SEED DEVELOPMENT IN TRIMENIA (TRIMENIACEAE) AND ITS BEARING ON THE EVOLUTION OF EMBRYO-NOURISHING STRATEGIES IN EARLY FLOWERING PLANT LINEAGES

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After the discovery (Mathews and Donoghue, 1999; Parkinson et al., 1999; Qiu et al., 1999; Soltis et al., 1999) that Amborellaceae, Nymphaeales (Cabombaceae, Nymphaeaceae), and Austrobaileyales (Austrobaileyaceae, Trimeniaceae, Illiciaceae, and Schisandraceae) were three of the four most ancient lineages of flowering plants (the other ancient clade being all other angiosperms, Fig. 1), the focus on reconstructing early angiosperm evolutionary history shifted abruptly to these understudied lineages. The additional phylogenetic insight that the aquatic family Hydatellaceae was not situated in the Poales, but was a member of the Nymphaeales (Saarela et al., 2007), only heightened interest in understanding patterns of biogeographical diversification among the earliest diverging clades of angiosperms (see Friedman et al., 2012, and references therein). It is not an overstatement to claim that the last fifteen years of work on Amborella and members of the Nymphaeales and Austrobaileyales has led to the near-global collapse of a century–old set of paradigms concerning the reproductive features of the earliest angiosperms (Floyd and Friedman, 2000, 2001; Williams and Friedman, 2002, 2004; Friedman and Williams, 2003, 2004; Friedman et al., 2003, 2008; Rudall, 2006; Friedman, 2006, 2008; Tobe et al., 2007; Rudall et al., 2008, 2009; Williams, 2008; Friedman and Ryerson, 2009; see also Friedman et al., 2012).

Yet, for all of the recent progress in characterizing the basic biological features of early divergent lineages of flowering plants, botanists still continue to puzzle over the key evolutionary transition from seed plants that nourish their embryos with haploid female gametophyte tissue (gymnosperms) to seed plants that typically nourish their progeny with a sexually formed endosperm tissue (angiosperms). We now know that in contrast with Amborella and most other flowering plants, which have triploid endosperms, the common ancestor of flowering plants is likely to have had a four-celled/four-nucleate female gametophyte that yielded a diploid genetically biparental endosperm, as is the case in all extant Nymphaeales and Austrobaileyales (Friedman and Ryerson, 2009).

In Nymphaeales, the diploid endosperm is minute and almost entirely devoid of reserves, and the main nutrient-storing and embryo-nourishing tissue in the seed is a copious starchy perisperm that is derived from the nucellus (see Friedman et al., 2012 and references therein). In contrast, in most Austrobaileyales, the diploid endosperm is typically described as being substantial, serving as the primary nutrient-storing and embryo-nourishing tissue (Austrobaileya, Endress, 1980; Yamada et al., 2003; Illicium, Hayashi, 1963a; Floyd and Friedman, 2000, 2001; Kadsura, Hayashi, 1963b; Schisandra, Hayashi, 1963b; Kapil and Jalan, 1964). Interestingly, previous studies of seeds of

1 Manuscript received 11 December 2012; revision accepted 28 February 2013.
2 The authors thank J. Bruhl for his help with the collection of plant material, Peter K. Endress for providing plant material, and A. B. Demarest, T. Eldridge, S. Holloway, T. S. McGillivray, R. A. Povilus, and E. I. Scherbitsky for help with some of the histology. We also thank two anonymous reviewers for their comments. This research was funded by a NSF grant (IOS–0919986) to W.E.F.
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doi:10.3732/ajb.1200632
extinct and extant *Trimenia* suggest that, as in Nymphaeales, members of this clade produce a perisperm along with a small endosperm (Prakash, 1998; Yamada et al., 2003, 2008). The combinations of different embryo-nourishing tissues (endosperm, perisperm) and levels of endosperm ploidy (diploid or triploid) among members of the Amborellales, Nymphaeales, and Austrobaileyales strongly suggest that the embryo-nourishing strategies of early flowering plants were likely much more diverse and evolutionarily labile than has traditionally been assumed.

With this in mind, we have been exploring the verity of the long-held view that the common ancestor of angiosperms exclusively depended upon a nutrient-storing endosperm to nourish the developing embryo within the seed and that the perisperm of Nymphaeales is apomorphic. As we have recently shown (Friedman et al., 2012), the hypothesis that a perisperm was primarily responsible for nutrient storage and nourishing the embryo in the first flowering plants is a plausible (although not most parsimonious) evolutionary scenario. As such, the perispermous seeds of Nymphaeales might represent this plesiomorphic condition, and endosperm would thus have gradually acquired its dominant nutrient-storing and embryo-nourishing behaviors after the earliest phases of angiosperm diversification (Friedman et al., 2012).

