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FEMALE GAMETOPHYTE AND EARLY SEED DEVELOPMENT IN *PEPEROMIA* (PIPERACEAE)[1]

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The evolution of female gametophyte development provides an example of how minor ontogenetic modifications can impact the functional biology of seeds. Mature Peperomia-type female gametophytes are normally depicted as 16-nucleate, nine-celled structures. However, recent ultrastructural data have demonstrated that many previous reports were incorrect, suggesting that our understanding of the Peperomia-type ontogeny is incomplete. In this investigation, female gametophyte and early seed development is described in *Peperomia dolabriformis*, *P. jamesoniana*, and *P. hispidula*. Nuclear positioning, nuclear division, and vacuole morphology are documented during the syncytial stages of development, and two mature female gametophyte cellular configurations are described. Endosperm ploidy is measured in each species using microspectrofluorometry. We conclude that a 10-celled construction is likely the most common cellular configuration in *Peperomia* and that a three-celled female gametophyte exists in *P. hispidula*. We also describe how developmental modifications of wall formation could produce the diverse cellular configurations observed throughout *Peperomia*. Interestingly, the onset of female gametophyte diversification within Piperales correlates with the origin of the perisperm in the common ancestor of Piperaceae + Saururaceae. We posit that the origin of the perisperm may have relaxed selection on endosperm genetic constructs, thereby promoting diversification of female gametophyte ontogeny.

Key words: development; evolution; female gametophyte; magnoliid; Piperales; Piperaceae; *Peperomia*.

The evolution of female gametophyte development in angiosperms provides an excellent system in which to investigate the developmental basis of morphological novelty. Piperales, a lineage within the larger ancient angiosperm clade eumagnoliids, are unique because they display a diverse array of female gametophyte developmental pathways. Six of the 10 basic female gametophyte ontogenies recognized in angiosperms (e.g., Maheshwari, 1950) are present within the clade. These include monosporic, bisporic, and tetrasporic patterns of megasporogenesis (Madrid and Friedman, 2008, 2009). All members of Piperaceae are tetrasporic and in *Peperomia*, female gametophytes contain 16 nuclei at maturity (Maheshwari, 1950; Davis, 1966). Dozens of researchers have investigated female gametophyte development in *Peperomia* (Campbell, 1899a, b, 1901, 1902; Johnson, 1900a, b, 1907, 1914; Brown, 1908, 1910; Fisher, 1914; Abele, 1923, 1924; Fagerlind, 1939; Martinoli, 1948; Murty, 1958; Nikiticheva, 1981; Nikiticheva et al., 1981; Plyushch, 1982a, b; Bannikova and Plyushch, 1984; Bannikova et al., 1987; Smirnov and Grakhantseva, 1988), and cumulatively, they have described 15 different cellular configurations at maturity.

All female gametophytes in *Peperomia* contain one egg cell; however, synergid number may range from zero (Martinoli, 1948) to four (Martinoli, 1948), and antipodal cell number may range from zero (Johnson, 1905, 1914) to 11 (Martinoli, 1948). Nuclei that do not become partitioned into the egg, synergid, or antipodal cells become polar nuclei within the central cell. Central cells in *Peperomia* may contain as few as four (Fisher, 1914; Martinoli, 1948) and as many as 14 polar nuclei (Johnson, 1914). However, comparative embryological reviews often depict Peperomia-type female gametophytes as nine-celled structures with one egg, one synergid, six antipodals, and an 8N central cell (e.g., Maheshwari, 1950; Gifford and Foster, 1974), even though many other cellular configurations have been reported in the clade.

Recent ultrastructural data in *Peperomia* demonstrate that several previous reports of nine-celled female gametophytes were incorrect and that these species actually produce 10-celled female gametophytes (Nikiticheva, 1981; Nikiticheva et al., 1981; Plyushch, 1982a, b). Although informative, these ultrastructural investigations focused on the formation of the egg apparatus and double fertilization and did not describe ontogenetic stages leading up to the formation of the mature female gametophyte (Nikiticheva, 1981; Nikiticheva et al., 1981; Plyushch, 1982a, b). Thus, photographic data documenting the syncytial stages of female gametophyte development in *Peperomia* do not currently exist.

In this investigation, we combined standard light microscopy with high-resolution three-dimensional computer reconstruction techniques to describe female gametophyte and early seed development in three species of *Peperomia*: *P. dolabriformis*, *P. jamesoniana*, and *P. hispidula*. We also set out to collect microspectrofluorometric data that would allow us to quantify endosperm ploidies in *Peperomia*. Female gametophytes of *P. hispidula* reportedly contain three cells at maturity: one egg, one synergid, and a 14-nucleate central cell (Johnson, 1905, 1914). Female gametophyte development has not been described in *P. dolabriformis* or *P. jamesoniana*.

MATERIALS AND METHODS

Plant collection—Developing inflorescences of *P. dolabriformis* and *P. jamesoniana* were collected from greenhouses at the University of Colorado in 2005, 2006, and 2007. Developing inflorescences of *P. hispidula*

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were collected from the Cerro de La Muerte biological field station in Costa Rica by Barry Hammel and shipped to the University of Colorado by overnight mail in a sealed and cooled container (USDA permit #37-79881; Republica de Costa Rica certificado fitosanitario #1016390). In the laboratory, each inflorescence was immersed in a modified PIPES buffer (PIPES 60 mM, HEPES 25 mM, $CaCl_2$ 5.4 mM, $MgCl_2$ 1.97 mM) where it was dissected into smaller pieces (each contained approximately 12 flowers) for chemical fixation.

Histology—The dissected flowers were placed in a fixative solution containing either 3 : 1 95% ethanol : acetic acid or 4% paraformaldehyde, 2.5% glutaraldehye, and 4% acrolein in the modified PIPES buffer for 12 h. Specimens were dehydrated in a graded ethanol series, infiltrated, embedded with glycol methacrylate (JB-4 embedding kit, Polysciences, Warrington, Pennsylvania, USA), and serially sectioned into 5-μm thick ribbons using a Microm HM330 rotary microtome (Microm International, Walldorf, Germany) and glass knives. Sections from flowers that were fixed in the 3 : 1 solution were stained with a solution of 0.25 $\mu g \cdot ml^{-1}$ of 4′,6-diamidino-2-phenylindole (DAPI) and 0.1 $mg \cdot ml^{-1}$ phylenediamine in 0.05 M Tris (pH 7.2). Sections from flowers that were fixed with paraformaldehyde, glutaraldehyde, and acrolein were stained with 0.1% aqueous toluidine blue. All slides were viewed with a Zeiss Axiophot microscope using bright field, differential interference contrast, and epifluoresence optics. Cross polarization optics were employed to detect the presence of starch in developing seeds. Digital micrographs were taken with a Zeiss Axiocam digital camera. Postprocessing of all images was done with the Adobe (San Jose, California, USA) Creative Suite 2 software package. Image manipulations were restricted to processes that were applied to the entire image unless otherwise noted in specific figure legends.

Three-dimensional computer reconstruction—Selected 5-μm thick serial sections of developing female gametophytes that had been fixed in paraformaldehyde, glutaraldehyde, and acrolein were photographed using a Leica TCS SP2 AOBS laser scanning confocal microscope. Images were created by exciting the toluidine blue stain at 633 nm with a helium/neon laser, with detectors set to absorb within the 640–700 nm range. Each 5-μm thick section was photographed along the *Z*-axis to create a stack of eight to ten 0.5-μm thick optical sections. *Z*-stacks from each section were aligned and modeled using the IMOD software package (Kremer et al., 1996).

Microspectrofluorometry—Sections from flowers fixed in the 3 : 1 solution were stained with DAPI (described earlier) for 1 h at room temperature in a light-free environment. Microspectrofluorometric measurements of relative DNA levels of DAPI-stained nuclei were performed within 2 h. Measurements were made with a Zeiss MSP 20 microspectrophotometer with digital microprocessor coupled to a Zeiss Axioskop microscope equipped with epifluorescence (HBO100-W burner). An ultraviolet filter set (model number 48702) with excitation filter (365 nm, bandpass 12 nm), dichroic mirror (FT395), and barrier filter (LP397) were used with a Zeiss Plan NeoFluar 40× objective. Before each recording session, the photometer was standardized by taking a reading of fluorescence emitted from a fluorescence standard (GG 17), and this reading was taken to represent 100 relative fluorescence units (RFU). At the completion of each session, an additional reading was made of the fluorescence standard to confirm that little or no deviation in the relative fluorescence value obtained from the fluorescence standard had occurred during the period of data recording. Relative DNA content for each nucleus was determined by summing the individual fluorescence values of each of the serial sections through that nucleus. A net photometric value for each section of a nucleus was obtained by recording an initial reading of the nucleus and subtracting a background value obtained from cytoplasm proximal to the nucleus. Thus, background fluorescence from the glycol methacrylate was removed from the photometric analysis of relative DNA content.

RESULTS

We collected developmental data during megasporogenesis, megagametogenesis, and early seed development in *P. dolabriformis*, *P. jamesoniana*, and *P. hispidula*. The syncytial stages of female gametophyte development are extremely similar in each of the species that we examined. Our data from *P. dolabriformis* are the most complete during the syncytial stages of development, and we will use these data to represent all three taxa up to the time of cellularization.

