adaxial groove that leads down into the ovary (Conrad, 1900; Camus, 1934; Reece, 1938; Langdon, 1939; Tillson and Muller, 1942; Mogensen, 1965; Soepadmo, 1972; Abbe, 1974; Nixon, 1984; Kaul, 1985, 1986; Cecich, 1997; Boavida et al., 1999; Borgardt and Nixon, 2003). In *Q. palmeri*, the stigmatic surfaces were only at the apex of the styles (Figures 43-44). The adaxial grooves below the stigmatic surfaces had abutted margins that stained the same as the rest of the non-receptive style tissue. This morphology is best described as capitate, and a review of other species in section *Protobalanus* shows a wide continuum of




Figures 43-44 Capitate stigmatic surface morphology: *Q. palmeri* flowers of current year. **43.** 3 May. Transverse section is above level of perianth insertion onto styles at a tangential angle. Four styles (S) in this specimen, two styles sectioned at their bases on the left and two styles sectioned at their apices on the right; perianth lobes (PL) visible at left. Stigmatic surfaces visible on the right two styles (darker blue edges), but no stigmatic surfaces on the left styles which also show the adaxial grooves mentioned in the text. **44.** 4 June. Longitudinal section is above level of perianth insertion onto styles. Two styles (S) show stigmatic surfaces restricted to apices.

this type of stigmatic morphology. The stigmatic surfaces as seen in *Q. palmeri* (elevated high above the perianth with discrete edges) can also be seen in *Q. chrysolepis*, but in the other species the styles are shorter and although the stigmatic surfaces are still discrete and the adaxial groove margins are still abutted, it can appear as if the stigmatic surfaces are connected where the styles meet before descending into the ovary through the perianth. More sampling with the aid of TBO staining would be useful to verify if capitate stigmatic surface morphology is also present in other species from this section. Stigmatic surface morphology of the pistillate flower within subgenus *Quercus* has historically not been an important character in distinguishing among the sections, but with the description of capitate stigmas in *Q. palmeri* (P), this feature deserves closer examination.

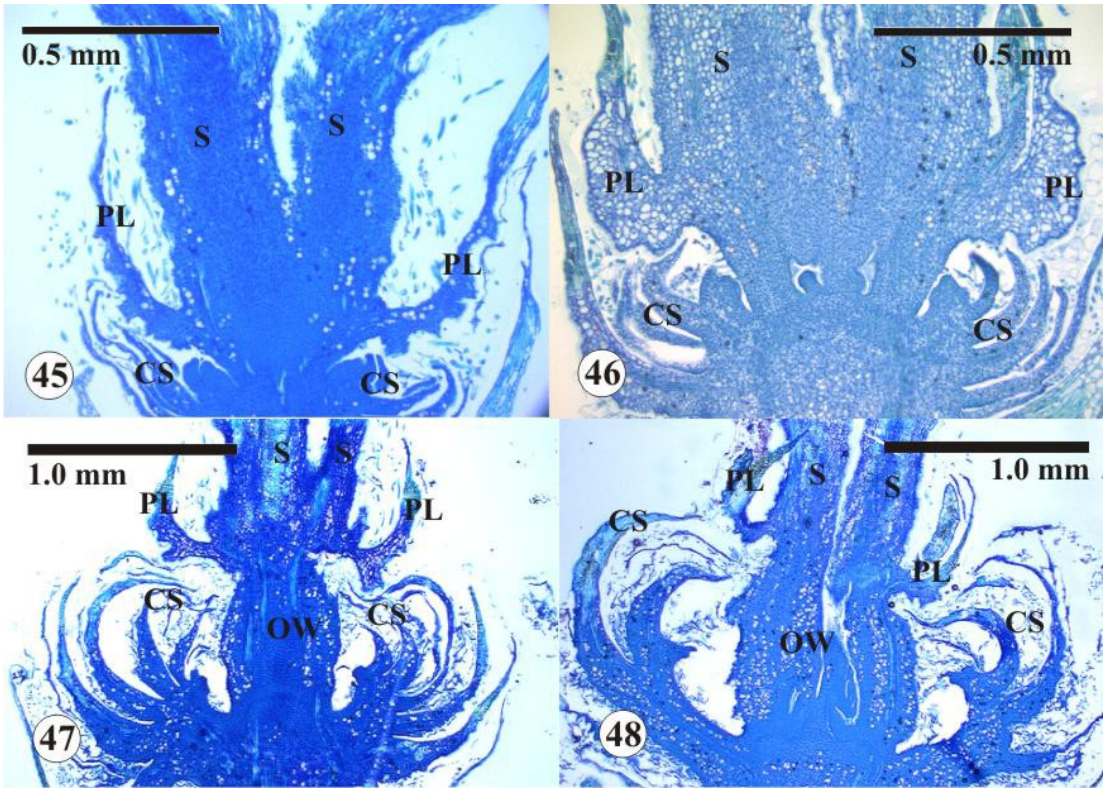
The styles and perianth in *Q. palmeri* (P) developed very thick epidermal cells

after pollination that extended from the apex of the flower down to the adaxial surface of the perianth (Figure 13). This extreme epidermal sclerification was not observed in *Q. acutissima* (C), and as style/perianth senescence was not documented in *Q. alba* (Q) or *Q. velutina* (L), it is unknown at this time whether this character is unique to *Q. palmeri* or section *Protobalanus*. It bears mentioning that this also could be an adaptation to extremely dry habitat to minimize moisture loss and may be a common feature to any oak species found in those conditions.

*Perianth flange – Q. palmeri* exhibits a "skirt" or flange at the base of the perianth that catches the tips of some of the cupule scales during quiescence (Figures 45-48). The flange in *Q. palmeri* results from the lowest part of the tepal lobes projecting out almost perpendicularly from their point of insertion on the fused style bases to form an undulating surface that wedges the tips of the cupule scales under the perianth. A perianth flange that interlocks the perianth and the cupule scales has been well documented in many of the biennial-fruiting oaks of section *Lobatae*, (Oersted, 1871; Trelease, 1924; Camus, 1934; Muller, 1942b; Soepadmo, 1968; Tucker, 1980; Nixon, 1984, 1993; Kaul, 1985), but is a new feature for section *Protobalanus*. In *Q. palmeri*, the depth of the flange is shallow (Figures 8, 15, 18, 45-48) when compared to the deep indentation seen in oaks of section *Lobatae*, and the apex of the nut of *Q. palmeri* was not indented from the tips of the cupules scales as sometimes happens in oaks of section *Lobatae*. The subtle nature of the flange in *Q. palmeri* relative to those seen in section *Lobatae* is probably the reason that this feature has not been described in section *Protobalanus* before now.