Reconstruction of the developmental characteristics of ovules/seeds of the members of the Austrobaileyales, as well as the common ancestor of Austrobaileyales, is central to the further...
evolutionary assessment of the plesiomorphic condition for embryo–nourishing behavior in the earliest angiosperms. Regrettably, the evidence previously presented for the presence of a perisperm in Trimeniaceae does not appear to be definitive. Yet, proper assessment of this key seed biological feature is critical to inferring the evolutionary history of embryo-nourishing strategies during the early diversification of angiosperms. In this paper, we re-evaluate the embryology and seed development of *Trimenia moorei* (Oliv.) Philipson and *T. neocaledonica* Baker f. to confirm or refute the previously reported presence of a perisperm in Trimeniaceae.

**MATERIALS AND METHODS**

**Material collection and fixation**—Floral buds, flowers, and fruits of *Trimenia moorei* were collected in September 2009 in the field from various locations in New South Wales, Australia, by W. E. Friedman, I. R. H. Telford, T. Eldridge, and J. J. Bruhl (see Appendix S1 in Supplemental Data with the online version of this article). Additional fruits were collected by J. J. Bruhl from October through December 2009 and January 2010 (see Appendix S1 in Supplemental Data with the online version of this article). Fruits of *T. neocaledonica* were collected in the field by P. K. Endress in 1981 in New Caledonia (see Appendix S1). All plant material collected in Australia (*T. moorei*) was fixed for 24 hours in 4% glutaraldehyde in a modified PIPES buffer adjusted to pH 6.8 (50mM PIPES and 1mM MgSO_4_ (BDH, London, UK); 5mM EGTA (Research Organics, Cleveland, Ohio, USA)) or FAA (10% formaldehyde (37%), 5% glacial acetic acid, and 50% ethyl alcohol). Fruits collected in New Caledonia (*T. neocaledonica*) were fixed in FAA. Material fixed in glutaraldehyde was rinsed a few times with the same modified PIPES buffer, dehydrated through a graded ethanol series and stored in 70% ethanol. Material fixed in FAA was rinsed a few times with 50% ethanol and stored in 70% ethanol. All spirit collections are deposited at the Weld Hill research facility at the Arnold Arboretum of Harvard University.

**Light microscopy**—Floral buds and flowers were dissected to collect carpels. Carpels and seeds from later stages of fruit development were dehydrated through another ethanol series up to 100%, then infiltrated and embedded with glycol methacrylate (JB–4 embedding kit [Polysciences, Warrington, Pennsylvania, USA]). Embedded materials were mounted and sectioned serially into 4μm–thick ribbons using a Microm HM360 rotary microtome (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with glass knives. Serial sections were mounted onto slides and stained using a periodic acid–Schiff’s reagent (PAS) to detect all insoluble carbohydrates including starch grains (Schiff’s reagent from Fischer Scientific, Pittsburgh, Pennsylvania, USA), 1% aniline blue-black (ABB, also called amido black; Harleco, Clearwater, Florida, USA) in 7% acetic acid (BDH, London, UK) to detect proteins, and 0.01% Auramine–O (Allied Chemical, New York, New York, USA) in 0.05M Tris (Mallinckrodt Chemicals, Phillipsburg, New Jersey, USA) / HCl buffer adjusted to pH 7.2 to detect lipids. Alternatively, 0.1% aqueous toluidine blue O (J. T. Baker, Philipsburg, New Jersey, USA) was used as a nonspecific dye, or as a counterstain after PAS.

**Digital imaging**—A Zeiss Axio Imager Z2 microscope equipped with a Zeiss High Resolution AxioCam digital camera (Carl Zeiss, Oberkochen, Germany) was used for bright field, differential interference contrast (DIC), and
only be observed prior to application of these stains or after
staining protocols that do not involve acids (e.g., toluidine blue)
(Fig. 3B).

Early seed development in Trimenia moorei—Pollen tube
discharge was observed in the synergids of several mature fe-
male gametophytes, but double fertilization of the haploid egg
cell and haploid central cell (syngamy) was not captured in any
of the histological preparations, as is usually the case. After
fertilization, a few additional small starch grains are formed in
the epidermal cells of the nucellus, in the zygote, and around
the diploid primary endosperm nucleus. The endosperm ex-
tends for the entire length of the nucellus but the first divisions
were not observed at the chalazal end. Successive transverse
cell divisions in the large micropylar chamber create a uniseri-
te endosperm (Fig. 4A, see also Fig. 8C).