Megasporogenesis—The megasporocyte in *P. dolabriformis* has dense cytoplasm and normally contains a single vacuole (Fig. 1A, 1B). When the megasporocyte is about 50 μm long and 30 μm wide, meiosis I is initiated in the center of the cell (Fig. 1C, 1D). During this division, the vacuole is situated near the micropyle, and a phragmoplast always forms perpendicular to the micropylar–chalazal axis of the cell between the two dyad nuclei (Fig. 1C, 1D). The phragmoplast eventually gives way to a cell plate that begins to expand outward from between the two nuclei toward the cell wall (Fig. 1E, 1F). However, expansion of the cell plate aborts before making contact with the side walls of the syncytium, and we never observed a female gametophyte in which each meiotic dyad nucleus was completely partitioned into a separate cell. By the time of meiosis II, the cell plate that formed during meiosis I (Fig. 1C–1F) is no longer visible (Fig. 2A–2C).

The axes of division in meiosis II are perpendicular, and megaspore nuclei immediately become placed in a tetrahedral configuration (Fig. 2A–2C). Phragmoplasts also form in meiosis II between each megaspore pair (Fig. 2A–2C), and go on to form cell plates that expand centrifugally toward the periphery of the female gametophyte (Fig. 2D–2F). Eventually, expansion of

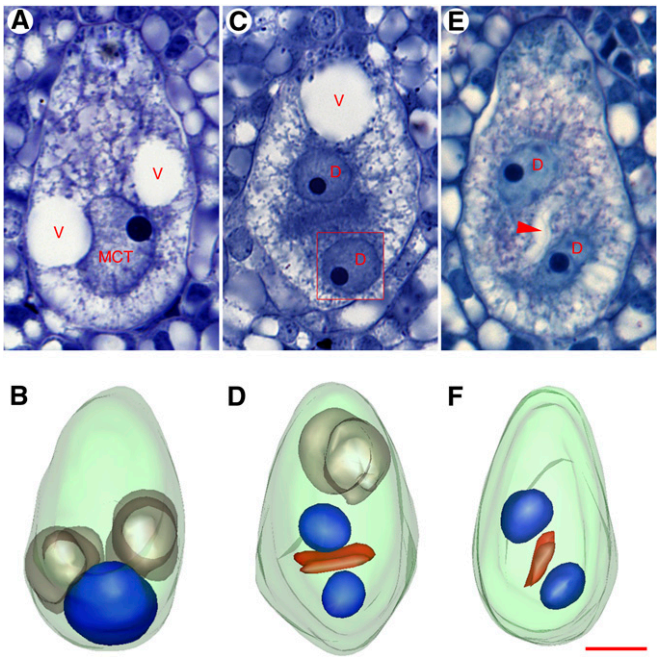

Fig. 1. Meiosis I in *Peperomia*. The computer reconstruction in each column has been created from the micrograph above it in the first row. The squares around nuclei in some of the micrographs indicate that the outlined area has been copied from adjacent serial sections to make a composite image. In each reconstruction, nuclei are blue, vacuoles are tan, cell plates and phragmoplasts are red, and the outer wall of the syncytium is green. Arrowhead indicates developing cell plate. Scale bar = 10 μm. *Figure abbreviations*: D, dyad nucleus; MCT, megasporocyte; V, vacuole. (A, B) Megasporocyte with two vacuoles. (C, D) Meiosis I with phragmoplast between dyad nuclei. A single vacuole is at the micropylar end of the cell. (E, F) Later stage of meiosis I, with a cell plate beginning to form between each dyad nucleus.

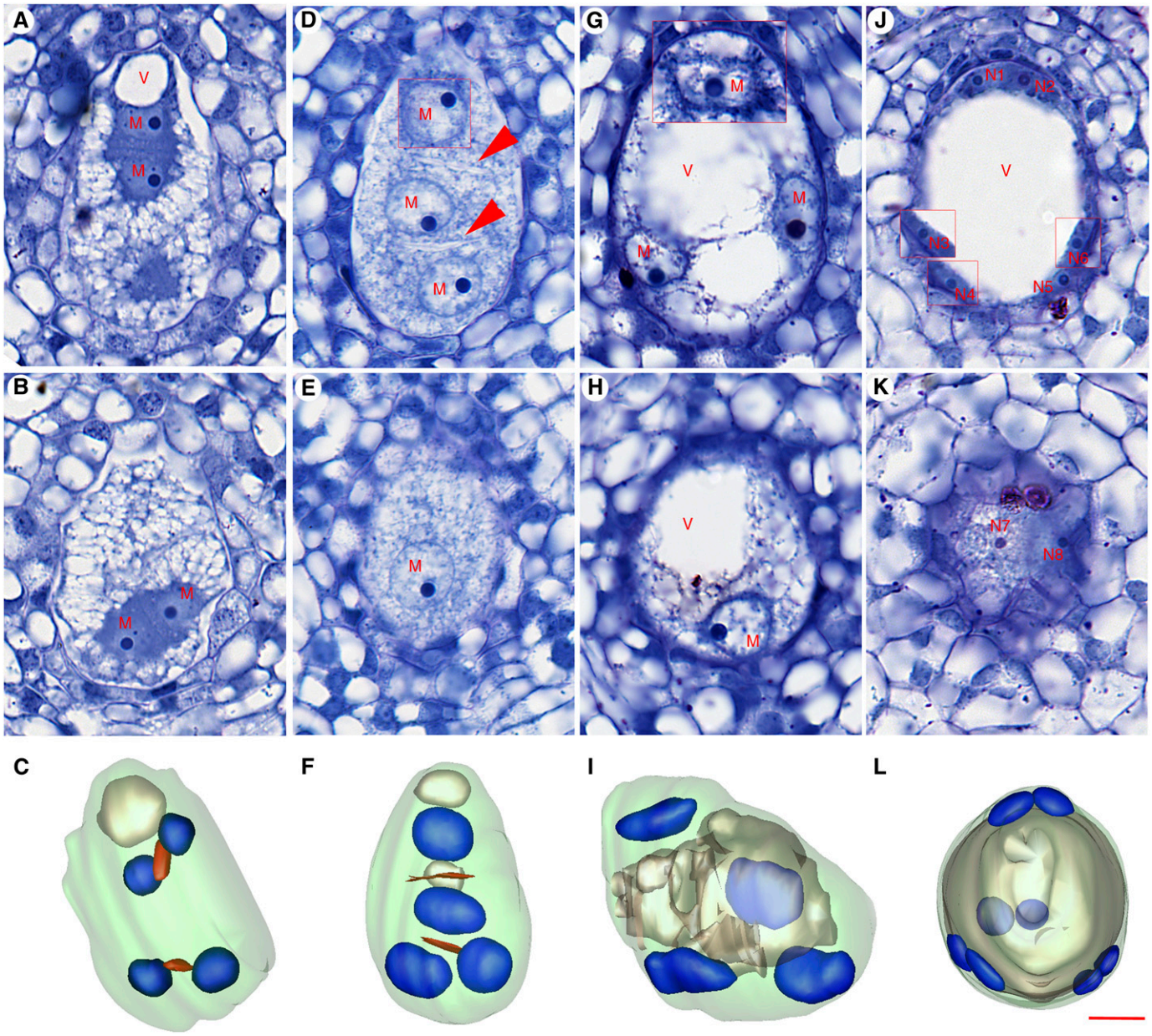

Fig. 2. Four- and eight-nucleate stages of female gametophyte development in *Peperomia*. The squares around some nuclei indicate that the outlined area has been copied from adjacent serial sections to make a composite image. The computer reconstructions in each column have been created from the serial micrographs above them. Each computer reconstruction has been placed in an identical orientation that allows for observation of the tetrahedral arrangement of the megaspore nuclei. In each reconstruction, nuclei are blue, vacuoles are tan, cell plates are red, and the outer wall of the syncytium is green. Arrowheads indicate developing cell plates. Scale bar = 10 μm. *Figure abbreviations*: M, megaspore; N, nucleus (each has been numbered); V, vacuole. (A–C) Meiosis II. The axes of division are perpendicular, and phragmoplasts form between megaspore pairs. (D–F) Later stage four-nucleate female gametophytes with cell plates between megaspore pairs. (G–I) Late stage four-nucleate female gametophyte with large central vacuole. (J–L) Eight-nucleate female gametophyte.

these walls aborts, and all four megaspore nuclei become situated in a single functional megaspore cell (Fig. 2G–2I).

Female gametophyte development—After meiosis, each megaspore nucleus initiates a free-nuclear mitotic division. The overall position of nuclei within the syncytium does not change as the female gametophyte expands, and the four pairs of nuclei that comprise the eight-nucleate female gametophyte retain a tetrahedral configuration (Fig. 2J–2L). By this time, the central vacuole takes up the majority of cell volume (Fig. 2J–2L).

