DOUBLE FERTILIZATION IN GNETALES: IMPLICATIONS FOR UNDERSTANDING REPRODUCTIVE DIVERSIFICATION AMONG SEED PLANTS

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The coupled processes of double fertilization and postfertilization endosperm formation have long been viewed as important and synapomorphic features of flowering plants. Recent developmental studies of fertilization in the nonflowering seed plants Ephedra and Gnetum clearly document a regular process of double fertilization. The condition for Welwitschia remains unknown. Unlike angiosperms, the product of the second fertilization event in Ephedra and Gnetum is diploid and expresses the developmental program of an embryo. Explicit criteria for the evaluation of evolutionary homology indicate that the processes of double fertilization in Gnetales and angiosperms are homologous, having first evolved in a common ancestor of these two lineages. It is hypothesized that the second fertilization product initially yielded a supernumerary embryo genetically identical to the normal embryo. This rudimentary process is expressed in relatively unmodified form in Ephedra. Other reproductive features of Ephedra that are conserved from the common ancestor of angiosperms and Gnetales are a monosporic female gametophyte with two or more eggs and the partial allocation of maternal resources, for subsequent embryo nourishment, to a large cellular female gametophyte in advance of fertilization. In Gnetum, evolutionary modification of reproduction resulted in a paedomorphic female gametophyte that is fertilized at a “juvenilized” free nuclear stage of development. In Gnetum gnemon, egg cells are not formed and maternal provisioning of the embryo-nourishing female gametophyte takes place entirely after fertilization. The biological significance of double fertilization that does not form endosperm, in Ephedra and Gnetum, is currently unknown. This process may be biologically neutral. However, due consideration must be given to the hypothesis that the second fertilization product in Ephedra and Gnetum, while expressing a structural/developmental program of an embryo, may “behave” as a rudimentary and cryptic endosperm and assist with the development of the normal embryo.

The Gnetales have been called the lure and the despair of the morphologist. (Thompson 1916)

Mais les Gnetophytes se présentent au botaniste, depuis longtemps, comme un ensemble d’un intérêt exceptionnel et comme une énigme particulièrement irritante. (Martens 1971)

Introduction

The Gnetales have intrigued developmental and evolutionary botanists for well over a century. Collectively, the nonflowering seed plants Ephedra, Gnetum, and Welwitschia possess more evolutionarily derived (apomorphic) features than any other group of land plants (Doyle and Donoghue 1992) and exhibit many angiosperm-like features. Much of the historical ambiguity surrounding the comparative and evolutionary interpretation of biological features of the Gnetales can be traced to a relative dearth of anatomical and morphological studies of Ephedra, Gnetum, and Welwitschia, a minimal fossil record that has hindered analysis of character evolution within the Gnetales and the absence of a well-resolved phylogenetic framework in which to interpret the structural features of the Gnetales vis à vis other seed plant clades, including the angiosperms.


Of equal significance, phylogenetic analyses of diverse data sets have yielded a broad consensus about the phylogenetic relationships of the Gnetales. All analyses of gene sequence data (Martin and Dowd 1986, 1991; Zimmer et al. 1989; Hamby and Zimmer 1992; Hasebe et al. 1992a, 1992b; Chase et al. 1993; Doyle et al. 1994; Chaw et al. 1995; Goremykin et al. 1996) and most cladistic analyses of morphological and anatomical features (Hill and Crane 1982; Crane 1985a, 1985b; Doyle and Donoghue 1986, 1992; Locicore and Stevenson 1990; Doyle et al. 1994; Rothwell and Serbet 1994) support the hypothesis that extant Gnetales are monophyletic, with Ephedra basal and Gnetum and Welwitschia as derived sister groups. Moreover, most of these analyses clearly indicate that Gnetales are the most closely related extant seed plants to flowering plants (figs. 1, 2). A minority of phyl-

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genetic analyses have suggested that Gnetales may be paraphyletic (Nixon et al. 1994; Taylor and Hickey 1996), although these studies still conclude that *Ephedra*, *Gnetum*, and *Welwitschia* are the three most closely related extant taxa to angiosperms (fig. 1). Two phylogenetic analyses have provided weak evidence of the monophyly of "gymnosperms" (Hasebe et al. 1992b; Goremykin et al. 1996). The extinct lineages Bennettitales, Caytoniales, and *Pentoxylon* have all been suggested to share recent common ancestors with the angiosperms and Gnetales. The precise interrelationships of these taxa, however, remain uncertain (fig. 1).