*Staminodia* – In the current study, 1-3 staminodia were observed in every *Q. palmeri* (P) flower/fruit sampled (Figures 5, 8, 12, 15-16, 18, 28, 39-41). They were enclosed within the perianth lobes and only visible after dissection or sectioning. Most were undifferentiated, cylindrical or club-shaped, but some had differentiated tissues that looked like filaments, pollen sacs, and/or endothecal tissue. One inflorescence from 2 August of the current year (Figures 39-41) had pistillate flowers with stamens that had differentiated filaments, pollen sacs, pollen grains, and evidence of tube growth within the pollen sacs. A preliminary survey of over 50 different specimens of the five species within section *Protobalanus (Q. cedrocensis*, *Q. chrysolepis*, *Q. palmeri*, *Q. tomentella*, and *Q. vaccinifolia*) in the Bailey Hortorium-Cornelius H. Muller (BH-CHM) Oak Collection showed that the presence of staminodia was a consistent feature in all of these species except *Q. vaccinifolia*. Since these staminodia are hidden under the perianth lobes and not visible except with careful inspection/dissection, I submit that this is probably why they have not been observed before now. This character could have important phylogenetic ramifications in understanding relationships within *Quercus* as well as other genera within Fagaceae, and deserves further study.

*Fruit wall epidermis appearance and "secondary" palisade layer* – After the ovary wall in *Q. palmeri* (P) differentiated into exocarp, mesocarp, and endocarp (Stage 3; ca. 22 May) the exocarp epidermis appeared to mature into two rows of sclerified epidermal cells over the ovary surface. Although an epidermis usually consists of a single layer of cells, periclinal divisions within the epidermis or the




Figures 45-48 Perianth flange: *Q. palmeri* flowers of current year and previous year. **45.** 22 May. Flower (dead) *ls* of current year showing two styles (S), perianth lobes (PL), and cupules scales (CS). Notice undulate surface of perianth apical to cupule scales. **46.** 19 June. Flower *ls* of current year showing two styles (S), perianth lobes (PL), and cupules scales (CS). Flange more developed than previous figure. **47.** 17 May. Flower (dead) *ls* of previous year showing two styles (S), perianth lobes (PL), ovary wall(OW) and cupules scales (CS). Still quiescent, showing cupules scales caught between ovary and perianth lobes. **47.** 4 June. Flower (dead) *ls* of previous year showing two styles (S), perianth lobes (PL), ovary wall (OW) and cupules scales (CS). Cupule scales still caught under perianth lobes, ovules forming in ovary.

modification of subtending tissues can add several cell tiers to an epidermis (Esau, 1977; Mauseth, 1988). Re-examination of ovary epidermis development revealed that the ovary epidermis in *Q. palmeri* (P) is uniseriate prior to ovary wall differentiation. As the ovary develops, the two-layered epidermis appearance appears only around the median of the fruit, with uniseriate epidermis persisting at the apex and base of the

ovary/fruit. It is probable that the two-layered appearance resulted from epidermal cell displacement and not from cell division or co-option, but this needs verification with more intensive sampling at the time of ovary wall differentiation. Sclerification and development of thick cell walls of the top cell layers in the exocarp occurred at this time as well, and may play a role in cell displacement and this appearance.

After megasporogenesis (ca. 19 June) in *Q. palmeri* (P), parenchymatous mesocarp cells immediately basal to the exocarp palisade layer became elongated and sclerified to form a cell tier (ca. 2-4 cells thick) that was similar in appearance to the exocarp palisade layer (Figure 30) As maturation of the ovary/fruit wall progressed through the year, more cells were recruited from the mesocarp into this "secondary" palisade layer to form at least three separate tiers at a medial level in the fruit. Fewer cells were recruited in the apex and base of the fruit, resulting in thinner tiers in those areas. The tiers were separated by small rounded cells that remained parenchymatous throughout fruit maturation (Figure 30). The rest of the mesocarp (internal to the tiers) contained isolated sclereid clusters surrounded by parenchyma (Figures 30, 38, 42) that became compressed between the seed and the exocarp at fruit maturity. This results in a pericarp at maturity that appears to be primarily a palisade layer with few sclereids. This is similar to the final appearance of the pericarp in *Q. velutina* (L), but the palisade layer in *Q. velutina* (L) is composed of exocarp tissue only. In the other three sections of subgenus *Quercus*, the mesocarp only has sclereid clusters differentiate internally, and are either a minor component of the mesocarp (sections *Cerris* and *Lobatae*), or combine to form a mostly sclerified mesocarp with a "mealy"

texture (section *Quercus s. s.*). The pattern of mesocarp sclerification in *Q. palmeri* (P) that results in a "secondary" palisade layer is a new feature in *Quercus* and the Fagaceae (Soepadmo, 1968) and may be a unique feature of *Q. palmeri* or the section *Protobalanus*.

**Comparison of flower and fruit anatomy and development across all four sections of *Quercus* subgenus *Quercus***

For the purposes of discussion, species have been selected to represent their section as example species based on both the work presented in this paper and previous anatomical work. *Q. palmeri* will function as an example species for section *Protobalanus,* and *Q. acutissima* will function as an example species for section *Cerris*, with previous works on other species in section *Cerris* supplementing the anatomy and development seen in the study by Borgardt and Nixon (2003). *Q. alba* of section *Quercus s. s.* and *Q. velutina* of section *Lobatae* will function as example species for their respective sections because there are numerous anatomical and comparative studies that exist for these two species, including the Mogensen (1965) work that provided a framework for the current thesis. To assist the reader in remembering these associations, an initial will follow the species name to indicate its respective section: e. g., *Q. alba* (Q) refers to *Q. alba* (section *Quercus s. s.*). When discussing all these taxa in various groupings, the sections (not the species) will be listed in alphabetical order.

Features or characteristics shared by all four sections of *Quercus* subgenus

*Quercus* will be discussed first to establish a framework for the remaining discussion. Mapping these shared features on a cladogram (not included) with outgroups would determine whether these are sympleisiomorphies or synapomorphies within this subgenus. The final discussion will be about unique characteristics as well as double- or triple-shared characteristics across the sections, and will be discussed within the context of both historical and/or newly described morphological and anatomical features. A summation is presented in Table 2.