Further cell divisions of the endosperm around the undivided
zygote yield smaller and more densely cytoplasmic cells (Fig. 4B,
see also Fig. 8D). Only after the completion of this first phase
of cellular endosperm development does the zygote undergo its
first mitotic and cytokinetic division (Fig. 5). With the excep-
tion of the very apex and epidermis, most of the nucellus re-
 mains devoid of stored nutrients (Fig. 5B, C). During this period
of early endosperm development, raphides continue to accumulate
in nucellar cells.

Seed maturation in Trimenia moorei and T. neocaledonica—
Initial development of the embryo proceeds at a much slower
rate than the endosperm (Figs. 5, 6). By the time the embryo is
globular, the endosperm has begun to expand radially and the
immediately surrounding cells of the nucellus are beginning to
Nutrient storage in mature seeds of *Trimenia moorei*—Large starch grains are found in the cells of the embryo suspensor, the protoderm, and the cotyledon primordia (Fig. 7C). The endosperm contains small starch grains at its periphery and larger grains in the cells at the center of the tissue (in line with the axis of the embryo). In addition to starch, the endosperm contains significant quantities of protein bodies and lipid droplets (Fig. 7A, D, E). Clusters of raphides (from nucellus cells that have been crushed during the expansion of the endosperm) are present between the endosperm and the persistent layer of nucellus tissue (Fig. 7F). The persistent layer of the nucellus, however, remains devoid of stored nutrients at seed maturity/dormancy (Fig. 7D, E). Thus, there is no evidence for a perisperm in *T. moorei*. Although we were unable to obtain mature seeds of *T. neocaledonica*, our developmental analysis indicates there is no evidence for the sequestration of embryo-nourishing reserves in the nucellus in this species either.

**DISCUSSION**

**Seed development and structure in *Trimenia moorei***—In *T. moorei*, previous studies reported that the majority of the tissue charged with storing and contributing nutrients to the embryo in mature seeds was the nucellus, functioning as a perisperm (Prakash, 1998; Yamada et al., 2003). In addition, these reports...
Thus, the findings of Prakash (1998) and Yamada et al. (2003, 2008), who concluded that the majority of the embryo-nourishing tissue within the mature seeds of Trimenia moorei is a perisperm (derived from the nucellus), cannot be sustained. To some extent, nutrients are stored in the small but well differentiated dicotyledonous embryo itself, but by the time of seed dormancy, the main nutrient-storing and embryo-nourishing tissue in seeds of Trimenia moorei is a genetically biparental diploid endosperm.

Our re-evaluation of the development of seeds in Trimenia moorei clearly shows that the previous report of a vermiform endosperm extending for the entire length of the nucellus, but occupying only a small central portion of the seed, was probably based on an immature stage of seed development (compare Figs. 4 and 5A in Prakash, 1998 with Fig. 8C, D in this study). Our developmental work on Trimenia also shows that while the nucellus is substantial and contains significant amounts of starch prior to fertilization, it does not play any role in nutrient storage and embryo-nourishing during seed development. Rather, the nucellus remains largely devoid of stored nutrients from the time of fertilization through seed maturation and is almost entirely obliterated by the expansion of the endosperm.

Thus, the findings of Prakash (1998) and Yamada et al. (2003, 2008), who concluded that the majority of the embryo-nourishing tissue within the mature seeds of Trimenia moorei is a perisperm (derived from the nucellus), cannot be sustained. To some extent, nutrients are stored in the small but well differentiated dicotyledonous embryo itself, but by the time of seed dormancy, the main nutrient-storing and embryo-nourishing tissue in seeds of T. moorei is a genetically biparental diploid endosperm.