Insight into the evolutionary relationships of *Ephedra*, *Gnetum*, and *Welwitschia* has led to a renewed interest in their biology and made possible the analysis of comparative vegetative and reproductive characters within a phylogenetic context. This article reviews re-
Fig. 2 Phylogenetic relationships of the major groups of extant seed plants in which the Gnetales are monophyletic and are the closest extant relatives of angiosperms. Relationships between the basal extant seed plants (cycads, conifers, and Ginkgo) are currently unresolved.

cent developmental studies of sexual reproduction in Ephedra and Gnetum and the documentation of regular double fertilization events that yield two diploid zygotes in members of these two taxa. It is not known whether double fertilization events occur in Welwitschia or extinct seed plant lineages that may be closely related to angiosperms (e.g., Bennettitales, Caytoniales, and Pentoxylon). Because the fertilization biology of extinct seed plant lineages is essentially unknowable, these taxa will not be further considered.

Evidence is presented that double fertilization events in Ephedra, Gnetum, and angiosperms are evolutionarily homologous and were inherited from a common ancestor. In its original and rudimentary form in the common ancestors of Gnetales and flowering plants, double fertilization yielded twin diploid embryos. Comparative reproductive biology is integrated with inclusive fitness theory to demonstrate how this rudimentary process of double fertilization that did not yield an embryo-nourishing tissue could have been modified to produce an endosperm in the flowering plant stem lineage. Finally, the biological significance of double fertilization in Ephedra and Gnetum is examined.

**Double fertilization in Ephedra**

Reproduction in Gnetales has been reviewed by Maheshwari and Vasil (1961), Lehmann-Baerts (1967), Martens (1971, 1973), Friedman (1990b, 1992b), and Carmichael and Friedman (1996). Development of the female gametophyte of Ephedra is fundamentally similar to that of the extant nonflowering seed plants cycads, conifers, and Ginkgo. The female gametophyte is monosporic and initially undergoes a period of free nuclear development followed by centripetal wall formation (alveolarization). A cellular phase of growth ensues, and the female gametophyte matures into a substantial multicellular organism with two or more archegonia (fig. 3; Martens 1971). In Ephedra, development of the embryo-nourishing female gametophyte is largely completed prior to fertilization. At the time of fertilization in *E. nevadensis* and *E. trifurca*, each egg cell contains an egg nucleus and a ventral canal nucleus (figs. 5, 16; Friedman 1990a, 1992a, 1992b).

Within 24 h of pollination, growth of a pollen tube in Ephedra results in the deposition of two sperm nuclei from a binucleate sperm cell into an archegonium (Moussel 1983). In *E. nevadensis* and *E. trifurca*, one sperm nucleus migrates to the egg nucleus, which invaginates as contact is established between the gamete nuclei (figs. 7, 16). Ultimately, fusion is completed and yields a typical diploid zygote nucleus (Friedman 1990a, 1992). Concurrent with the first fertilization event, a process of fusion is initiated between the ventral canal nucleus and the second sperm nucleus. The ventral canal nucleus invaginates as contact is made with the second sperm nucleus and these two nuclei fuse (fig. 7; Friedman 1990a, 1990b, 1991, 1992b).

At the end of double fertilization in *E. nevadensis* and *E. trifurca*, two diploid nuclei reside within the cytoplasm of the former egg cell: a “normal” zygote nucleus, derived from the fertilization of the egg nucleus by a sperm nucleus, and a “supernumerary” zygote nucleus, formed from the fusion of the ventral canal nucleus with a second sperm nucleus (figs. 8, 16; Friedman 1990b, 1991). In *E. trifurca*, microspectrophotometric data confirm that both products of double fertilization are diploid and that each nucleus contains a 4C quantity of DNA (Friedman 1991). The fates of the first and second fertilization products in *E. nevadensis* and *E. trifurca* are identical: each diploid nucleus will ultimately form embryos (Friedman 1992b, 1994).

Shortly after the completion of double fertilization in Ephedra, synchronous mitotic divisions of both the normal zygote nucleus and the supernumerary zygote nucleus result in the production of two sets of daughter nuclei. A second wave of mitosis is initiated by each of the four nuclei, typically yielding eight nuclei that are arranged in two groups within the former egg cell cytoplasm: a basal set of four nuclei derived from the normal zygote nucleus and an apical set of four nuclei descended from the supernumerary zygote nucleus (Friedman 1992a, 1992b). Subsequent to the free nuclear proliferation of each of the two zygotes in Ephedra, elaborate arrays of microtubules (phragmoplasts) form around each of the individual nuclei (Friedman 1994) and cell walls are deposited (Land 1907; Khan 1943; Lehmann-Baerts 1967; Moussel 1977). Cellularization typically results in the production of two sets of four uninucleate cells (Friedman 1994). Because each of the eight cells establishes an individual proembryo, the separate products of the normal zygote and the supernumerary zygote are referred to as “clonal zygotes” in Ephedra (fig. 12; Friedman 1994).
Figs. 3, 4  Fig. 3, Longitudinal section of female gametophyte of *Ephedra* near the time of fertilization. The gametophyte is monosporic in origin, undergoes free nuclear development, cellularizes, and forms two or more archegonia in advance of fertilization. Two archegonia are visible. Fig. 4, Longitudinal section of female gametophyte of *Gnetum gnetanum* just prior to fertilization. The gametophyte is tetrasporic in origin, and at the time of fertilization the micropylar end contains a parietal band of free nuclei with a single large central vacuole. The chalazal end of the female gametophyte is also free nuclear but lacks a central vacuole. *AR*, archegonium; *CE*, chalazal end of female gametophyte; *FG*, female gametophyte; *FN*, nuclei of female gametophyte; *ME*, micropylar end of female gametophyte; *N*, nucellus. Scale bars = 100 μm.
In *Ephedra*, each of the proembryos initiates a pattern of filamentous development (fig. 13; Berridge and Sanday 1907; Land 1907; Khan 1943; Lehmann-Baerts 1967; Moussel 1977; Friedman 1990b). In *E. trifurca* and *E. nevadensis* the first cell plate within a proembryo forms in conjunction with or slightly after the first mitosis (Land 1907; Lehmann-Baerts 1967; Moussel 1977; Friedman 1994). In either case, free nuclear development, defined as successive mitotic divisions without intervening cytokinesis (Chamberlain 1935, p. 405), does not occur during normal development of individual proembryos of *Ephedra*.