**Shared features among all the sections in *Quercus* subgenus *Quercus*: *Cerris*, *Lobatae*, *Protobalanus*, and *Quercus s. s.***

For all of the representative species, the meristems of the current years' pistillate inflorescence axes (and staminate inflorescence axes not followed in this study) differentiate at the end of the previous years' growing season and remain quiescent in terminal buds until the next growing season (usually spring). These reproductive meristems (usually basal in the bud) are mostly indistinguishable from vegetative meristems until a few weeks before the buds break. The pistillate inflorescences, which can range in length from almost sessile up to several centimeters long when mature, are formed in the axils of leaves on the main branch and are distal on the branch relative to the staminate inflorescences. Each pistillate inflorescence axis bears one to several mostly sessile flowers that are usually arranged on the axis as subopposite pairs, with each pair perpendicular to adjacent pairs in a decussate-like orientation (Trelease, 1924; Langdon, 1939; Muller, 1942a; Turkel, 1950; Corti, 1954,

1955, 1959; Bianco, 1961; Sharp and Sprague, 1967; Soepadmo, 1968; Fey, 1981; Kaul, 1985; Boavida et al., 1999; Borgardt and Nixon, 2003).

With the vast morphological variation among the cupules in *Quercus*, characterizing the development and maturation of this structure is beyond the scope of this dissertation, but some generalizations are possible to allow gross comparisons. In *Quercus* subgenus *Quercus*, the cupule subtends and encloses the pistillate flower (except for the styles, perianth and the apex of the ovary) from the initial stages of floral development through early fruit maturation. The cupule is attached only at the base of the fruit in this group, with the pericarp differentiating into a specialized abscission zone at the point of attachment with the cupule. In the last stages of fruit and seed maturation, the fruit expands out of the distal end of the cupule to form the familiar "acorn and cap" morphology. The fruit is either shed with the cupule attached, drops separated from the cupule, or is removed by animals. Most cupules in this group do not grow or interlock together into a single propagule as is seen in other acorns producing taxa such as *Quercus* subgenus *Cyclobalanopsis* and *Lithocarpus* (Camus, 1934; Soepadmo, 1972). Cupules usually appear hemispherical at maturity with scales that cover the exterior surface in an imbricate phyllotaxy. These scales can become inconspicuous and/or submerged in the cupule ground tissue as is seen in some species in section *Protobalanus* (e.g., *Q. palmeri, Q. chrysolepis*), or enlarge and reflex back as seen in section *Cerris*. Cupule morphology is also derived from ground tissue that subtends and supports the scales. This ground tissue can range in thickness from very thin or inconspicuous below the scales to very thick and comprise the majority of the

cupule volume.

When the pistillate flowers are initially exposed on the inflorescence axis, each flower is predominantly three styles subtended by a perianth and an undifferentiated ovary enclosed by the cupule that is present even at this early stage as a torus-like structure with at least two cycles of cupule scales (Turkel et al., 1955; Scaramuzzi, 1960; Bianco, 1961; Fey, 1981; Cecich, 1997; Boavida et al., 1999, 2001). A primary floral bract and two flanking, ephemeral secondary floral bracts enclose each pistillate flower on the inflorescence axis until the styles are exposed. Stigmatic surfaces consist of the nonpapillate, "dry" type with a pellicle (Heslop-Harrison, 1971). The styles senesce within a month after pollination. Pollen tube penetration into the styles has been documented for *Q. alba* (Q) and *Q. velutina* (L) (Turkel et al., 1955; Stairs, 1964; Cecich, 1997), was observed in the current *Q. palmeri* (P) study, but was not observed in the *Q. acutissima* (C) study.

Species with annual fruit maturation have a delay of approximately one month between pollination and syngamy, while species with biennial fruit maturation delay pollination and syngamy by around 13 months. Despite the difference in time of syngamy, ovule initiation and maturation are similar in all four taxa. Typically, there are three locules in the pistillate flowers, with each locule containing two collateral ovules that at maturity are oriented with the micropyle toward the apex of the flower (epitropous) with the raphe on the ventral side of the ovule. This final ovule orientation is best described as semianatropous (Corti, 1954). Soon after the nucellus is distinguishable within the developing ovule, one or more megaspore mother cells,

or megasporocytes, can be seen within the apex of the nucellus (Benson, 1893; Langdon, 1939; Turkel, 1950; Rebuck, 1952; Hjelmqvist, 1953; Corti, 1954, 1955, 1959; Turkel et al., 1955; Scaramuzzi, 1960; Bianco, 1961; Stairs, 1964; Mogensen, 1965; Fey, 1981; Boavida et al., 1999). Only one megaspore mother cell was found in each ovule in *Q. acutissima* (C), and no megaspore mother cells could be identified in the *Q. palmeri* (P) sections probably due to the coarse-grained sampling protocol. Although not directly observed in the current studies, megagametogenesis is of the monosporic, Polygonum-type with a 7-celled, 8-nucleate embryo sac (Hjelmqvist, 1953; Corti, 1959; Bianco, 1961; Stairs, 1964), although several authors have mistakenly described the process as tetrasporic, Adoxa-type (Conrad, 1900; Bagda, 1952; Turkel et al., 1955). At least one ovule could be found with a tri-cellular egg apparatus in both the *Q. acutissima* (C) and *Q. palmeri* (P) studies, and the large central cells were easy to locate in many of the embryo sacs in both studies. The ephemeral antipodals were not detected in the *Q. acutissima* (C) study, but were found in the *Q. palmeri* (P) study apical to the senescing nucellus or hypostase (Benson, 1893; Langdon, 1939; Turkel, 1950; Rebuck, 1952; Hjelmqvist, 1953; Corti, 1954, 1955, 1959; Turkel et al., 1955; Scaramuzzi, 1960; Bianco, 1961; Stairs, 1964; Mogensen, 1965; Fey, 1981; Boavida et al., 1999). A few studies that examined *Q. rubra* (L), *Q. alba* (Q), and other species from section *Cerris* reported tracheids or procambium-like tissue in the hypostase or surrounding tissue (Benson, 1893; Langdon, 1939; Corti, 1954, 1955; Turkel et al., 1955), but this was not observed in the *Q. acutissima* (C) or the *Q. palmeri* (P) studies.