**Endosperm development in Trimenia and Austrobaileyales**—

In Trimenia, endosperm is *ab initio* cellular (Prakash, 1998). This pattern of endosperm development appears to be general to all other members of the Austrobaileyales, including Austrobaileyax (Endress, 1980), Illicium (Hayashi, 1963a; Floyd and Friedman, 2000, 2001), Kadsura (Hayashi, 1963b), and Schisandra (Hayashi, 1963b; Kapil and Jalan, 1964). Endosperm development begins with a division of the primary endosperm cell that yields two large cells in *T. moorei* (Prakash, 1998), and a large micropylar and a smaller chalazal cell in *Illicium* (Floyd

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Fig. 6. Immature fruits and seeds of *Trimenia neocaledonica* with large multiseriate endosperms and multicellular embryos. Median longitudinal sections of younger (A, B) and older stages (C, D, E) stained with toluidine blue. Large arrowheads point to endosperm. (A) Endosperm is multiseriate, but has only just begun to expand radially. (B) Higher magnification view of tissue within the red box in (A) showing the embryo and immediately surrounding endosperm. (C) Endosperm has continued to expand radially. Black boxes indicate digital insets from adjacent histological sections to show the full longitudinal extent of the endosperm. (D) Higher magnification view of tissue within the red box in (C) showing a cluster of raphides in the nucellus. (E) Late stage of endosperm development with multicellular embryo. At this point, inner portions of the nucellus have been crushed by the radial expansion of the endosperm. Abbreviations: ch, chalaza; emb, embryo; end, endosperm; fun, funicle; ii, inner integument; nuc, nucellus; oi, outer integument; r, raphide. Bars = 500 μm (A, C), 100 μm (E), 20 μm (B, D).
present in the mature endosperm of *Trimenia moorei*. Although most of the earlier studies of seed development in members of the Austrobaileyales did not undertake extensive histological analyses of endosperm contents, in *Illicium*, the endosperm contains proteins and lipids, but starch is reported to be absent (Floyd and Friedman, 2001). In *Schisandra*, both starch and oils are present in the mature endosperm but it is unknown whether proteins are accumulated (Kapil and Jalan, 1964). In *Austrobaileya*, starch has been reported in the large ruminate endosperm, although tests for lipids and proteins were not performed (Endress, 1980). Thus, there may be a modest amount of variation in the nature and relative proportion of the different types of storage compounds among genera of Austrobaileyales. Given the different functional and nutritional roles of starch, proteins, and lipids in seeds (Kitajima and Myers, 2008; Soriano et al., 2011), it would be interesting to examine how the different proportions of these storage reserves in the endosperms of the diverse members of the Austrobaileyales might correlate with patterns of seedling germination and establishment.
Modest amounts of starch are also accumulated in the nucellus of some Calycanthaceae (Laurales; Mathur, 1968), Annonaceae (Magnoliales; Lora et al., 2010), and Piperaceae (Piperales; Lei et al., 2002) prior to fertilization. In Hydatellaceae and Piperaceae, the nucellus persists after fertilization and differentiates into the main nutrient storage and embryo-nourishing tissue (perisperm) in the seed. In Calycanthaceae (Mathur, 1968) and Annonaceae (Lora et al., 2010), the nucellus does not accumulate significant reserves after fertilization and the endosperm is the primary embryo-nourishing tissue within the seed. For now, the developmental and evolutionary history of storage compounds in nucellar tissues during the early diversification of angiosperms remains opaque.

Conclusions —

More than a century after the discovery that endosperm is formed as a consequence of a second fertilization event in angiosperms, the evolutionary and developmental transition from seeds with a maternally derived haploid nutrient-storing and embryo-nourishing tissue (the female gametophyte of gymnosperms) to seeds with a biparental full-fl edged endosperm (most angiosperms) remains poorly understood. Among early angiosperm lineages, endosperm provisions for...
and nourishes the embryo in Amborella and members of the Austrobaileyales. In the case of Amborella, the endosperm is triploid, while in Austrobaileyales, it is diploid. As first suggested by Williams and Friedman (2002), current evidence points to a diploid condition for endosperm as being plesiomorphic for angiosperms as a whole (see Friedman et al., 2012).

In Nymphaeales, however, the diploid endosperm is minute, and the entirety of nutrient storage and embryo-nourishing within the seed is associated with the nucellus and its formation into a perisperm. Previous reports of a minute endosperm coupled with a perisperm in the seeds of Trimenia moorei (Prakash, 1998; Yamada et al., 2003, 2008) raised the possibility that the common ancestors of the Nymphaeales and Austrobaileyales could both have used a perisperm as the primary embryo-nourishing tissue within the seed, but our current findings for Trimenia diminish the likelihood that the common ancestor of Austrobaileyales formed an embryo-nourishing perisperm. In either case, the developmental and functional biology of the diploid endosperm of Trimenia (and other Austrobaileyales) differs markedly from the diploid endosperms of Nymphaeales, and is fundamentally similar to the triploid endosperms of most other angiosperms.

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