As a consequence of the formation of two or more archegonia per female gametophyte in *Ephedra*, multiple double fertilization events may occur within a single ovule, a phenomenon referred to as “complex simple polyembryony” (Friedman 1995). Since each double-fertilized archegonium yields eight clonal zygotes/proembryos and each of the two or more egg cells within a female gametophyte may be double fertilized, 16, 24, or a greater number of clonal zygotes/proembryos may be initiated within a single seed (Friedman 1995). However, only one embryo will ultimately survive during the maturation of the seed.

Although double fertilization is a regular feature of reproduction in *E. nevadensis* and *E. trifurca*, the second fertilization product does not develop into a specialized embryo-nourishing structure, as in angiosperms. In *Ephedra*, embryo nourishment depends upon maternal reserves accumulated by the large cellular female gametophyte (fig. 3), as in extant basal seed plants such as cycads, conifers, and Ginkgo.

**Double fertilization in Gnetum**

There have been many studies of reproduction in *Gnetum* over the course of the past century (reviewed in Maheshwari and Vasil 1961; Carmichael and Friedman 1996). Although unconfirmed, a few of these studies suggested that double fertilization events might occur in certain species of *Gnetum* (Lotsy 1899; Vasil 1959; Sanwal 1962). However, “investigators differ in their descriptions, even for the same species” (Gifford and Foster 1989), and most aspects of female gametophyte development, fertilization biology, and early embryo development in *Gnetum* have been poorly understood. As a consequence, the fertilization biology of *Gnetum gnemon* was carefully reinvestigated (Carmichael and Friedman 1995, 1996). It was predicted that if a pattern of double fertilization evolved or was present in the common ancestors of the Gnetales, twin
fertilization events would characterize species of *Gnetum*, barring any character reversals.

From the initial stages of megasporogenesis to the final formation of female gametophyte, the female gametophyte of *G. gnemon* is unique among seed plants. Female gametophyte development begins with the differentiation of several megaspores per ovule, as in other species of *Gnetum* (Waterkeyn 1954; Vasil 1959), and this is probably a synapomorphy of the genus (Carmichael and Friedman 1996). Meiosis is free nuclear and megasporogenesis results in the production of several tetrasporic coenomesporas within an individual ovule (Carmichael and Friedman 1996). In *G. gnemon*, development of each coenocytic megaspore is free nuclear and similar to the initial free nuclear phase of female gametophyte development among basal seed plants (cycads, *Ginkgo*, conifers) and *Ephedra*. Typically, only a single female gametophyte per ovule undergoes significant development; the remaining female gametophytes are crushed and eventually degenerate (Carmichael and Friedman 1996).

The successful/dominant female gametophyte of *G. gnemon* continues to undergo extensive free nuclear development similar to the female gametophytes of all nonflowering seed plants. This phase of development lasts for approximately 2 wk and concludes just prior to fertilization, when approximately 1000 nuclei have formed within the coenocytic female gametophyte (fig. 4). In *G. gnemon*, no archegional structures or egg cells differentiate in association with sexual maturation of the female gametophyte (Carmichael and Friedman 1995, 1996). Instead, any of the free nuclei at the micropylar end of the female gametophyte appear to be able to participate in fertilization events.

The lack of differentiated egg cells in *G. gnemon* and the free nuclear nature of the female gametophyte at sexual maturity can be ascribed to the evolutionary effects of heterochrony. Compared with conifers, cycads, and *Ginkgo*, where free nuclear development is followed by cellularization and the subsequent formation of defined egg cells, the female gametophyte in *G. gnemon* becomes sexually mature at a juvenile stage of somatic ontogeny (Carmichael and Friedman 1995). In *Welwitschia*, archegonia are also absent and free nuclei of the female gametophyte are reported to have little ability to participate in fertilization events (Martens 1973). The loss of differentiated egg cells and the involvement of free female nuclei in sexual reproduction in *Gnetum* and *Welwitschia* are unique among land plants and are likely to be synapomorphies of these two taxa.