When the female gametophyte is about 75 μm in diameter, each nucleus initiates another round of mitosis. Sixteen total nuclei are produced and cell walls form within the female gametophyte (Fig. 3A–3C). In all three species of *Peperomia* that we investigated, cell walls are initially faint, and individual cells are difficult to distinguish within the egg apparatus and at the chalazal pole (Figs. 3A–3C, 4A–4C). However,

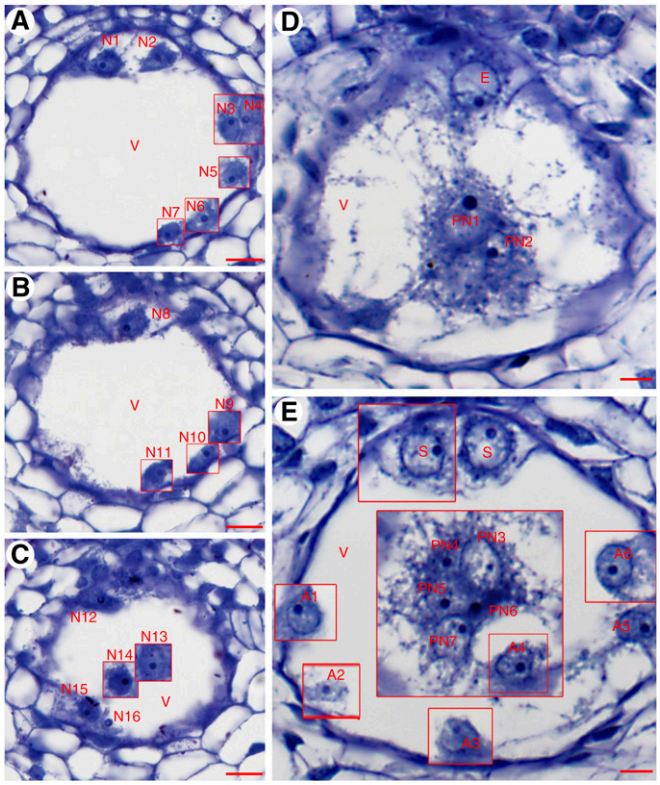

Fig. 3. Sixteen-nucleate female gametophytes of *Peperomia dolabriformis*. The squares around nuclei in some of the micrographs indicate that the outlined area has been copied from adjacent serial sections to produce a composite image. Scale bar = 10 µm. *Figure abbreviations*: A, accessory cell (each has been numbered); E, egg cell; N, female gametophyte nucleus (each has been numbered); PN, polar nucleus (each has been numbered); S, synergid; V, vacuole. (A–C) Young 16-nucleate female gametophyte. Faint walls are visible, but individual cell types cannot be identified. (D, E) Mature 16-nucleate female gametophyte with 10 cells: two synergid cells, one egg cell, six accessory cells, and a 7N polar nucleus. A4 may appear to be part of the central cell; however, it is actually located against the wall of the female gametophyte behind the polar nuclei.

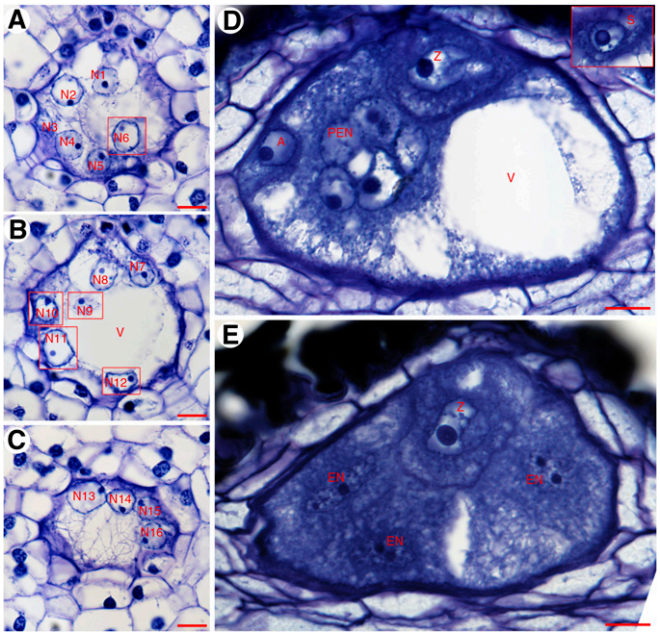

Fig. 4. Mature and fertilized female gametophytes in *Peperomia jamesoniana* with developing endosperm. The squares around nuclei in some micrographs indicate that the outlined area has been copied from adjacent serial sections to make a composite image. Scale bar = 10 µm. *Figure abbreviations*: A, accessory cell; EN, endosperm nucleus; N, female gametophyte nucleus (each has been numbered); PEN, primary endosperm nucleus; S, synergid; V, vacuole; Z, zygote. (A–C) Young 16-nucleate female gametophyte. Cell walls are visible around the cells that comprise the egg apparatus (N1, N7, and N8), but their individual identity cannot be discerned. (D) Fertilized female gametophyte with nine cells: one remaining synergid cell (the other is destroyed at fertilization), one zygote cell, six accessory cells (one shown), and an 8N primary endosperm nucleus. The synergid has been placed in the top corner because in the original serial section it was located directly on top of the zygote cell. (E) Zygote and endosperm in seed of *P. jamesoniana*.

as the female gametophyte expands, cell walls become more apparent.

By the time of fertilization, each cell within the female gametophyte can be identified (Figs. 3D, 3E, 4D, 5A, 5B). The micropylar-most nuclear tetrad goes on to produce the egg apparatus. Egg cells were identified by their position near the micropyle, their large size relative to the other cells within the egg apparatus, and their 2C content of DNA (discussed later). Cells within the egg apparatus that did not take part in fertilization were labeled synergids. Nuclei in the other three nuclear tetrads either became partitioned into the common cytoplasmic space of the central cell or were compartmentalized into sterile cells positioned around the periphery of the female gametophyte. These cells resemble antipodals in a typical Polygonum-type female gametophyte and have been referred to as such in many previous investigations. However, we do not wish to assert that these cells are homologous to antipodals. For the purposes of this investigation, we will refer to sterile cells that are not located within the egg apparatus as "accessory cells." We do not use this term in the same way as Campbell (1899a, b; 1901, 1902), who believed that accessory cells were homologous to prothallial cells in gymnosperms.

In *P. dolabriformis* (Figs. 3D, 3E, 6A, 6B) and *P. jamesoniana* (Fig. 4A–4D, 6A, 6B), the mature female gametophyte contains 10 cells: an egg cell, two synergid cells, six accessory cells, and a central cell. We consistently counted seven polar nuclei within the central cells of *P. dolabriformis* (Figs. 3D, 3E, 6A) and *P. jamesoniana* (Fig. 4D). In *P. hispidula*, the mature female gametophyte contains three cells: an egg cell, one synergid cell, and a central cell (Figs. 5A, 5B, 6C, 6D). Accessory cells are absent in *P. hispidula*, and the secondary nucleus is the fusion product of 14 polar nuclei (Figs. 5A, 5B, 6C, 6D).

Fertilization—We did not observe fertilized ovules in *P. dolabriformis*. This species is apparently self-incompatible, and the individuals in our greenhouse, which are clones of a single plant, did not produce seed. We did observe fertilization and early seed development in *P. jamesoniana* and *P. hispidula*.

After fertilization in *P. jamesoniana*, the six accessory cells have prominent cell walls (Fig. 4D). One synergid cell persists beside the zygote, and seven individual polar nuclei remain visible in the primary endosperm cell (Fig. 4D). In *P. hispidula*, the fertilized female gametophyte has a zygote, one persistent synergid cell, and a very large primary endosperm nucleus (Figs. 5A, 5B, 6C, 6D). The zygote in both species has a prominent cell wall and is densely cytoplasmic (Figs. 4D, 5B). Polar

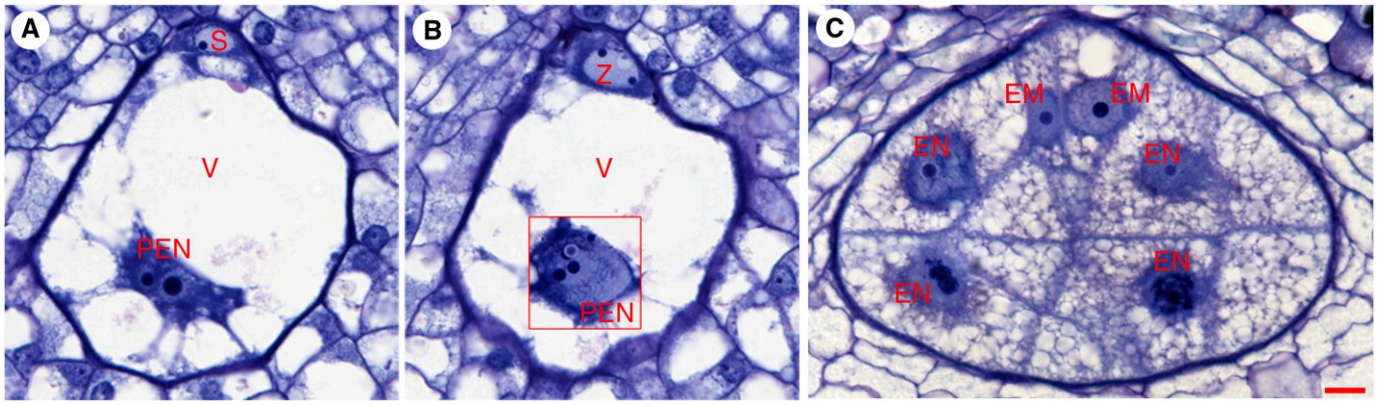

Fig. 5. Fertilization and endosperm development in *Peperomia hispidula*. The squares around nuclei in some of the micrographs indicate that the outlined area has been copied from adjacent serial sections to make a composite image. Scale bar equals 10 μm. *Figure abbreviations*: EM, embryo nucleus; EN, endosperm nucleus; PEN, primary endosperm nucleus; S, synergid; V, vacuole; Z, zygote. (A, B) Fertilized female gametophyte of *P. hispidula* with persistent synergid cell, zygote, and primary endosperm nucleus. (C) Embryo and endosperm in seed of *P. hispidula*. Cell types have been determined by measuring the DNA content of each nucleus.

nuclei in female gametophytes of *P. hispidula* often fuse before fertilization (Fig. 5A, 5B), while in *P. jamesoniana* polar nuclei fuse after fertilization (Fig. 4D). Fertilized female gametophytes of *P. jamesoniana* contain one synergid (Fig. 4D), while unfertilized female gametophytes contain two synergids (Fig. 4A–4C), indicating that one synergid degenerates just before or during fertilization in *P. jamesoniana*. Female gametophytes of *P. hispidula* contain one synergid cell before and after fertilization (Fig. 5A). Occasionally, we observed one or two accessory cells in developing seeds of *P. jamesoniana*, but normally they would degrade shortly after the first division of the primary endosperm nucleus.

