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 Nymphaeales (Saarela et al., 2007), only heightened interest in understanding patterns of biological 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 endosperm, as is the case in all extant Nymphaeales and Austrobaileyales. The nutrient-storing and embryo-nourishing perisperm with a minute endosperm, as in Nymphaeales, remains a plausible plesiomorphic condition for angiosperms as a whole. In either case, the developmental and functional biology of the diploid endosperm of Trimenia (and other Austrobaileyales) differs markedly from the diploid endosperm of Nymphaeales, and is fundamentally similar to the triploid endosperms of most other angiosperms.

Key words: Austrobaileyales; basal angiosperms; diploid endosperm; female gametophyte; Nymphaeales; perisperm.
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 4 μm– 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, Philipsburg, 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

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**Fig. 3.** Mature ovule of *Trimenia moorei* at the time of fertilization. (A) Median longitudinal section stained with PAS and toluidine blue. Large arrowheads identify mature tube-like four-celled/four-nucleate female gametophyte. (B) Higher magnification view of tissue within the red box in (A). Three of the four cells of the female gametophyte can be seen (one of the two synergids is not visible in this section). Small arrow points to a few of the many small starch grains in the nucellus. (C) Median longitudinal section of the nucellus of an ovule stained with toluidine blue and observed with polarized light. Raphides can be seen in several of the cells which are otherwise highly vacuolate. **Abbreviations:** ccn, central cell nucleus; ch, chalaza; egg, egg cell; fg, female gametophyte; fun, funicle; ii, inner integument; nuc, nucellus; oi, outer integument; r, raphide; syn, synergid cell; zm, zone of megasporogenesis. Bars = 200 μm (A), 50 μm (B), and 10 μm (C).
EMBRYO-NOURISHING STRATEGIES IN SEEDS OF EARLY ANGIOSPERMS

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 female 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 extends 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 uniseriate 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 exception of the very apex and epidermis, most of the nucellus remains 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

RESULTS

Ovule development prior to fertilization in Trimenia moorei—The ovule of T. moorei is crassinucellate (sensu Endress, 2011), anatropous (syntropous), and bitegmic. Both the annular inner integument and the thicker hood-shaped outer integument are involved in the formation of the micropyle. Starch is accumulated prior to fertilization in the center of the massive nucellus tissue, but it is almost entirely consumed during megasporogenesis and female gametophyte development (Fig. 2, see also Fig. 8A; for detail see Figs. 1, 2, and 3 in Bachelier and Friedman, 2011).

At fertilization, there are only a few small starch grains remaining around the tip of the dominant mature female gametophyte (more than one female gametophyte can develop in each individual ovule). Most cells of the nucellus are devoid of starch and highly vacuolated (Fig. 3A, see also Fig. 8B). Polarized light reveals, however, that many nucellus cells start to accumulate raphides (Fig. 3B). These crystals, however, are dissolved by the acids during PAS and ABB staining, and can

polarized microscopy, as well as digital imaging. Fluorescence of Auramine-O was visualized with an HBO 100W burner with excitation filter (BP-530-585 nm), dichroic mirror (FT600), and barrier filter (LP615) (Zeiss). Pictures, line drawings, and figures were all processed and edited using Adobe Creative Suite 5 (Adobe Systems, San Jose, California, USA). Image manipulations were restricted to operations that were applied to the entire image, except as noted in specific figure legends. Boxes outlined in solid black lines in a micrograph indicate a digital inset from an adjacent section of the same series of histological sections. Insets outlined with a solid red line are linked to a higher magnification image of the boxed portion of a section. All ovules and seeds are oriented with the apex of the nucellus upwards (Figs. 2-8).
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.

Endosperm development in Trimenia and Austrobaileyales—

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. 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 Austrobaileyia (Endress, 1980), Ilicium (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 Ilicium (Floyd
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.

Fig. 7. Mature seed and embryo of *Trimenia moorei*. Median longitudinal sections stained with PAS (A, B), PAS and aniline blue black (D), Auramine-O (E), or toluidine blue (F). Large black arrowheads in D and large white arrowheads in E point to contact zone between endosperm and persistent nucellus tissue. Small black arrows in B identify starch grains and small white arrows in E indicate lipids. Asterisks mark cotyledons. (A) Seed with a small dicotyledonous embryo and a full-fledged endosperm surrounded by a persistent layer of nucellus tissue. (B) Higher magnification view of tissue within the upper red box in (A) of the embryo with differentiated protoderm and procambial strand, and cotyledonary primordia crushing the surrounding endosperm. (C) Whole embryo with two cotyledon primordia (asterisks) and adherent endosperm, dissected from a seed. (D-F) Contact zone between endosperm and the persistent layer of nucellus. Each of these panels is taken from the general area denoted by the lower red box in (A). (D) Protein bodies in the endosperm (indicated by small white arrows) but not in the nucellus layer. (E) Lipid droplets in the endosperm (indicated by small white arrows) but not in the nucellus layer. (F) Raphides (indicated by large white arrowheads) between the endosperm and the persistent nucellus epidermal layer visualized with crossed polarizing filters. Abbreviations: emb, embryo; end, endosperm; nuc, nucellus; s, suspensor. Bars = 500 μm (A), 200 μm (B, C), 20 μm (D-F).
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.

**Storage compounds in the nucellus in early diverging angiosperms**—Given the complete absence of a perisperm in the mature seeds of extant *Trimenia* and all other members of the Austrobaileyales studied to date (this study; Hayashi, 1963a, 1963b; Kapil and Jalan, 1964; Endress, 1980; Chien et al., 2011; Floyd and Friedman, 2000, 2001), the presence of a nutrient-storing and embryo-nourishing biparental endosperm remains the most parsimonious reconstruction of the ancestral condition for angiosperms as a whole.

However, the accumulation of starch prior to fertilization in the ovule of *Trimenia moorei* (Bachelier and Friedman, 2011) raises the question of whether this might represent an ancient developmental feature of the nucellus of angiosperms. In Amborella, there is no evidence for the accumulation of any significant storage compounds in the nucellus prior to or after fertilization (Tobe et al., 2000; Floyd and Friedman, 2001; Friedman, 2006). Among other early divergent lineages of flowering plants, the accumulation of starch in the nucellus prior to fertilization has been reported in Hydatellaceae (Nymphaeales; Friedman, 2008; Rudall et al., 2008), but not in members of the Nymphaeaceae and Cabombaceae (Seaton, 1908; Khanna, 1967; Schneider, 1978; Van Miegroet and Dujardin, 1992).

**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-fledged 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 ancestor 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 Nymphaeae, and is fundamentally similar to the triploid endosperms of most other angiosperms.

LITERATURE CITED