At the time of fertilization, the female gametophyte of *G. gnemon* is approximately 1 mm in length and consists of an enlarged micropylar region and a constricted chalazal region (figs. 4, 16). The micropylar portion of the sexually mature female gametophyte includes a large central vacuole and a parietal band of cytoplasm with free nuclei (figs. 4, 6). It is within the coenocytic micropylar region that fertilization takes place. The chalazal end of the female gametophyte contains many free nuclei but no central vacuole (Carmichael and Friedman 1996).

Within 5 d of pollination, pollen tubes containing binucleate sperm cells are found in close proximity to the female gametophyte in *G. gnemon*. Shortly thereafter, one to several pollen tubes enter the functional female gametophyte at the micropylar pole (figs. 6, 16). Pollen tube entry into the female gametophyte entails a significant protrusion into the coenocytic cell followed by the migration of female cytoplasm containing nuclei around the apex of the pollen tube (Carmichael and Friedman 1996; figs. 9, 16).

Discharge of the binucleate sperm cell from a pollen tube in *G. gnemon* leads to the deposition of two sperm nuclei within the cytoplasm of the female gametophyte (fig. 10). Each sperm nucleus fuses rapidly with a female nucleus to yield two zygote nuclei that lack cell walls. The zygote nuclei formed from double fertilization events in *G. gnemon* can be clearly distinguished from unfertilized female nuclei by size, appearance, and DNA content (figs. 11, 16; Carmichael and Friedman 1995).

Shortly after double fertilization in *G. gnemon*, a spherical cell wall forms around each of the previously unwalled zygote nuclei (figs. 11, 14). This process is fundamentally similar to the cellularization of each of the eight nuclei (clonal zygotes) derived from double fertilization in *Ephedra* and involves the cleavage of neighboring female cytoplasm into enucleate as well as nucleate cells. Germination of each proembryo begins with the formation of a tubular primary suspensor (fig. 15). In *G. gnemon*, proembryo development is ab initio cellular: free nuclear development does not occur (Carmichael and Friedman 1996). If two or more pol-

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**Figs. 7-11** All sections viewed with fluorescence microscopy (DNA stained with DAPI). Fig. 7, Double fertilization in *Ephedra trifurca* (longitudinal section). A sperm nucleus is fusing with an egg nucleus, while a second sperm nucleus has established contact with the ventral canal nucleus. Scale bar = 50 μm. Fig. 8, End result of double fertilization in *E. trifurca* with two diploid zygotes within the former egg cell (longitudinal section). The normal zygote from the fusion of a sperm nucleus with the egg nucleus is situated at the chalazal end of the former egg cell. The supernumerary zygote is positioned within the micropylar end of the former egg cell. Scale bar = 50 μm. Fig. 9, Double fertilization in *Gnetum gnemon*. The pollen tube (seen in transverse section and delimited by arrows) is surrounded by free nuclei of the female gametophyte, two of which will be fertilized by sperm from a binucleate sperm cell. Scale bar = 10 μm. Fig. 10, Two sperm nuclei in *G. gnemon* within a pollen tube just prior to release into the cytoplasm of the coenocytic female gametophyte. Scale bar = 10 μm. Fig. 11, End result of double fertilization in *G. gnemon* with two diploid zygotes at the tip of a pollen tube. EC, egg cell; EN, egg nucleus; FG, female gametophyte; FN, nuclei of female gametophyte in *Gnetum*; JC, jaccard cells of female gametophyte in *Ephedra*; NZ, normal zygote; PT, pollen tube; SP1, first sperm nucleus; SP2, second sperm nucleus; SZ, supernumerary zygote; VCN, ventral canal nucleus; Z1, first zygote; Z2, second zygote.
Fig. 12-15  Fig. 12, Two of eight unicellular proembryos (clonal zygotes) surrounded by enucleate cellular structures within the former egg cell in *Ephedra nevadensis*. Scale bar = 50 μm. Fig. 13, Initiation of filamentous growth in a proembryo of *Ephedra*. Fig. 14, Two unicellular zygotes formed from double fertilization in *Gnetum gnemon*. The zygotes are surrounded by nucleate and enucleate cellular structures that form from the neighboring cytoplasm of the female gametophyte following double fertilization. Scale bar = 50 μm. Fig. 15, Initiation of filamentous growth in a proembryo of *G. gnemon*. Scale bar = 10 μm. CFC, cellularized female cytoplasm; CZ, clonal zygote; FG, female gametophyte; FPE, filamentous proembryo; JC, jacket cells; Z, zygote.
len tubes enter a female gametophyte, most—if not all—will discharge the contents of their binucleate sperm cells and produce two zygotes. As in *Ephedra* and other nonflowering seed plants, only a single embryo typically will mature within a seed.