Microspectrofluorometry—We determined the DNA content of various cell types throughout development in mature and fertilized *Peperomia* female gametophytes by measuring the relative fluorescence of nuclei stained with DAPI, a DNA-binding fluorochrome (Tables 1, 2). To calibrate our photometric measurements in *P. jamesoniana* and *P. hispidula*, we used zygote nuclei during prophase. By definition, these nuclei contain 4C content of DNA.

Prophase zygote nuclei in *P. hispidula* had an average RFU value of 75.90 ± 7.61 (N = 7). Therefore, in *P. hispidula* 4C DNA is equivalent to roughly 76 RFU, 2C is equal to about 38 RFU, and 19 RFU corresponds to 1C of DNA. Before fertilization, egg cell nuclei in *P. hispidula* fluoresced with 40.16 ± 8.56 RFU (N = 5), suggesting that they contain 2C DNA. If secondary nuclei in *P. hispidula* contain 14 nuclei (as our micrographs suggest, see Fig. 5A, 5B), then primary endosperm nuclei in *P. hispidula* (that contain a 14N secondary nucleus and one sperm nucleus) should contain 30C DNA in prophase, or roughly 574 RFU. Primary endosperm nuclei had an average value of 531.6 ± 73.23 RFU (N = 8) during prophase, and prophase endosperm nuclei had an almost equivalent value of 580.02 ± 120.29 RFU (N = 32). When these data are combined, they yield an average value of 570.34 ± 113.35 RFU (N = 40) for prophase endosperm nuclei in *P. hispidula*. All these values correspond closely to 30C DNA and indicate that endosperm in *P. hispidula* is 15N (Table 1).

Prophase zygote nuclei in *P. jamesoniana* had an average relative fluorescence of 73.11 ± 8.08 (N = 2) RFU, indicating that 2C DNA is equal to about 37 RFU and 1C corresponds to about 18 RFU. Our micrographic data indicate that central cells of *P. jamesoniana* contain seven polar nuclei and yield an octoploid primary endosperm nucleus containing 16C

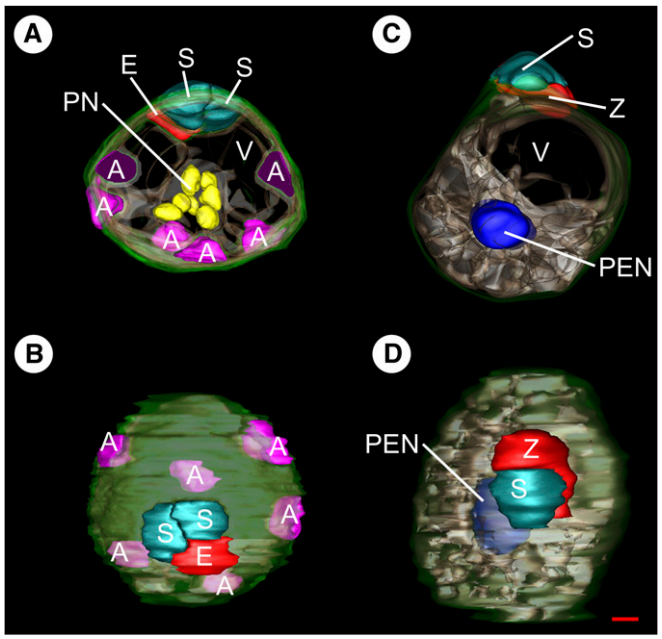

Fig. 6. Three-dimensional reconstructions of three- and 10-celled female gametophytes in *Peperomia dolabriformis* and *P. hispidula*. In each reconstruction, synergids are teal, the egg/zygote is red, accessory cells are pink, polar nuclei are yellow, the primary endosperm nucleus is blue, the central vacuole is tan, and the outer wall of the central cell / primary endosperm nucleus is green. Scale bar = 10 μm. *Figure abbreviations*: A, accessory cell; E, egg; PN, polar nuclei; PEN, primary endosperm nucleus; S, synergid; V, central vacuole; Z, zygote. (A, B) Mature female gametophyte of *P. dolabriformis* that contains 10 cells: an egg, two synergids, six accessory cells, and a 7N central cell. Polar nuclei have been removed in (D) so that all six accessory cells are visible. (C, D) Female gametophyte of *P. hispidula* at fertilization. The structure contains three cells: a zygote, one synergid, and a primary endosperm nucleus.

TABLE 1. Photometric data from (A) *Peperomia hispidula* and (B) *P. jamesoniana*. Predicted and observed relative fluorescence units (RFU) are provided for each cell type.

Species/Object measured	Predicted DNA content		Mean observed DNA content (RFU), *N*	Difference from predicted (%)
	C	RFU		
A) *P. hispidula*				
Egg cell nucleus	2	37.95	40.16 ± 8.56, 5	5.82
Zygote cell nucleus (prophase)	4	—	75.90 ± 7.61, 7	—
Primary endosperm cell nucleus (prophase)	30	569.25	531.60 ± 73.23, 8	−6.61
Endosperm cell nucleus (prophase)	30	569.25	580.02 ± 120.29, 32	1.89
B) *P. jamesoniana*				
Egg nucleus	2	36.56	43.10 ± 8.64, 2	17.90
Zygote cell nucleus (prophase)	4	—	73.11 ± 8.08, 2	—
Endosperm cell nucleus (prophase)	16	292.44	286.79 ± 59.39, 14	−1.93

DNA in prophase (Table 1). We did not observe primary endosperm nuclei in prophase in *P. jamesoniana*; however, we did measure the fluorescence of prophase nuclei in cellularized endosperm. These nuclei had an average value of 286.79 ± 59.39 RFU (N = 14). This fluorescence value closely corresponds to 16C of DNA and indicates that endosperm in *P. jamesoniana* is 8N.

Endosperm/perisperm development—Starch begins to form in the nucellus immediately after fertilization (Fig. 7A, 7B, 7D, 7E), even before the primary endosperm nucleus has divided. Starch grains first accumulate in the cytoplasm of nucellus cells closest to the female gametophyte (Fig. 8B, 8E). As the seed develops (Fig. 8), starch grains become larger, more oval-shaped (Fig. 7C, 7F), and appear in nucellus cells farther away from the female gametophyte (Fig. 8C, 8F). In the oldest seeds of *P. jamesoniana* and *P. hispidula* that we observed, starch grains were about 17 μm wide.

Endosperm is *ab initio* cellular and does not contain starch (Figs. 4E, 5C). In our preparations, we observed endosperm up to the eight-celled stage in *P. jamesoniana* and the 20-celled stage in *P. hispidula*. Perisperm made up the majority of seed volume in both species, and development of the embryo lagged behind that of the endosperm (Fig. 8C, 8F). The most mature embryos we observed were in *P. hispidula*, and they contained four cells (Fig. 8F). The zygote cell had not divided in seeds of *P. jamesoniana* with eight endosperm cells (Figs. 4E, 8C).

DISCUSSION

Megasporogenesis—During meiosis I, we observed phragmoplasts and cell plates between meiotic dyad nuclei (Fig. 1C–1F) and in meiosis II between megaspore nuclei (Fig. 2A–2H). Phragmoplasts and cell plates have been described in almost every investigation of megasporogenesis in *Peperomia* (Johnson, 1900b, 1905, 1907, 1914; Brown, 1908, 1910; Fisher, 1914). In all these reports and in the data we present here (Figs. 1, 2), cell walls degrade between megaspore nuclei soon after being formed. It is interesting to note that *Peperomia* is the only lineage in Piperaceae that appears to form cell plates/cell walls during megasporogenesis. Embryological investigations in *Piper* (Joshi, 1944; Swamy, 1944; Maugini, 1953; Kanta, 1962; Prakash et al., 1994; Madrid and Friedman, 2009), *Zippelia* (Lei et al., 2002), and *Mannekia* (Arias and Williams, 2008) have described tetrasporic patterns of female gametophyte development, but have shown no evidence of cell plates or cell walls during any part of megasporogenesis.