During the maturation of the ovules and embryo sacs, the pollen tubes are in a basal area of the styles that remains parenchymatous during quiescence (~1 month to ~13 months). After megasporogenesis and during megagametogenesis, the pollen tubes resume growth and enter the ovules through the micropyle (Benson, 1893; Kerner Von Marilaun, 1895; Conrad, 1900; Klebelsberg, 1910; Hjelmqvist, 1953; Corti, 1954; Mogensen, 1972; Cecich, 1997; Boavida et al., 1999). Pollen tube growth into the ovules as well as the resulting syngamy was not directly observed in the *Q. acutissima* (C) or the *Q. palmeri* (P) studies, but there was no evidence of ovular disruption that would indicate that the pollen tubes entered the embryo sac other than through the micropyle. For all of the representative species, all of the ovules in the ovary usually have embryo sacs of various developmental stages at the time of syngamy, which sometimes results in simultaneous syngamy of several ovules in each flower, but typically only one ovule matures into a functional seed. The determination of which ovule becomes the seed appears to be competitive, not positional within the ovary, as the embryo sac that matures the fastest usually becomes the seed. The non-surviving ovules show embryo sac disintegration initially, but eventually the inner integument and disrupted embryo sac coagulate together, resulting in each aborted ovule being composed of an outer integument enclosing a densely staining mass. The aborted ovules remain attached to their respective septal remnants, with the final aborted ovule position (apical, lateral or basal) dependent on many factors that will be discussed later in this thesis (Conrad, 1900; Langdon, 1939; Corti, 1954, 1955, 1959; Turkel et al., 1955; Scaramuzzi, 1960; Bianco, 1961; Stairs, 1964; Mogensen, 1965,

1972, 1973, 1975a; b; Brown, 1971; Brown and Mogensen, 1972; Fey, 1981; Boavida et al., 1999).

For the remainder of the discussion, any ovule with a multicellular embryo will be referred to as a seed, with its respective ovary now a fruit. Multicellular embryos were common in the *Q. acutissima* (C) and *Q. palmeri* (P) studies, and are assumed to be the result of syngamy, and not apomixis or parthenogenesis (Johri, 1984). In the embryo sac, the free-nuclear or coenocytic endosperm proliferates before the first division of the zygote and the embryo sac can have vacuoles of various sizes (Hjelmqvist, 1953)., but was not documented in the *Q. acutissima* (C) and *Q. palmeri* (P) studies. Maturing seeds could be identified by the presence of coenocytic endosperm (and usually cellular endosperm) seen in section. Zygotes could not be unequivocally located in any of the sections, although circular structures that could be either zygotes or globular embryos were identified in both studies.

In the *Q. acutissima* (C) study, a concentration of cellular endosperm was observed at the chalazal end of the embryo sac in the more mature fruits and may be haustorial. Adjacent to this chalazal concentration of cellular endosperm is the hypostase, and several authors have discussed the possibility that the hypostase functions as a haustorium for the growing embryo (Benson, 1893; Langdon, 1939; Hjelmqvist, 1953; Corti, 1954; Mogensen, 1973). Although there was not an obvious concentration of cellular endosperm in the base of the seeds in *Q. palmeri* (P), there was always a layer of endosperm (until total assimilation) at least several cells thick basal to the embryo in the seed. Whether the chalazal cellular endosperm or the

hypostase function separately or together as a haustorium is not known at the present time.

In one collection (29 August) of *Q. acutissima* (C) and one collection (2 August) of *Q. palmeri* (P), fruits with almost mature seeds were collected at the same time as a fruit that had a heart-shaped embryo, supporting the observation made by other authors that the level of maturity in fruits from an individual tree can vary widely at any given time (Conrad, 1900; Langdon, 1939; Corti, 1954, 1955, 1959; Turkel et al., 1955; Scaramuzzi, 1960; Bianco, 1961; Stairs, 1964; Mogensen, 1965; Fey, 1981).

A feature within the exocarp that may be of important taxonomic value for understanding relationships among genera in the Fagaceae was first observed in *Q. palmeri* (P) study. After ovary wall differentiation, an interrupted line of small cells containing soft-edged, rhomboidal crystals (Carlquist, 1988) that could also be described as prisms or cubes (Esau, 1977) was present between the exocarp epidermis and palisade layer (Figure 35). In wood anatomy, the position and morphology of crystals have proved to be taxonomically important. Similar crystals have been described in both rays and axial parenchyma of *Quercus* and *Notholithocarpus* wood. These wood crystals are described as "chambered" which means that they are isolated by cell division into smaller cells after formation (Carlquist, 1988). The crystals in *Q. palmeri* were sometimes found within the palisade layer that subtends the epidermis (Figure 35), which may indicate that these ovary/fruit crystals are also chambered. A re-examination of *Q. acutissima* sections prepared using the same methods as the current study (Borgardt and Nixon, 2003) revealed crystals in the same

position, but they were less common and appeared smaller than those in *Q. palmeri*. Re-examination of slides prepared by Mogensen (1965) of *Q. alba* (Q) and *Q. velutina* (L) using paraffin embedding techniques revealed crystals in these taxa as well, but are very hard to see on those slides. The GMA methods used in the *Q. palmeri* (P) and *Q. acutissima* studies emphasized these crystals by allowing light to pass through them and they sparkled as the sections were moved around on the microscope stage. The more opaque appearance of paraffin-embedded material may be why these crystals have not been described before. The occurrence of these rhomboidal crystals is currently unknown in the fruits of *Quercus* subgenus *Cyclobalanopsis*. It would be interesting to see if these crystals are present in the fruits of species in the genus *Notholithocarpus*, which also has this wood crystal anatomical character.

**Potential Synapomorphies within *Quercus* subgenus *Quercus***

To minimize any repetition that would occur by comparing the sections in various groupings, the discussion will be organized within the framework of both historical and/or newly described morphological and anatomical features presented in order from the apex to the base and from the exterior to the interior of the flower/fruit (Table 2). Within each character discussion, unique states will be described first followed by shared states among the sections, with a summary of the states ending the character discussion.

As always, determining the phylogenetic significance of shared and unshared characters and whether they are apomorphies or pleisiomorphies must be made in the

context of a phylogenetic tree or cladogram.

***Syngamy – Annual- and biennial-fruiting patterns***

As mentioned previously in *Q. acutissima* (C) study (Borgardt and Nixon, 2003), *Quercus* acorns mature either the same year the pistillate flowers emerge (annual-fruit maturation) or the next year after the flowers emerge (biennial-fruit maturation). In almost all cases, a species will exhibit only one of these patterns, although section *Cerris* has been reported to contain a few species with populations that contain individuals that exhibit annual-fruit maturation in addition to individuals that exhibit biennial fruit maturation (Corti, 1955; Elena-Rossello et al., 1993).

Section *Cerris* – Contain species that exhibit either annual -or biennial-fruit maturation patterns or both.

Section *Lobatae* – Contain species that exhibit either annual -or biennial-fruit maturation patterns.

Section *Protobalanus* – All species exhibit biennial-fruit maturation only.

Section *Quercus s. s.* – All species exhibit annual-fruit maturation only.