After fertilization in *G. gnemon*, numerous multinucleate cells form in the previously free nuclear chalazal portion of the female gametophyte. The multiple nuclei of each coenocytic cell then fuse to form a single highly polyploid nucleus (confirmed by microspectrophotometry) within each cell of the chalazal end of the female gametophyte (Carmichael and Friedman 1995). For the next 2–3 mo, the chalazal region undergoes cellular growth and develops into a large embryo-nourishing tissue. Thus, although fertilization in *G. gnemon* occurs at a free nuclear stage of development, the female gametophyte goes on to complete the cellular phase of somatic development similar to that expressed among the female gametophytes of more primitive seed plants (fig. 17). *Gnetum gnemon* and probably other species of *Gnetum* are the only nonflowering seed plant taxa in which maternal commitment of embryo-nourishing provisions is an exclusively postfertilization phenomenon. This is directly analogous to the evolution of postfertilization development of an embryo-nourishing endosperm in flowering plants (figs. 17, 18; Carmichael and Friedman 1995).

**Determination of the homologies of double fertilization in *Ephedra*, *Gnetum*, and angiosperms**

There are three explicit and fundamentally different ways in which the expression of double fertilization events in *Ephedra*, *Gnetum*, and angiosperms can be explained from an evolutionary perspective (figs. 19–21): (1) Double fertilization events involving the interactions of sperm from a single pollen tube with female nuclei within the female gametophyte evolved three times—separately in *Ephedra*, *Gnetum*, and angiosperms (fig. 19). (2) Double fertilization events in-
volving the interactions of sperm from a single pollen tube with female nuclei within the female gametophyte evolved twice—once in a common ancestor of Gnetales and once in a common ancestor of angiosperms. As such, double fertilization in Gnetum and Ephedra is evolutionarily homologous, but such events represent a convergence with angiosperms (fig. 20). (3) Double fertilization events involving the interactions of sperm from a single pollen tube with female nuclei within the female gametophyte evolved once in a common ancestor of Gnetales and angiosperms. As such, double fertilization is evolutionarily homologous in Ephedra, Gnetum, and angiosperms (fig. 21). In order to evaluate the issue of evolutionary homology among the double fertilization processes represented in Ephedra, Gnetum, and angiosperms, explicit developmental and genetic criteria must be employed (Friedman 1990a, 1992, 1994, 1995). These criteria clearly favor the hypothesis that double fertilization events in Ephedra and Gnetum are evolutionarily homologous and that the pattern of double fertilization present in angiosperms is very likely to have evolved from a rudimentary condition similar to that expressed in the Gnetales.

**Homology of double fertilization in Ephedra and Gnetum**

In spite of the significant differences in female gametophyte development and organization in Ephedra and Gnetum, double-fertilization events in these two taxa are developmentally similar. In *E. nevadensis*, *E. trifurca*, and *G. gnemon* the two sperm that participate in double fertilization are derived from a single binucleate sperm cell (Friedman 1994; Carmichael and Friedman 1996). In both genera, double fertilization yields two diploid products that will ultimately develop into embryos. While it is possible that double fertilization events in Ephedra and Gnetum evolved separately (and are homoplasious), the total evidence is most congruent with the interpretation that double fertilization in these two taxa is homologous and was present in a common ancestor of the Gnetales.

When reproductive development in *G. gnemon* is compared with Ephedra, it is evident that many modifications have occurred during the evolution and diversification of the Gnetales. In Gnetum, the basic structure of the female gametophyte has undergone radical modification in structure and development (tetrasporic origin, loss of archegonia, free nuclear con-
dation at sexual maturity), while most aspects of the female gametophyte in *Ephedra* represent plesiomorphic character states similar to those of basal seed plants. In *Gnetum*, as a consequence of the tetrasporic and free nuclear nature of the female gametophyte, the products of double fertilization will have maternal gamete contributions that are, on average, meiotically related. Hence, the two zygotes derived from double fertilization in *Gnetum* may not be genetically identical, even though they share identical paternal genomes and are formed within a single female gametophyte. Nevertheless, it is clear that a rudimentary process of double fertilization that yields two zygotes has been retained in *Gnetum*.

As a consequence of the basal phylogenetic position of *Ephedra* within the Gnetales (fig. 2) and its retention of many plesiomorphic reproductive features of seed plants (such as a monosporic female gametophyte and presence of archegonia), it is likely that most developmental attributes of double fertilization in extant *Ephedra* are relatively unchanged from the process that was present in the common ancestors of the Gnetales. It is hypothesized that in the common ancestors of Gnetales, double fertilization events involved the interactions of two sperm from a single pollen tube with the egg and its sister nucleus within an archegonium of a cellular monosporic female gametophyte. The products of double fertilization would have been two diploid zygotes that were genetically identical (as in *Ephedra*) and directly initiated embryo development (the proliferation of four clonal zygotes/proembryos from each fertilization in *Ephedra* is apomorphic for the genus) (Friedman 1994). As in all extant seed plants, only a single embryo ultimately would have matured within the ovule; embryos in excess of one would have ceased to develop.