***Female gametophyte development in* Peperomia**—After meiosis, the four megaspore nuclei are tetrahedrally arranged within the female gametophyte (Fig. 2A–2L) and initiate two rounds of mitotic divisions (Figs. 2M–2P, 3A–3C, 4A–4C, 5A, 5B). These divisions have been described with great consistency in all previous investigations in *Peperomia* (Campbell, 1899a, b, 1901, 1902; Johnson, 1900a, b, 1905, 1907, 1914; Brown, 1908, 1910; Martinoli, 1948; Murty, 1958; Smirnov and Grakhantseva, 1988). All species exit the last division of female gametophyte development with 16 nuclei that are distributed among four tetrahedrally arranged nuclear tetrads (Figs. 3A–3C, 4A–4C). However, previous reports of wall formation (cellularization) have been variable (Table 2). Earlier studies of female gametophyte development in 18 species of *Peperomia* cumulatively describe 15 different cellular configurations at maturity, with corresponding endosperm ploidies that are predicted to range from 5N to 14N (Table 2). Reports of mature female gametophyte structure differ within and between species. For example, we observed two different patterns of mature female gametophyte structure in the three species of *Peperomia* we examined, while Martinoli (1948) reported six different mature female gametophyte configurations in *P. maculosa* alone (Table 2). The full extent of this diversity is not always emphasized, and certain cellular configurations are cited more often than others in comparative reviews of female gametophyte development in *Peperomia* (e.g., Maheshwari, 1950; Gifford and Foster, 1974).

Nine-celled female gametophytes with one egg, one synergid, six accessory cells, and a seven-nucleate central cell have been frequently reported in *Peperomia* since their apparent discovery in *P. pellucida* (Johnson, 1900a, b; Campbell, 1901, 1902). Subsequent embryological investigations have described a nine-celled female gametophyte in 10 other *Peperomia* species (Table 2). In species with reported intraspecific variation in patterns of cellularization such as *P. blanda*, *P. maculosa*, and *P. pellucida* (Table 2), the nine-celled configuration was the most commonly observed structure at maturity (Johnson, 1900a; Fisher, 1914; Murty, 1958). Although this cellular conformation is referenced in many classic embryological reviews (e.g., Maheshwari, 1950; Gifford and Foster, 1974) as the "typical" Peperomia-type female gametophyte, micrographic evidence of this configuration surprisingly does not exist, and recent ultrastructural studies demonstrate that previous reports of this nine-celled configuration in *P. blanda*, *P. maculosa*, and *P. pellucida* are incorrect (Table 1). Instead, these female gametophytes actually contain two synergid cells and one egg cell with variable numbers of accessory cells and central cell nuclei (Nikiticheva, 1981; Nikiticheva et al., 1981; Plyushch, 1982a, b). Nikiticheva (1981), Nikiticheva et al. (1981), and Plyushch (1982a, b) suggest that the mistakes of previous researchers regarding the number of cells in the egg apparatus are likely due to limitations of resolution inherent in paraffin embedding and light microscopy (for a discussion of wall formation events in *Peperomia* at the ultrastructural level, see Nikiticheva [1981], Nikiticheva et al. [1981], and Plyushch [1982a, b]).

TABLE 2. Female gametophyte development in *Peperomia*. Fifteen different cellular configurations have been reported at maturity. The three-celled configuration reported in *P. hispidula* is shaded pink, reports of the nine-celled configuration are shaded gray, and reports of the 10-celled configuration are shaded purple.

Species	Egg cells	Synergid cells	Accessory cells	Polar nuclei	Relevant citations
P. arifolia, *P. blanda*	1	1	6	8	Brown, 1908
	1	2	8	5	Nikiticheva, 1981; Nikiticheva et al., 1981
	1	1	8	6	Fisher, 1914
	1	2	7	6	Nikiticheva, 1981; Nikiticheva et al., 1981
	1	2	6	7	Nikiticheva, 1981; Nikiticheva et al., 1981; Plyushch, 1982a, b
	1	1	6	8	Murty, 1958
	1	2	5	8	Murty, 1958; Nikiticheva, 1981; Nikiticheva et al., 1981
	1	2	4	9	Murty, 1958; Nikiticheva, 1981; Nikiticheva et al., 1981
	1	2	3	10	Nikiticheva, 1981; Nikiticheva et al., 1981
	1	2	2	11	Nikiticheva, 1981; Nikiticheva et al., 1981
	1	2	1	12	Nikiticheva, 1981; Nikiticheva et al., 1981
	1	2	0	13	Nikiticheva, 1981; Nikiticheva et al., 1981
P. clusiifolia	1	1	6	8	Smirnov and Grakhantseva, 1988
P. comarapana	1	1	6	8	Murty, 1958
	1	2	5	8	Murty, 1958
P. dolabriformis	1	2	6	7	This study
P. eburnia	1	2	6	7	Nikiticheva et al., 1981
P. hispidula	1	1	0	14	Johnson, 1914; this study
P. jamesoniana	1	2	6	7	this study
P. langsdorffii	1	2	6	7	Smirnov and Grakhantseva, 1988
	1	1	6	8	Smirnov and Grakhantseva, 1988
P. maculosa	1	0	11	4	Martinoli, 1948
	1	2	8	5	Nikiticheva, 1981; Nikiticheva et al., 1981
	1	2	7	6	Nikiticheva, 1981; Nikiticheva et al., 1981
	1	3	6	6	Martinoli, 1948
	1	2	6	7	Martinoli, 1948; Nikiticheva, 1981
	1	1	6	8	Martinoli, 1948; Smirnov and Grakhantseva, 1988
	1	2	5	8	Nikiticheva, 1981; Nikiticheva et al., 1981
	1	4	3	8	Martinoli, 1948
	1	2	4	9	Nikiticheva, 1981; Nikiticheva et al., 1981
	1	1	4	10	Martinoli, 1948
	1	2	3	10	Nikiticheva, 1981; Nikiticheva et al., 1981
	1	2	2	11	Nikiticheva, 1981; Nikiticheva et al., 1981
	1	2	1	12	Nikiticheva, 1981; Nikiticheva et al., 1981
	1	2	0	13	Nikiticheva, 1981; Nikiticheva et al., 1981
P. magnofolia	1	2	6	7	Smirnov and Grakhantseva, 1988
P. obtusifolia	1	2	8	5	Nikiticheva, 1981; Nikiticheva et al., 1981
	1	2	7	6	Nikiticheva, 1981; Nikiticheva et al., 1981
	1	2	6	7	Nikiticheva et al., 1981
	1	2	5	8	Nikiticheva, 1981; Nikiticheva et al., 1981
	1	2	4	9	Nikiticheva, 1981; Nikiticheva et al., 1981
	1	2	3	10	Nikiticheva, 1981; Nikiticheva et al., 1981
	1	2	2	11	Nikiticheva, 1981; Nikiticheva et al., 1981
	1	2	1	12	Nikiticheva, 1981; Nikiticheva et al., 1981
	1	2	0	13	Nikiticheva, 1981; Nikiticheva et al., 1981
P. ottoniana	1	1	6	8	Brown, 1908
P. pellucida	1	2	8	5	Nikiticheva, 1981; Nikiticheva et al., 1981
	1	2	7	6	Nikiticheva, 1981; Nikiticheva et al., 1981
	1	2	6	7	Nikiticheva, 1981; Fagerlind, 1939
	1	1	6	8	Johnson, 1900, 1905; Murty, 1958; Fagerlind, 1939
	1	2	5	8	Murty, 1958;
	1	2	5	8	Campbell, 1899, 1901
	1	2	4	9	Nikiticheva, 1981; Nikiticheva et al., 1981
	1	2	3	10	Nikiticheva, 1981; Nikiticheva et al., 1981
	1	2	2	11	Nikiticheva, 1981; Nikiticheva et al., 1981
	1	2	1	12	Nikiticheva, 1981; Nikiticheva et al., 1981
	1	2	0	13	Nikiticheva, 1981; Nikiticheva et al., 1981
P. reflexa	1	1	6	8	Murty, 1958
	1	2	5	8	Murty, 1958
P. scandens	1	1	6	8	Smirnov and Grakhantseva, 1988
P. sintensii	1	1	6	8	Brown, 1908
P. verticillata	1	2	9	4	Fisher, 1914
	1	2	8	5	Fisher, 1914
	1	2	7	6	Fisher, 1914
	1	2	6	7	Fisher, 1914

Our data confirm that female gametophytes in *Peperomia* contain a three-celled egg apparatus at maturity; however, we did not observe corresponding levels of interspecific variability in the number of accessory cells and central cell nuclei as reported by Nikiticheva (1981), Nikiticheva et al. (1981), and Plyushch (1982a, b). Female gametophytes in *P. dolabriformis* and *P. jamesoniana* are exclusively 10-celled and contain one egg, two synergids, six accessory cells, and seven polar nuclei. Currently, no data specifically refute the possibility of nine-celled female gametophytes with one synergid cell and eight polar nuclei in *P. arifolia*, *P. clusiifolia*, *P. comarapana*, *P. langsdorffi*, *P. ottonia*, *P. reflexa*, *P. scandens*, and *P. sintensii*