***Syngamy – Delay between pollination and syngamy***

In order to compare the time delay between pollination and syngamy across all four sections, the year of quiescence seen in biennial species will be subtracted. The remaining one month observed time delay can now be compared across most annual-fruit maturation taxa such as *Q. alba* (Q) as well as some biennial-fruit maturation taxa such as *Q. velutina* (L) and *Q. palmeri* (P) (Conrad, 1900; Corti, 1954; Turkel et al., 1955; Stairs, 1964; Mogensen, 1965; Sharp and Sprague, 1967; Cecich, 1997). In the

*Q. acutissima* (C) study, no zygotes or embryos could be located for at least two months after pollination occurred, from 16-23 May to 18 July (Borgardt and Nixon, 2003). A 3–5 months delay in syngamy for section *Cerris* has been reported previously for an annual-fruiting form of *Q. aegilops* (Scaramuzzi, 1960), but it is unknown at this time if a delay in syngamy of longer than one month is typical for this section.

Section *Cerris* – Delay of greater than one month in syngamy reported for one annual-fruiting and one biennial-fruiting species (subtracting one year quiescence).

Section *Lobatae* – Delay of about one month (subtracting one year quiescence).

Section *Protobalanus* – Delay of about one month (subtracting one year quiescence).

Section *Quercus s. s.* – Delay of about one month.

***Primary floral bract***

This character has not been well documented in previous works, so it is difficult to make conclusions based on the following observations: the primary floral bract was ephemeral on *Q. acutissima* (C) and was not found after pollination occurred, but the primary floral bract was persistent on *Q. palmeri* (P), and could be found on the inflorescence after pollination occurred.

Section *Cerris* – Primary floral bract ephemeral

Section *Lobatae* – Unknown.

Section *Protobalanus* – Primary floral bract persistent.

Section *Quercus s. s.* – Unknown.

***Cupule scales***

In *Quercus* subgenus *Quercus,* the cupule scales are formed from scale primordia at apex of the cupule (the cupule scale meristem) in a tight imbricate series in which the bases of the scales are tightly appressed to adjacent scales. Final scale morphology associated with each section are listed below, but in general, each scale is flattened, with the distal portion sclerified into a papery, two-dimensional structure or an awl-shaped spine. The basal portion of the scales either remain appressed to the cupule or become submerged into the cupule (Oersted, 1871; Camus, 1934; Muller, 1942b; Soepadmo, 1968, 1972; Maleev, 1970; Kaul, 1985; Nixon, 1997; Borgardt and Nixon, 2003).

Section *Cerris* – In *Q. acutissima* (C), cupule scales emerge from the cupule meristem in an open imbricate series as three-dimensional, awl-shaped, parenchymatous structures that are separate and distinct from adjacent scales, and mature into a three-dimensional bur-like structure. The cupule scale meristem is active in both the first and second year of flower and fruit development.

Section *Protobalanus* – In *Q. palmeri* (P), cupule scales emerge from the cupule meristem in a tight imbricate series the first year and these flattened scales eventually become spread out or sink into the cupule in the second year (Tucker and Haskell, 1960; Manos, 1993, 1997; Nixon, 1997). The cupule scale meristem is active only in the first year of flower and fruit development. The remaining species in section *Protobalanus* have

hemispherical cupules with tightly abutted imbricate scales (although *Q. chrysolepis* can have submerged scales), but it is unknown at this time whether these species have an active cupule scale meristem in their second year of growth.

Section *Lobatae* – In contrast to the other three sections, the cupule scale morphology typically seen in section *Lobatae* is less robust with flattened scales with paper tips. Some cupules in section *Lobatae* are thickened from ground tissue growth. It is currently unknown whether the cupule meristem in biennial-fruiting species from this section resumes growth in the second year.

Section *Quercus s. s.* – The cupule scale morphology in section *Quercus s. s.* is arguably the most famous for the genus *Quercus* in northern temperate areas. The basally thickened, warty-looking scales are elevated above the ground tissue of the cupule and form a bumpy spiral/imbricate surface that is commonly illustrated in popular literature. This morphology contrasts with the flattened scales of section *Lobatae*, the protruding bur-like scales of section *Cerris*, and the sometimes submerged scales of section *Protobalanus*.

***Styles***

The characters of style length and width in subgenus *Quercus* have historically been important distinguishing among the major groups. Style length can vary from over twice the height of the ovary to styles that are barely elevated above the ovary.

The style width is usually measured at the widest point of the flared end of the styles (stigmatic surface), not the bases of the styles as they emerge from the perianth. Styles from sections *Cerris* and *Lobatae* are characterized as long and thin, while styles from sections *Protobalanus* and *Quercus s. s.* are described as short and broad (Oersted, 1871; Trelease, 1924; Camus, 1934; Reece, 1938; Muller, 1942b; Tillson and Muller, 1942; Soepadmo, 1968; Tucker, 1980; Nixon, 1984, 1993; Kaul, 1985). A morphological feature that has not been emphasized in prior studies and may have phylogenetic implications is an adaxial groove or seam that can be visible at the base of each style. These adaxial grooves are either "closed" with abutted margins at the base of the style and flare out distally to expose the stigmatic surfaces, or these grooves can be "open" the whole length of the style and sometimes present uninterrupted stigmatic surfaces from base to apex of the style (Nixon, 1984).

Section *Cerris* – In *Q. acutissima*, the styles are slender and appear flattened, with adaxial grooves open the whole length of the styles, but the stigmatic surfaces do not merge or form a continuous surface at the center appressed joining of the styles. The styles curve or reflex away from the center of the flower as they senesce. Styles in section *Cerris* are described as long (relative to sections *Protobalanus* and *Quercus s. s.*), and these styles can appear to be cradled within the equally long perianth lobes.

Section *Lobatae* – The styles in this section are dramatically longer than the other sections (in some cases the styles are more than twice as long as the entire flower before pollination occurs), and seem to attenuate below the

stigmatic surfaces at the apex of the styles. The adaxial grooves have abutted margins at the bases of the styles and open up (usually midway up the style) to expose stigmatic surfaces that continue up to the apices of the styles. As these styles senesce, they often twist, reflex, or become curved, and remain a prominent feature at the apex of the nut.

Section *Protobalanus* – *Q. palmeri* (P) styles are stouter and shorter than those seen in section *Cerris* and section *Lobatae*, with the margins of the adaxial grooves abutted at the base of the styles, and the styles do not twist or reflex as they senesce. A review of other species in section *Protobalanus* reveals a wide continuum of morphology with some species having squat and wide styles (as seen in section *Quercus s. s.)*, and others develop styles that are longer like *Q. palmeri* (P). Styles seen in section *Protobalanus* are often coded as similar to section *Quercus s. s.* in morphological analyses, but the combination of longer styles and abutted margins of the adaxial grooves that limit the stigmatic surfaces to the apex of the styles (not seen in *Quercus s. s.*) demonstrate that this characterization could be too simplistic and may need to be revisited.