**Comparison of double fertilization in Gnetales and angiosperms**

In order to analyze double fertilization events in angiosperms and Gnetales, it is necessary to make comparisons between the patterns of double fertilization that are likely to have been present in the common ancestors of each clade. Phylogenetically based analysis of character evolution indicates that double fertilization in *Ephedra* is very likely to be representative of the plesiomorphic condition within Gnetales, while many aspects of double fertilization in *Gnetum* are apomorphic. In angiosperms, the plesiomorphic condition for double fertilization involves a monosporic "*Polygonum*-type" embryo sac (Stebbins 1974) in which a diploid zygote and a triploid endosperm are formed following double fertilization by two sperm from a single pollen tube.

Double fertilization events in basal angiosperms and basal Gnetales (*Ephedra*) share several critical developmental relationships among the sets of male and female nuclei involved in double fertilization (table 1). In angiosperms with monosporic embryo sacs, one of the two polar nuclei with which the second sperm nucleus fuses is the sister nucleus of the egg nucleus (Thomas 1907; Brink and Cooper 1947; Huang and Russell 1992). In *Ephedra*, the ventral canal nucleus (which fuses with the second sperm) is also the sister nucleus of the egg nucleus. In both groups of seed plants, the two sperm that engage in double fertilization are typically derived from a single pollen tube (Friedman 1992b; Mogensen 1992). Thus, from a developmental perspective, double fertilization events in basal angiosperms and basal Gnetales (*Ephedra*) involve similar sets of male and female nuclei (table 1).

From a genetic perspective, the coefficients of relatedness of the first fertilization product to the second fertilization product in basal angiosperms and basal Gnetales (*Ephedra*) are identical. In angiosperms with a plesiomorphic monosporic embryo sac, the two female nuclei with which the second sperm fuses are genetically identical to the egg nucleus. In *Ephedra*, the single female nucleus involved in the second fertilization event is also genetically identical to the egg nucleus (Friedman 1992, 1994). In both groups of seed plants, the sperm involved in double fertilization are genetically identical and derived from a single pollen tube (Friedman 1992, 1994). Thus, although endosperm in basal angiosperms and the supernumerary zygote in *Ephedra* differ with respect to ploidy and developmental fate, the first fertilization product (zygote) in each of these groups of seed plants is identical (at the level of alleles) to the zygote or endosperm resulting from the second fertilization event; i.e., the co-
efficient of relatedness of the first fertilization product to the second fertilization product is 1.0 (table 1).

The key differences between double fertilization in basal Gnetales (Ephedra) and basal angiosperms are twofold. (1) In basal flowering plants, an additional female nucleus (the chalazal polar nucleus) that is not a sister nucleus of the egg nucleus (Thomas 1907; Brink and Cooper 1947; Huang and Russell 1992) participates in the second fertilization event, and through its involvement results in a triploid fusion product. (2) In angiosperms, the second fertilization product develops into endosperm, whereas in Gnetales, the second fertilization product is a supernumerary zygote that develops into an embryo.

Homology of double fertilization in Gnetales and angiosperms

Comparative developmental and genetic analyses of double fertilization in angiosperms and Gnetales (table 1) strongly support the hypothesis that a rudimentary process of double fertilization evolved once in a common ancestor of Gnetales and angiosperms and hence is a synapomorphy of the anthophyte clade (fig. 22; Friedman 1994, 1995). Similar sets of nuclei participate in double-fertilization events (i.e., two genetically identical sperm from a single pollen tube and the egg and its genetically identical sister nucleus), and the genetic outcome of double fertilization is one in which the coefficient of relatedness of the zygote to the second fertilization product is 1.0. While many points of developmental and genetic comparison between double fertilization in angiosperms and Gnetales are congruent with the hypothesis that double fertilization is homologous in these two lineages, it is important to note that this conclusion was not inevitable and that alternative findings might have falsified this hypothesis.

For example, if two sperm from different pollen tubes in Ephedra and Gnetum were involved in double fertilization, this “finding” would have argued against an interpretation of homology between double fertilization in Gnetales and angiosperms, since the genetic relatedness of the first fertilization product to the second fertilization product (coefficient of relatedness = 0.5) would have differed from the situation in basal angiosperms (coefficient of relatedness = 1.0). A similar rejection of the hypothesis of homology would have held true if double fertilization in the tetrasporic Gnetum had been determined to represent the plesiomorphic condition within Gnetales, since this would have yielded first and second fertilization products with different relatedness coefficients in the ancestors of Gnetales (most often 0.75) and in the ancestors of angiosperms (always 1.0) (table 1).