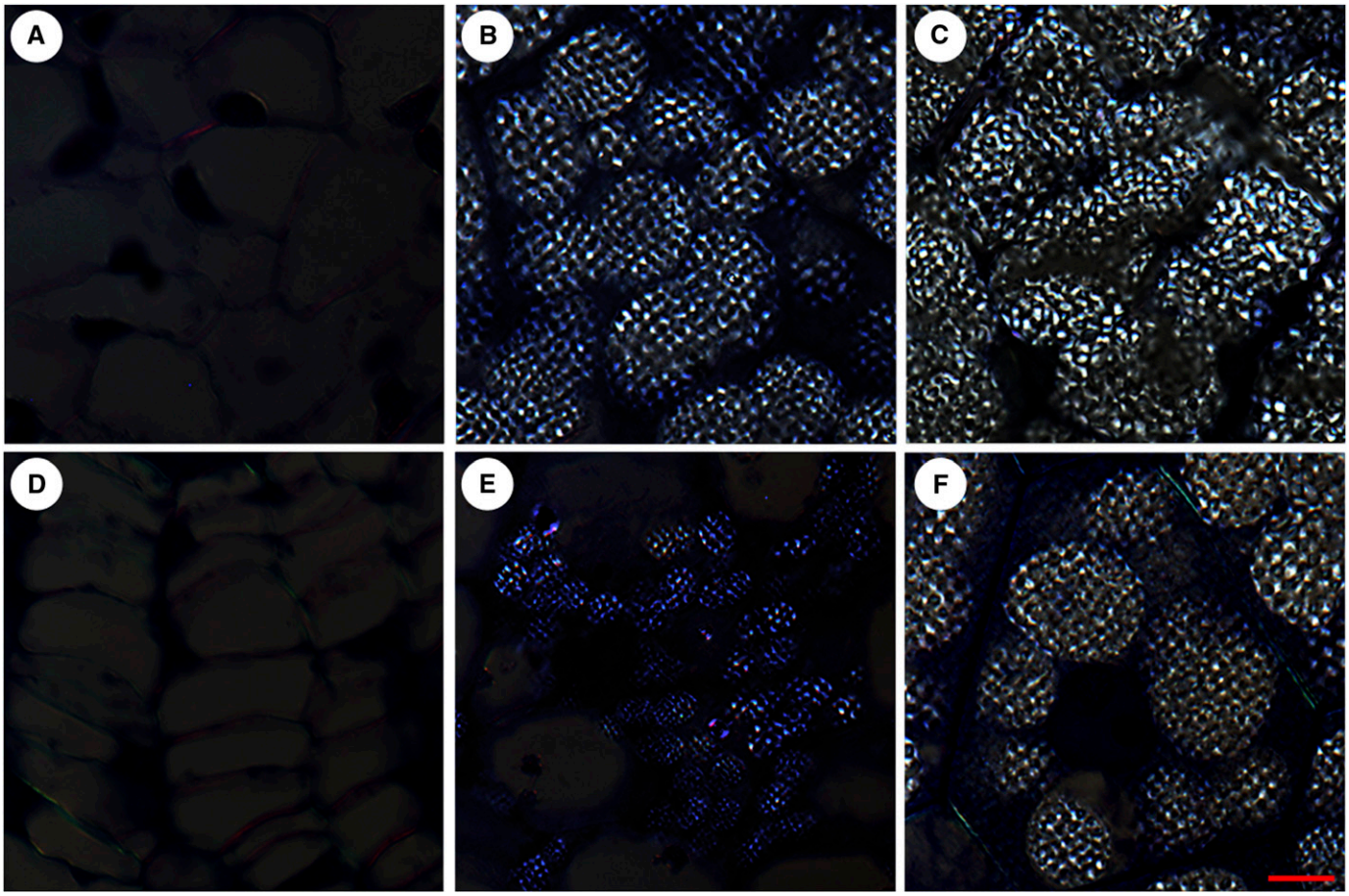

Fig. 7. Starch in nucellus cells of (A–C) *Peperomia jamesoniana* and (D–F) *P. hispidula*. These images were acquired from the areas of the nucellus outlined with red boxes in Fig. 7. Scale bar = 100 μm. Images in first column (A, D) are from mature, unfertilized female gametophytes, images in second column (B, E) are from female gametophytes at the time of fertilization, and those in third column (C, F) are from developing seeds.

(Table 2); however, these data were also obtained with paraffin-embedding techniques and may have been misdiagnosed.

Fertilization—There have been conflicting reports about how male gametes are transmitted from the pollen tube to the egg cell in *Peperomia*. Early studies using light microscopy describe fertilization of the egg nucleus in *Peperomia* through direct penetration by the pollen tube (Campbell, 1899b; Johnson, 1900a, b, 1914; Campbell, 1901; Brown, 1908, 1910). However, ultrastructural studies have documented fertilization of the egg nucleus by a male gamete that is released from the pollen tube into the cytoplasm of a synergid cell in *P. blanda*, *P. eburnia*, *P. maculosa*, *P. obtusifolia*, and *P. pellucida* (Table 2) (Nikiticheva, 1981; Nikiticheva et al., 1981; Plyushch, 1982a, b). This is the same fertilization pathway taken by the majority of angiosperm species and results in the degeneration of one synergid cell at the time of fertilization (Huang and Russell, 1992; Ge et al., 2007).

Our data from *P. jamesoniana* agree with these ultrastructural data in that we observed a three-celled egg apparatus before fertilization (Fig. 4A–4C) and a two-celled egg apparatus after fertilization (Fig. 4D). One synergid cell appears to degenerate at fertilization (Fig. 4D), consistent with the idea that it aids in the transmission of male gametes. The synergid cell that persists after fertilization (Fig. 4D) only remains for a short period of time and is absent by the first division of the primary endosperm nucleus (Fig. 4E). Female gametophytes of *P. dolabriformis* also contain two synergid cells at maturity (Fig. 3D, 3E); however, we were unable to determine if one degenerates at fertilization.

We are the first to reinvestigate female gametophyte development in *P. hispidula* since the original claim that egg cells are fertilized through direct penetration of the pollen tube in this species (Johnson, 1914). Here we have documented one synergid cell both before and after fertilization in *P. hispidula* (Fig. 5A) and indirectly support Johnson's conclusion because transmission of sperm nuclei normally destroy synergid cells (Huang and Russell, 1992; Ge et al., 2007). The persistence of the synergid cell in *P. hispidula* (Fig. 5A) may indicate that it does not play a functional role in fertilization.

***Developmental evolution of 16-nucleate female gametophytes in* Peperomia**—Syncytial stages of female gametophyte development are similar across all species of *Peperomia* that have been examined. Thus, differences among mature female gametophyte cellular configurations in *Peperomia* are the result of variation in late stage wall formation patterns in which nuclei become positioned into peripheral cells (an egg cell, accessory cells, and synergids) or the central cell that will be fertilized to