Section *Quercus s. s.* – Styles do not lengthen appreciably in *Q. alba* (Q) and flare out to form wide, spreading apices that can be oriented in an almost horizontal plane, with open adaxial grooves the whole length of the styles. These styles do not reflex or twist as they senesce.

***Stigmatic surfaces***

As mentioned previously, the stigmatic surfaces in genus *Quercus* are found on the style apices and along adaxial grooves (when open) on the styles and consist of the nonpapillate, "dry" type with a pellicle (Heslop-Harrison, 1971). The metachromatic Toluidine Blue O stain, used in the *Q. acutissima* (C) and *Q. palmeri* (P) studies, confirmed that all surfaces exposed by open margins of the adaxial grooves are stigmatic.

Section *Cerris* – The stigmatic surfaces of *Q. acutissima* (C) cover the spreading surfaces at the apices of the styles and along open adaxial grooves almost to where the styles are appressed.

Section *Lobatae* – The stigmatic surfaces in section *Lobatae* are concentrated at the apices of their very long styles, but also cover the open areas of the adaxial grooves.

Section *Protobalanus* – In *Q. palmeri* (P), the stigmatic surfaces are restricted to the apex of the styles. The adaxial grooves have abutted margins and are closed for most of the style length. This morphology is more similar to the capitate stigmas of subgenus *Cyclobalanopsis*, and needs to be explored more thoroughly.

Section *Quercus s. s.* – The stigmatic surfaces of *Q. alba* (Q) cover the widely spreading apices of the styles and open adaxial grooves. The stigmatic surface often appears uninterrupted over the top surface of of the styles.

***Perianth***

The perianth has been treated in much the same way as the styles, with sections *Cerris* and *Lobatae* described as long and slender, and sections *Protobalanus* and *Quercus* described as short and broad. The perianth can form a flange and interlock with cupule scales during pistillate flower quiescence (Oersted, 1871; Trelease, 1924; Camus, 1934; Muller, 1942b; Soepadmo, 1968; Tucker, 1980; Nixon, 1984, 1993; Kaul, 1985).

Section *Cerris* – The pistillate flowers in *Q. acutissima* (C) have perianth lobes that are longer than they are wide and can be mistaken for styles in longitudinal anatomical sections. The tips of the perianth lobes can extend higher than the apices of the styles (when viewed from the side). No flange is formed.

Section *Lobatae* – *Q. velutina* (L) has perianth lobes that are longer than they are wide, but the lobes are usually not higher than the styles. The base of the perianth forms an obvious and thick flange that traps some of the apical cupule scales between the perianth and the style column as well as beneath the perianth during pistillate flower quiescence.

Section *Protobalanus* – The perianth in *Q. palmeri* (P) has tepals as long as they are wide. The tips of the perianth lobes do not extend higher than the apices of the styles. A shallow flange traps cupule scales beneath the perianth lobes but not between the style column and perianth during pistillate flower quiescence.

Section *Quercus s. s.* – The perianth in *Q. alba* (Q) has tepals as long as they are wide. The tips of the perianth lobes do not extend higher than the apices of the styles. No flange.

***Staminodia***

The presence of well-developed staminodia in pistillate flowers in genus *Lithocarpus* (which also produces fruits described as acorns) has been used in Malesian floras to distinguish between the two genera, *Quercus* and *Lithocarpus*, where they co-occur (Soepadmo, 1972). Although staminodia have been described in some species of *Quercus* subgenus *Cyclobalanopsis* in southeastern Asia and Malesia, their presence in *Quercus* subgenus *Quercus* has been considered inconsistent and an exception (Oersted, 1871; Camus, 1934; Tillson and Muller, 1942; Soepadmo, 1972; Nixon, 1984, 1993; Kaul, 1985, 1986). The discovery of consistent staminodia in pistillate flowers from species in section *Protobalanus* does not resolve any relationships within *Quercus* subgenus *Quercus* at this time as it is currently unique within this group of taxa, but it could prove useful at the genus level and also for resolving other closely related taxa in the Fagaceae.

Section *Cerris* – No staminodia.

Section *Lobatae* – No staminodia.

Section *Protobalanus* – Staminodia consistently present in *Q. palmeri*, cursory examination of other species in this section reveals presence of staminodia as well.

Section *Quercus s. s.* – No staminodia.

***Exocarp***

As mentioned in the *Q. acutissima* study (Borgardt and Nixon, 2003), distinguishing between the different layers in the fruit wall can be arbitrary, particularly in the early stages of ovary development, but for discussion purposes, the mature fruit layers as defined by Harz (1885) and Soepadmo (1968) for the genus *Quercus* are used. The exocarp is composed of the epidermis, which is one cell thick for all sections in *Quercus* subgenus *Quercus*, and a palisade layer which can be several to many cells layers thick and is composed of cells oriented perpendicularly to the external surface of the ovary/fruit.

Section *Cerris* – The palisade layer in *Q. acutissima* (C) is a few cell layers thick, and at maturity is thin relative to the other sections.

Section *Lobatae* – The extremely thick and highly sclerified palisade layer of *Q. velutina* (L) is massive compared to the other sections, and becomes the primary component of the fruit wall at maturity.

Section *Protobalanus* – The palisade layer in *Q. palmeri* (P) is thicker relative to sections *Cerris* and *Quercus s. s.*, but not as massive as in section *Lobatae*.

Section *Quercus s. s.* – The palisade layer in *Q. alba* (Q) is thin, and not a major component of the fruit wall at maturity.

***Mesocarp***

The mesocarp is mostly parenchymatous during ovary/fruit development until it becomes compressed at maturity, and contains scattered sclereid clusters and vascular tissue.

Section *Cerris* – The mesocarp in *Q. acutissima* (C) has scattered groups of sclereids, is thin (relative to the very thick mesocarp in section *Quercus s. s.*), and is one of the two main components of the mature pericarp at maturity.

Section *Lobatae* – The mesocarp in *Q. velutina* (L) has scattered groups of sclereids, is thin, and is not the main component of the mature pericarp at maturity.

Section *Protobalanus* – The mesocarp in *Q. palmeri* (P) has scattered groups of sclereids, as well as cell tiers oriented parallel and just below the exocarp palisade layer, and forming a "secondary" palisade layer, and is one of the two main components of the pericarp at maturity.

Section *Quercus s. s.* – The main component of the pericarp at maturity, the mesocarp in *Q. alba* (Q) is crowded with dense clusters of sclereids forming a thick layer that appears glistening and mealy when the fruit wall is torn open.