Although never explicitly stated within the literature, comparisons of precise developmental and genetic relationships within a seed have been implicitly
Table 1

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<th>Female participants in second fertilization event</th>
<th>Products</th>
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<td>Angiosperm double fertilization (pleisiomorphic) ................................ 1.00</td>
<td>Two sperm from single pollen tube</td>
<td>Sister nucleus of egg + nonsister nucleus of egg</td>
<td>Diploid embryo + diploid embryo</td>
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<tr>
<td>Ephedra double fertilization .......... 1.00</td>
<td>Two sperm from single pollen tube</td>
<td>Sister nucleus of egg</td>
<td>Diploid embryo + diploid embryo</td>
</tr>
<tr>
<td>Gnetum double fertilization .......... Usually 0.75</td>
<td>Two sperm from single pollen tube</td>
<td>Probably nonsister nucleus of “egg”</td>
<td>Diploid embryo + diploid embryo</td>
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<tr>
<td>Conifer simple polyploidy ............ 0.50</td>
<td>Two sperm from different pollen tubes</td>
<td>Nonsister nucleus of egg</td>
<td>Diploid embryo + diploid embryo</td>
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<tr>
<td>Conifer cleavage polyploidy .......... 1.00</td>
<td>No second fertilization event</td>
<td>No second fertilization event</td>
<td>Diploid embryo + more diploid embryos</td>
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</tbody>
</table>

Note. Explicit developmental and genetic criteria for analysis of homology of double fertilization in angiosperms with other phenomena that yield two or more developmental entities within an individual seed. In angiosperms with monosporic embryo sacs, the embryo and endosperm are genetically identical but differ in ploidy levels; the two sperm are from a single pollen tube and the egg nucleus and two polar nuclei are mitotically related. In Ephedra, which is basal within Gnetales, the two fertilization products are also genetically identical since the two sperm are from a single pollen tube and the egg and ventral canal nuclei are mitotic relatives. With double fertilization in Gnetum and simple polyploidy in conifers, the embryos produced within a single seed are not genetically identical to each other; hence these processes could not directly have given rise to double fertilization in angiosperms. While the genetic constructs of cleavage polyploidy are consistent with double-fertilization products in angiosperms, the developmental features are very different. Only the pleisiomorphic and rudimentary pattern of double fertilization in basal Gnetales (as manifest in Ephedra, and likely expressed in the common ancestors of angiosperms and Gnetales) could have given rise to the process of double fertilization in flowering plants. Bold typeface denotes features that are shared in common with pleisiomorphic angiosperm double fertilization.

used to exclude various reproductive phenomena as possible antecedent processes for double fertilization and endosperm formation in angiosperms. For example, simple polyploidy in conifers (where two or more embryos form within a single female gametophyte from fertilization of separate eggs by sperm from different pollen tubes) has never been suggested to be potentially homologous with the process of double fertilization in angiosperms. When simple polyploidy occurs, the nuclei involved are developmentally and genetically discordant with those that participate in pleisiomorphic angiosperm double fertilization. Simple polyploidy typically yields embryos that are genetically identical on the maternal side and hence have a coefficient of relatedness of 0.5, assuming that separate unrelated pollen tubes deliver a sperm to each archegonium (table 1).

Multiple embryos within a single seed of conifers may also result from cleavage polyploidy, which involves clonal initiation of supernumerary embryos from a single fertilization product (proembryo). While embryos produced from cleavage polyploidy are genetically identical (similar to a zygote and endosperm in basal angiosperms) with a coefficient of relatedness of 1.0, there are no points of developmental similarity between the cleavage of a single fertilization product into two or more (usually conjoined) proembryos and the initiation of two entirely separate developmental entities following double fertilization, as occurs in Gnetales and angiosperms. Indeed, of all described phenomena in which two or more developmental entities are initiated within a single seed (double fertilization in Ephedra and Gnetum, simple polyploidy, cleavage polyploidy), only the pattern of double fertilization representative of the pleisiomorphic condition within Gnetales could have given rise to the process of double fertilization in angiosperms.

Reproductive features of the common ancestors of angiosperms and Gnetales and the origin of endosperm

The discovery that regular double-fertilization events in Ephedra and Gnetum yield supernumerary zygotes that express the developmental program of an embryo has profound implications for reconstruction of the evolutionary origin of endosperm. The specific developmental and genetic constructs associated with double-fertilization events in basal Gnetales (Ephedra) are congruent with the hypothesis that double fertilization in Gnetales and angiosperms is homologous. This indicates that the products of the second fertilization events in Gnetales and angiosperms are homologous and provides strong support for the hypotheses of Sargent (1900) and Friedman (1992b) that endosperm represents a highly modified evolutionary derivative of a supernumerary embryo (fig. 22).

Based upon comparative analysis of the pleisiomorphic features of reproduction in basal angiosperms and basal Gnetales (Ephedra), it is possible to reconstruct the characteristics of gametophyte structure, fertilization, and embryogeny that are likely to have defined the common ancestors of these two clades. The female gametophyte of this common ancestor was monosporic, initiated two or more genetically identical archegonia during the cellular phase of development, and ultimately functioned in the nourishment of embryos.
This pattern of development is plesiomorphic among seed plants and fundamentally similar to what is found among extant basal seed plants (cycads, conifers, Ginkgo) and Ephedra (Friedman 1995). The male gametophyte produced two viable and genetically identical sperm.