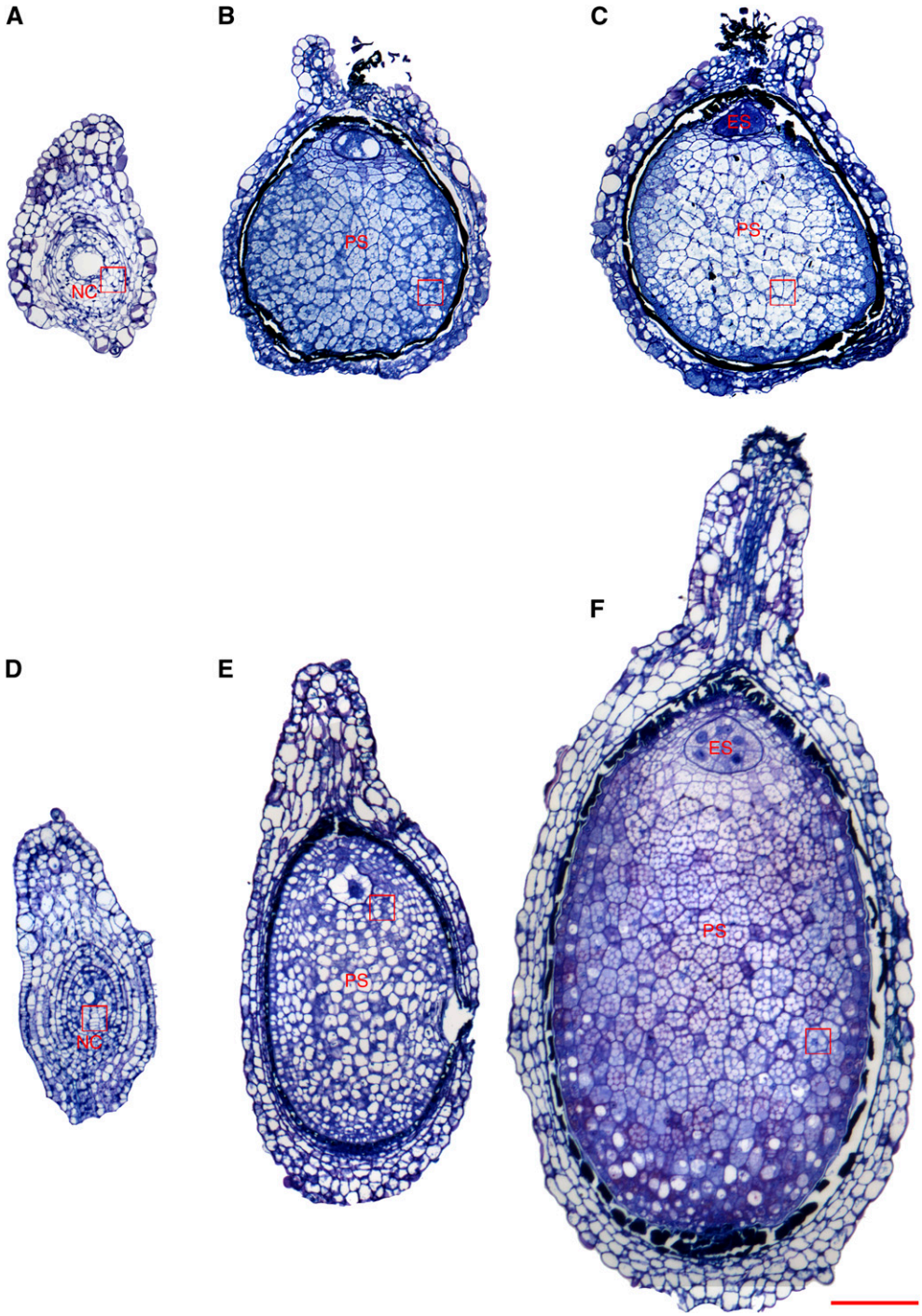

Fig. 8. Seed development in (A–C) *Peperomia jamesoniana* and (D–F) *P. hispidula*. Red boxes depict the approximate region within the nucellus used to create Fig. 8. Scale bar = 100 µm. *Figure abbreviations*: ES, endosperm; NC, nucellus; PS, perisperm. (A) An ovule of *P. jamesoniana* with a 16-nucleate female gametophyte. (B) Ovule of *P. jamesoniana* at fertilization. (C) The most mature seed of *P. jamesoniana* that we observed. This seed contains eight endosperm cells, and the zygote has not yet divided. (D) Ovule of *P. hispidula* with an eight-nucleate female gametophyte. (E) Ovule of *P. hispidula* at fertilization. (F) Seed of *P. hispidula* with 16 endosperm cells and a four-celled embryo.

create endosperm. As the number of accessory and synergid cells goes down, the number of polar nuclei in the central cell will go up, and vice-versa.

The 16 nuclei that comprise female gametophytes of *Peperomia* are initially positioned within four tetrahedrally arranged nuclear quartets. Nuclear quartets have long been viewed as the foundation of angiosperm female gametophyte developmental diversity (Favre-Ducharte, 1977; Battaglia, 1989; Haig, 1990), and recent comparative studies in ancient angiosperm lineages indicate that these tetrads of nuclei are fundamentally modular

entities (Friedman and Williams, 2003; Williams and Friedman, 2004). The modular hypothesis states that the plesiomorphic angiosperm female gametophyte developmental module proceeds through three critical ontogenetic stages: (1) positioning of a single nucleus within a developmentally autonomous cytoplasmic domain of the female gametophyte, (2) two free-nuclear mitoses in each cytoplasmic domain to yield four nuclei, and (3) the partitioning of three nuclei per domain into uninucleate cells and the fourth nucleus to the common cytoplasmic space of the central cell of the female gametophyte (Friedman and Williams 2003).

According to the basic tenets of the modular hypothesis, tetrads of nuclei in female gametophytes of *Peperomia* can be viewed as autonomous developmental units. The plesiomorphic developmental fate of three nuclei from each developmental module is to contribute to a set of three accessory cells or an egg plus two synergid cells, while the fourth nucleus from each module is contributed to the common cytoplasm of the central cell where it will function as a polar nucleus. Female gametophytes of *Peperomia* contain four tetrads/developmental modules, and in its plesiomorphic manifestation, this developmental pathway should produce a female gametophyte with four polar nuclei and 12 peripheral cells (nine accessory cells and a three-celled egg apparatus) at maturity. Martinoli (1948) and Fisher (1914) described this cellular configuration in *P. maculosa* and *P. verticillata*, respectively, and these female gametophytes possess the lowest ploidy (4N) central cells in *Peperomia* (Table 2).

Every other cellular configuration described in *Peperomia* includes the formation of a female gametophyte with more than four polar nuclei and fewer than 12 peripheral cells (Table 2). If we assume that these alternative cellular configurations are also produced within a modular framework, it is straightforward to imagine how ontogenetic modifications to the plesiomorphic cellularization/compartmentalization pathway could result in the absence of cell walls around individual nuclei, and different female gametophyte cellular configurations at maturity. If two nuclei from each module become partitioned into peripheral cells, and two nuclei are contributed to the central cell, a nine-celled structure will be produced with eight peripheral cells and an 8N central cell. This is the same nine-celled configuration reported in many classical reviews but that has never been confirmed with micrographic evidence (Table 2). If wall formation events are altered such that only one nucleus from each developmental module becomes a peripheral cell and three nuclei from each module are placed in the central cell, a five-celled female gametophyte would be produced with four peripheral cells and a 12N central cell. This female gametophyte cellular configuration has been reported in four species of *Peperomia* (Table 2). In each of these examples, each developmental module behaves similarly.

Developmental modules might evolve ontogenetic modifications in wall formation autonomously. For example, three of the developmental modules in female gametophytes of *P. hispidula* appear to have lost the ability to form peripheral cells at all, while the fourth developmental module (the micropylar-most module) retains a 2+2 pattern of wall formation that produces two peripheral cells (an egg and a synergid) and contributes two nuclei to the central cell. The mature three-celled female gametophyte thus has a 14N central cell (Figs. 5A, 5B, 6C, 6D). The more common 10-celled female gametophytes of *Peperomia* also appear to be the result of autonomous ontogenetic modifications to individual developmental modules. Three developmental modules have a 2+2 pattern of compartmentalization (two peripheral cells and a contribution of the other two nuclei to the central cell), while the developmental module that gives rise to the egg apparatus retains the plesiomorphic 1+3 compartmentalization pathway.

It is important to note that there is no evidence to suggest that cell walls in female gametophytes of *Peperomia* degrade from an initial 1+3 arrangement into an alternate cellular configuration. Embryological data describing female gametophyte development in *Peperomia* indicate that nuclei become partitioned into their final cellular configuration at cytokinesis, suggesting that ontogenetic modification appears to occur at the level of the developmental module itself rather than additional developmental steps being appended onto the plesiomorphic developmental pathway.

TABLE 3. Patterns of megasporogenesis and embryo-nourishing strategies in magnoliids.

Taxon	Megasporogenesis	Embryo-nourishing strategy	Relevant citations
Magnoliales	Monosporic	Endosperm	Davis, 1966
Laurales	Monosporic	Endosperm	Davis, 1966
Cannelales	Monosporic	Endosperm	Davis, 1966
Piperales			
Aristolochiaceae			
Aristolochia	Monosporic	Endosperm	Jacobsson-Stiasny 1918; Johri and Bhatnagar, 1955; González and Rudall, 2003; Madrid and Friedman, 2008
Thottea	Monosporic	Endosperm	Nair and Narayanan, 1961; González and Rudall, 2003
Asarum	Monosporic	Endosperm	Hofmeister, 1858; Jacobsson-Stiasny, 1918; Wyatt, 1955; González and Rudall, 2003
Saruma	Monosporic	Endosperm	González and Rudall, 2003
Lactoridaceae			
Lactoris	Monosporic	Endosperm	Tobe et al., 1993; González and Rudall, 2003
Hydnoraceae			
Prosopanche	Bisporic	Unknown	Chodat, 1916; Coccuci, 1976
Hydnora	Uncertain*	Unknown	Dastur and Bombay, 1921
Saururaceae			
Houttuynia	Monosporic	Perisperm	Murty, 1960; Raju, 1961
Anemopsis	Bisporic	Perisperm	Quibell, 1941; Raju, 1961
Gymnotheca	Unknown	Unknown	
Saururus	Bisporic	Perisperm	Raju, 1961
Piperaceae			
Verhuellia	Unknown	Unknown	
Peperomia	Tetrasporic	Perisperm	Johnson, 1900a, 1914; Martinoli, 1948
Piper	Tetrasporic	Perisperm	Maheshwari and Gangulee, 1942; Joshi, 1944; Swamy, 1944; Maugini, 1953; Murty, 1959; Yoshida, 1960; Kanta, 1962
Manekia	Tetrasporic	Unknown	Arias and Williams, 2008
Zippelia	Tetrasporic	Perisperm	Lei et al., 2002

The* Peperomia*-type female gametophyte ontogeny—The conceptual framework we outline describing female gametophyte developmental evolution in *Peperomia* predicts that a nine-celled configuration could exist; however, our review of the available literature (Table 2) and data from *P. dolabriformis* and *P. jamesoniana* (Figs. 3–6) do not provide any empirical support for this configuration—the so-called Peperomia-type of textbook fame. We confirm and extend the findings of recent investigations that describe 10-celled female gametophytes in *Peperomia* with two synergids, one egg cell, six accessory cells, and a 7N central cell (Figs. 3, 4, 6) and also of a three-celled configuration in *P. hispidula*. In the absence of evidence supporting the existence of nine-celled female gametophytes in *Peperomia*, it is probably best to describe the Peperomia-type ontogeny as a tetrasporic developmental pathway that goes on to produce four tetrahedrally arranged nuclear tetrads. These tetrads have inter- and intraspecific variation in wall formation events that lead to many cellular configurations at maturity (Table 2), of which a 10-celled configuration is likely to be the most common.

Interestingly, Peperomia-type female gametophyte development appears to have evolved at least two other times in angiosperm history (Maheshwari, 1950; Davis, 1966). Nine- and 10-celled Peperomia-type female gametophytes have been reported in *Gunnera* (Gunneraceae) (Ernst, 1908; Samuels, 1912; Modilewski, 1908), and three-celled Peperomia-type female gametophytes have been reported in *Acalypha* (Euphorbiaceae) (Thathachar, 1952). In light of our findings, female gametophyte development in *Gunnera* and *Acalypha* may warrant careful reinvestigation.