***Endocarp***

The absence or presence of a pubescent endocarp, historically cited as a useful character in differentiation among the sections of subgenus *Quercus*, can be problematic unless applied consistently. For example, when the fruit of a species in section *Quercus s. s.* is opened, the seed coat often detaches from the seed, adhering to the fruit wall, and the inside of the seed coat is then mistaken for the endocarp (Nixon, 1984, 1997). In some species of section *Lobatae*, the septa become woody as the fruit

matures and eventually intrude into the seed as it expands to fill the fruit.

Section *Cerris* – The endocarp in *Q. acutissima* (C) is pubescent, with early lignification of trichomes observed (before megasporogenesis).

Section *Lobatae* – Extremely pubescent, the endocarp can intrude into the seed. Trichome lignification begins after syngamy.

Section *Protobalanus* – Pubescent, sometimes extremely pubescent, the endocarp is lined with trichomes that begin to lignify after syngamy.

Section *Quercus s. s.* – The true endocarp in *Q. alba* (Q) is mostly glabrous at maturity, although patches of short and sparse pubescence can be found. Trichome lignification begins after syngamy.

***Pericarp***

The exocarp and mesocarp of all four representative species can be compared as separate layers during ovary and fruit wall differentiation, but at fruit maturity the two layers are compressed together to form the "shell" of the nut (Soepadmo,1968; Mogensen, 1965). In some cases, only one of these pericarp layers is visible at maturity, while in others, both layers are compressed more or less equally and appear to both contribute to the final fruit wall. The innermost layer, the endocarp, does not contribute to the thickness or hardness of the pericarp in *Quercus* subgenus *Quercus*, but it can influence the final shape of the seed. Differing patterns in maturation results in three different fruit wall compositions across the four species: the exocarp in *Q. velutina* (L), the mesocarp in *Q. alba* (Q), and a combination of both layers in *Q. acutissima* (C) and *Q. palmeri* (P).

Section *Cerris* – Mature pericarp is composed of both the exocarp palisade layer and mesocarp compressed together form the mature "shell".

Section *Lobatae* – The pericarp at maturity in *Q. velutina* (L) is composed almost entirely of the exocarp palisade layer. The enlargement and sclerification of cells in this thick outer layer begins very early in the ovary wall differentiation, and continues until fruit maturation.

Section *Protobalanus* – Mature pericarp is composed of both the exocarp palisade layer and mesocarp compressed together to form the mature "shell".

Section *Quercus s. s.* – In section *Quercus s. s*., the mature pericarp is composed primarily of the mesocarp that has packed clusters of sclereids forming a "mealy" texture.

***Locules***

One developmental pattern found in the present study that may be taxonomically useful among the sections of subgenus *Quercus* is whether the locules are filled by the ovules/seed during most of the flower/fruit development or the locules are mostly empty until the seed expands to fill the space at the end of fruit maturation (Conrad, 1900; Corti, 1954; Turkel et al., 1955; Mogensen, 1965; Borgardt and Nixon, 2003).

Section *Cerris* – In *Q. acutissima* (C), the ovules occupy most of the volume in the locules except near the time of megasporogenesis when the ovary expands faster than the ovules.

Section *Lobatae* – The ovules in *Q. velutina* (L) occupy less than half of the

volume of the locules until late in maturity when the seed finally fills the fruit.

Section *Protobalanus* – The ovules in *Q. palmeri* (P) occupy less than half of the volume of the locules until late in maturity when the seed finally fills the fruit.

Section *Quercus s. s.* – In *Q. alba* (Q), the ovules occupy most of the volume in the locules except near the time of megasporogenesis when the ovary expands faster than the ovules.

***Septal remnants***

The septa, which originally delimited the locules in the ovary, are displaced to the sides of the fruit as the embryo expands and can be flattened against the inner fruit wall or remain intruded into the interior of the fruit. The septal remnants can be present from the apex to the base of the seed at maturity, or they can be present only at the base of the seed. Sclerification at the base of the septa can result in a woody post-like structure between the fruit and seed at the base of the fruit that has been described as a "columella" (Nixon 1984, 1993, 1997).

Section *Cerris* – In *Q. acutissima* (C), the septal remnants can be found from the apex to the base of the fruit as thin, papery structures.

Section *Lobatae* – In *Q. velutina* (L), the septal remnants are a significant feature in the fruit in which they become woody and form ridges extending from the apex to the base of the fruit. As the seed expands to fill the locule of the fruit, these ridges can become pressed into the seed.

Section *Protobalanus* – In *Q. palmeri* (P), the septal remnants can be found from the apex to the base of the fruit as thin, papery structures. Woody post-like structure sometimes found in base of fruit.

Section *Quercus s. s.* – The septal remnants are almost indistinguishable at the base of the fruit with the basal aborted ovules in *Q. alba* (Q).

***Aborted ovules***

Two ovules are formed in each carpel for all species in *Quercus*, but only one survives to form the seed. The other ovules abort, and developmental changes in the ovary/fruit (primarily the septa) and the seed (if the septal remnants fuse to the seed coat), determine where these aborted ovules are positioned at maturity. As mentioned previously, all other taxa in the family Fagaceae develop apical aborted ovules, but some species in *Quercus* subgenus *Quercus* exhibit non-apical aborted ovules that are referred to as basal or lateral. Basal aborted ovules are located in the fruit either below the seed or at the base of the fruit on the side of the seed. Lateral aborted ovules are found only in the lower half of the fruit, on the side of the seed and are never below the seed (Conrad, 1900; Langdon, 1939; Corti, 1954, 1955, 1959; Turkel et al., 1955; Scaramuzzi, 1960; Bianco, 1961; Stairs, 1964; Mogensen, 1965, 1972, 1973, 1975a; b; Brown, 1971; Brown and Mogensen, 1972; Fey, 1981; Boavida et al., 1999). It must be stressed that identifying the character state for this position can only be made at fruit maturity, as positional changes within the fruit during the final stages of maturation can change orientation of structures within the fruit dramatically.

Section *Cerris* – Aborted ovules in *Q. acutissima* (C) study were found basal in

the fruit, on the side or below the seed in the mature fruit and usually clustered together.

Section *Lobatae* – For *Q. velutina* (L), aborted ovules are apical and can be clustered together or spread out in pairs along the top of the seed. Most of the species in this section develop apical aborted ovules, but a few develop lateral aborted ovules and others have been described with basal aborted ovules.

Section *Protobalanus* – The *Q. palmeri* (P) study had two main configurations: all of the aborted ovules clustered together and attached to a sclerified post-like compound structure at the base and side of the seed, or spread out along a short distance on the seed coat still attached to the septa remnants and just apical to the sclerified compound structure.

Section *Quercus s. s.* – The aborted ovules in *Q. alba* (Q) are basal, are found completely beneath the seed, and clustered together.