Evolution of female gametophyte ontogeny and embryo-nourishing strategies in Piperales—The genetic constitution of endosperm is directly tied to patterns of female gametophyte ontogeny that determine the nuclear composition of the central cell (Friedman et al., 2008). A large body of theory suggests that there may be potentially adaptive benefits for endosperms with higher levels of ploidy and heterozygosity and reduced levels of interparental and/or parent–offspring genetic conflict in the seed (Westoby and Rice, 1982; Queller, 1983, 1989. 1994; Wilson and Burley, 1983; Bulmer, 1986; Haig, 1986, 1987, 1990; Haig and Westoby, 1988, 1989a, b). Therefore, patterns of female gametophyte development that result in endosperms with progressively higher ploidies, higher levels of heterozygosity, and lower genetic conflict ought to be selectively favored over developmental patterns that produce endosperms with lower ploidies, lower heterozygosities, and higher genetic conflict (Friedman et al., 2008).

Polygonum-type female gametophytes are plesiomorphic in magnoliids and are produced from the mitotic derivatives of one megaspore, while Peperomia-type female gametophytes are formed from the mitotic derivatives of four meiotically related megaspore nuclei. Endosperms produced from the central cells of Peperomia-type female gametophytes will be highly heterozygous, relative to endosperm genetic constructs that result from the fertilization of the central cell of a monosporic Polygonum-type female gametophyte. Endosperm ploidy in *Peperomia* species ranges from pentaploid to decapentaploid, and calculations of genetic conflict for endosperms derived from Peperomia-type female gametophytes are among the lowest in angiosperms (Friedman et al., 2008). It is therefore reasonable to posit that the various genetic constructs of endosperms produced by Peperomia-type female gametophyte ontogenies may have been selectively favored during their evolutionary history and that this genetic selective advantage could have driven the evolutionary radiation of female gametophyte ontogeny in *Peperomia* and more generally throughout the Piperales. However, this hypothesis assumes that endosperm plays a dominant embryo-nourishing role in the seed, that endosperm is under significant selection, and that endosperm genetic constructs in *Peperomia* have evolved to become more heterozygous with higher ploidies and lower levels of genetic conflict. Recently published species-level phylogenetic analyses in *Peperomia* (Wanke et al., 2006, 2007b) will allow future embryologists to

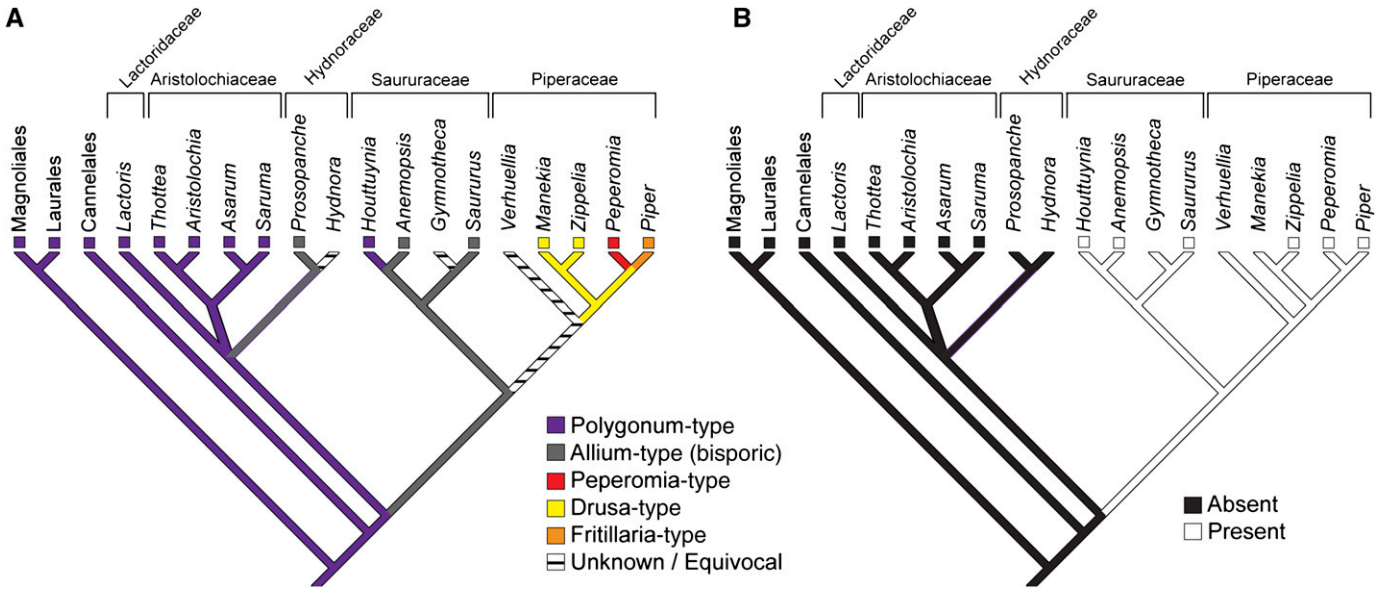

Fig. 9. (A) Female gametophyte and (B) perisperm evolution in magnolliids. Female gametophyte ancestral state reconstruction from Madrid and Friedman (2009). Phylogenetic relationships from Nickrent et al. (2002), Zanis et al. (2003), Neinhuis et al. (2005), Wanke et al. (2007a, b).

specifically discuss evolutionary transitions between the myriad subtypes of Peperomia-type female gametophytes, and their corresponding levels of endosperm ploidy, endosperm heterozygosity, and genetic conflict. However, these genetic properties of endosperm in *Peperomia* are only relevant if they confer a selective advantage to their compatriot embryo in the seed.

In most angiosperms, endosperm acquires nutrients from the maternal plant on behalf of its compatriot embryo, stores these nutrients, and ultimately transfers them to the developing embryo. In *Peperomia*, endosperm never develops beyond a few dozen cells (Fig. 7). Endosperms in *Peperomia* are densely cytoplasmic with prominent cytological features (just as in other angiosperms), but the majority of seed volume is taken up by maternal nucellus tissue that functions as an embryo-nourishing perisperm (Hill, 1906; Johnson, 1905, 1914; Fisher, 1914). This perisperm stores nutrients for the embryo until germination, when it transfers nutrients through the endosperm to the embryo (Johnson, 1914; Batygina, 2006). Thus, nutrient acquisition and nutrient storage functions in *Peperomia* appear to be carried out by the perisperm, but endosperm (which surrounds the developing embryo) presumably retains a basic nutrient transfer role at least during early stages of embryogeny.

Interestingly, all members of Piperales with bisporic and tetrasporic patterns of female gametophyte development have reduced endosperms and large genetically maternal perisperms (Table 3). The majority of Piperales with polysporic female gametophytes are found in Saururaceae and Piperaceae (Table 3), and unweighted parsimony analysis of embryo-nourishing strategies in Piperales reveals that the origin of polyspory in these two clades (Fig. 9A) correlates with the origin of perisperm in the common ancestor of Saururaceae + Piperaceae (Fig. 9B).

Given the developmental and functional relationship between female gametophyte ontogeny, the genetic constitution of endosperm, and the embryo-nourishing roles of endosperm and perisperm, this correlated onset of a significant diversification of all three structures (Fig. 9) could be indicative of an underlying evolutionary developmental relationship. A potential evolutionary scenario is that with the transfer of significant nutrient acquisition and nutrient storage functions from endosperm to perisperm in the common ancestor of Saururaceae + Piperaceae, selection on the genetic and physiological properties of endosperm (and thus selection on patterns of female gametophyte development) may have been relaxed. Therefore, the overall pronounced developmental radiation of female gametophyte ontogeny in Saururaceae and Piperaceae might be the result of an innovation, namely perisperm, in the common ancestor of Saururaceae + Piperaceae that diminished the intensity of selection on the nutrient acquisition and nutrient storage functions of endosperm.

Even if the origin of perisperm in the common ancestor of Piperaceae + Saururaceae led to a relaxation of selection on female gametophyte and endosperm development and genetic patterns, it is possible that during its earliest stages of development the embryo may be nearly exclusively reliant on the endosperm. Thus, early embryo ontogeny may still be closely regulated by the genetic and physiological relationships that exist between the embryo and endosperm. However, as the embryo enlarges and the minute endosperm is consumed, the perisperm may supplant the endosperm and become the primary tissue interacting with the developing embryo.

Conclusions—More than a century after its initial discovery, the nine-celled Peperomia-type female gametophyte widely depicted in embryology texts, in actuality, appears to lack empirical evidence of its very existence. Instead, micrographic evidence supports the existence of several female gametophyte cellular configurations in *Peperomia*, ranging from those with 13 cells to as few as three cells, with a 10-celled configuration appearing to be common (Table 2).

Nevertheless, *Peperomia* displays one of the most extreme examples of female gametophyte structural diversity in angiosperms, and this diversification has occurred within a larger evolutionary radiation of female gametophyte ontogeny throughout Piperales as a whole. Given the downstream functional properties of female gametophyte ontogeny in angiosperm breeding systems and reproduction, it is likely that further investigation of female gametophyte ontogeny and embryo nourishing strategies in Piperales will yield additional evolutionary historical insights that revolve around the interplay of developmental processes, genetic conflict, and natural selection.

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