***Connection of seed to fruit***

The surviving ovule that matures into the seed is attached to the fruit wall by a compound structure [the "umbilical complex" of Borgardt and Pigg (1999); the "columella" of Nixon (1984, 1993, 1997)] that is composed of the ovule placenta, the funiculus, and part of a septal remnant or remnants. Both the *Q. acutissima* (C) and the *Q. palmeri* (P) studies show that the seed attachment to the fruit wall is correlated with the location of the aborted ovules. Although the location of seed attachments for *Q. velutina* (L) and *Q. alba* (Q) were not documented in the Mogensen works, personal

observation of several related taxa confirm that this is the case for these taxa as well.

Section *Cerris* – In *Q. acutissima* (C), the compound structure connecting the seed to the fruit is at the base of the fruit, either underneath the seed or on the side.

Section *Lobatae* – In *Q. velutina* (L), the structure that attaches the seed to the fruit is presumed to be apical.

Section *Protobalanus* – In *Q. palmeri* (P), the compound structure connecting the seed to the fruit is at the base of the fruit.

Section *Quercus s. s.* – In *Q. alba* (Q), the compound structure that attaches the seed to the fruit is basal in the fruit, and usually found underneath the seed.

***Seed coat***

As mentioned previously in the endocarp discussion, some species in section *Quercus s. s.* appear to have a glabrous endocarp when the seed is removed from the fruit. But closer examination of the seed and fruit reveals that the seed coat is missing from the seed and adhered to the endocarp, so the "inside" of the fruit is actually the inside of the seed coat (Oersted, 1871; Trelease, 1924; Camus, 1934–1954; Muller, 1942b; Soepadmo, 1968; Tucker, 1980; Nixon, 1984; Kaul, 1985; Nixon, 1993). This unusual feature has not been well documented in taxa outside of *Quercus* subgenus *Quercus*, and needs more sampling.

Section *Cerris* – At maturity, the seed coat is adherent to the embryo.

Section *Lobatae* – At maturity, the seed coat is adherent to the embryo.

Section *Protobalanus* – At maturity, the seed coat is adherent to the embryo.

Section *Quercus s. s.* – At maturity, the seed coat is adherent to the endocarp.

***Embryo***

The embryo at fruit maturity can have a naked shoot apex, buttresses on the sides of the shoot apex, or have several leaf primordia formed on the shoot apex (Mogensen, 1965; Sutton, 1969; Sutton and Mogensen, 1970; Borgardt and Nixon, 2003).

Section *Cerris* – *Q. acutissima* (C) had buttresses on the embryo shoot apex in the most mature acorns, but is unknown at this time if the embryo shoot apex forms leaf or scale primordia before falling the tree.

Section *Lobatae* – In *Q. velutina* (L), the shoot apex was naked at maturity.

Section *Protobalanus* – The most mature acorn found in the *Q. palmeri* (P) study had an embryo with a naked shoot apex in the seed.

Section *Quercus s. s.* – *Q. alba* (Q) formed several leaf or scale primordia on the shoot apex before the seed completed maturation.

## CONCLUSIONS

This study both confirms previously described morphological features and also reveals new morphological and anatomical features for *Q. palmeri* and for section *Protobalanus*. Adding these discoveries to previously published work that compared sections within *Quercus* subgenus *Quercus* (Borgardt and Nixon, 2003) emphasizes the mosaic of morphological and anatomical characteristics among the four sections of *Quercus* subgenus *Quercus*. Table 2 summarizes these morphological and anatomical features for the example species of the sections, with some variability within the

sections noted in parentheses. It is interesting to note that although there are many features that unite *Q. palmeri* and section *Protobalanus* singly with the other sections, there are few that unite *Q. palmeri* with two sections at the same time. Features such as fruit maturation pattern, endocarp pubescence, and septal remnants unite section *Protobalanus* with sections *Cerris*/*Lobatae.* Palisade layer thickness unites section *Protobalanus* with sections *Cerris*/*Quercus s. s*., and time in syngamy delay unite section *Protobalanus* with sections *Lobatae*/*Quercus s. s*. There are also features, such as stigmatic surface morphology, presence of staminodia, and pericarp structures, that appear to be autapomorphic for section *Protobalanus* and do not provide any grouping information among the four sections.

The lateral aborted ovules that are found in *Q. palmeri* and section *Protobalanus* seem to add a third character state to the apical and basal abortive ovules found throughout subgenus *Quercus*, but this study and the previously published *Q. acutissima* (C) study (Borgardt and Nixon, 1993) demonstrate that the "lateral" position is the result of the same processes that form basal abortive ovules. An early sclerification/vascularization of the lower portion of the septal remnants during seed/fruit development results in a shift of the attachment of the seed and aborted ovules to a basal position in the fruit. In the *Q. acutissima* (C) study with basal abortive ovules, the bases of the septa became heavily vascularized and/or sclerified during megagametogenesis, while in the *Q. palmeri* (P) study with lateral abortive ovules, this vascularization occurred after syngamy. Although not recorded in the *Q. alba* (Q) study, the basal abortive ovules found in this section would seem to

indicate that sclerification/vascularization of the bases of the septa occurs very early (or even earlier) as seen in *Q. acutissima* (C). For species in section *Lobatae* that have basal aborted ovules, I predict that similar processes are occurring during maturation to result in a shift of ovules to the base of the fruit. For comparative purposes, sampling of species with apical abortive ovules would be needed to confirm this trend (early vascularization shifts aborted ovules to the base of the fruit) and ascertain whether the apical abortive ovule position is the result of septal vascularization at the base of the seed occurring after syngamy or it not occurring at all.

It is not clear at this time as to whether this innovation that results in a sequestering of aborted ovules and other sclerified structures outside the seed at the base of the fruit originated once in *Quercus* or multiple times, but it is interesting to note that this has remained a feature in all sections of *Quercus* subgenus *Quercus* to the present day. Early vascularization at the base of the septa not only clusters the seed and aborting ovules at the base of the fruit, it also results in the fruit-seed connection being basal. Since some oaks produce very large seeds, a short and thick conduit between the seed and the fruit to maximize nutrient flow could be an explanation for this trend to shift everything basally, but other taxa in the Fagaceae also produce large fruits/seeds and have apical aborted ovules with no basal fruit-seed connection.

Further comparative flower and fruit anatomical and developmental work on other groups within the genus *Quercus*, especially subgenus *Cyclobalanopsis* and the oaks of subgenus *Quercus* section *Lobatae* that have basal aborted ovules, as well as related genera in the family Fagaceae should provide more resolution on the

significance of these interesting features as well as a broader framework in which to pinpoint where this innovation and shift in fruit developmental patterns may have originated.

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