A rudimentary process of double fertilization characterized the common ancestors of angiosperms and Gnetales and involved the fusion of two sperm from a single pollen tube with the egg nucleus and its sister nucleus (fig. 22). The product of this second fertilization event was diploid, initiated embryo development and was genetically identical to the first fertilization product, as in Ephedra. As a consequence of the formation of multiple archegonia within a female gametophyte, multiple double fertilization events within a single seed ("complex simple polyembryony") could occur, assuming sufficient pollen was received (Friedman 1995). If this took place, each of the two or more eggs within a female gametophyte was double fertilized by different pollen tubes. Finally, although two or more embryos were always initiated in each seed, only a single embryo fully matured; abortion of embryos in excess of one is an essentially universal feature of seed development among almost all seed plants.

According to this hypothesis, the rudimentary pattern of double fertilization that yields two zygotes (and was first manifest in a common ancestor of angiosperms and Gnetales) has been maintained in essentially unmodified form in Ephedra. While genetic and structural patterns associated with female gametophyte development in Gnetum are highly apomorphic, it is evident that the formation of two viable zygotes from double fertilization has been conserved during the establishment and evolution of this taxon (fig. 22). Within the angiosperm lineage, rudimentary double fertilization to yield two diploid zygotes has been modified to produce a second fertilization product (endosperm) that is triploid and serves to nourish the compatriot embryo derived from the first fertilization event (fig. 22).

With an explicit reconstruction of the reproductive features of the common ancestors of Gnetales and angiosperms established (Friedman 1994, 1995), two critical and interrelated questions can be addressed. Could a rudimentary process of double fertilization, similar to what occurs in Ephedra, have been modified over the course of time to yield a double-fertilization process similar to what characterizes angiosperms? Under what conditions could potential development of an embryo, with subsequent production of progeny, have been sacrificed to a developmental program of altruism, determinate growth, and programmed death, the three characteristics that ultimately define "endosperm"?

To address questions associated with the evolution of embryo-nourishing behavior by an endosperm, the constructs of inclusive fitness theory have been em-
ployed. These ideas, first formulated by Charnov (1979), were later expanded upon by Cook (1981), Westoby and Rice (1982), Willson and Burley (1983), Law and Cannings (1984), Bulmer (1986), Haig and Westoby (1989a, 1989b), Queller (1989), and Donoghue and Scheiner (1992). However, these analyses preceded the development of an explicit historical hypothesis for the origin of endosperm (Friedman 1992b, 1994, 1995) and were unable to address the fundamental question of how the product of a second fertilization event initially acquired the development characteristics now associated with endosperm (Friedman 1995).

Advances in phylogenetic reconstruction, definitive documentation of double fertilization in the Gnetales, and constructs of inclusive fitness theory were recently integrated to elucidate the evolutionary events associated with the establishment of endosperm. The main conclusions of this analysis (Friedman 1995) are as follows: (1) The origin of endosperm lies in the developmental modification of an embryo. (2) Acquisition of embryo-nourishing behavior with accompanying loss of individual fitness by a supernumerary embryo was dependent upon compensatory gains in the inclusive fitness of the altruist embryo and its associated genetically identical beneficiary embryo. (3) The end result of the loss of individual fitness by one of the two original products (embryos) of a rudimentary pattern of double fertilization was the establishment of endosperm, a highly modified embryo/organism that reproduces cryptically through behavior that enhances the fitness of its associated embryo within a seed. (4) It cannot be determined which of the two original fertilization products (embryos) in the ancestors of flowering plants was subsequently modified into endosperm. It is entirely possible that the normal first fertilization product that is homologous with the fertilization products and embryos of all other nonflowering seed plants was ultimately modified into endosperm and that the evolutionarily novel second fertilization product has survived into the angiosperm lineage as the embryo. (5) Although triploid endosperm remains a synapomorphy of angiosperms, inclusive fitness analysis demonstrates that the embryo-nourishing properties of endosperm initially evolved in a diplloid condition.

Integrative developmental, historical, and theoretical analysis of endosperm evolution provides an explicit hypothesis for the origin of endosperm from a progenitor supernumerary embryo in which individual fitness of the original second fertilization product declines, but inclusive fitness of the products of double fertilization increases. Thus, the evolutionary origin of endosperm can be characterized as having resulted from organismal duplication, in which the establishment of a rudimentary double-fertilization process led to the regular formation of a supernumerary embryo that was genetically identical to and redundant with the normal embryo in the common ancestors of angiosperms and Gnetales; divergence of function, in which aberrant development of one of the two fertilization products resulted in nourishing behavior to assist with the developmental of the normal embryo; and increased inclusive fitness through cooperative developmental behavior, whereby genes that promoted nourishing behavior by one of the two fertilization products with an associated loss of individual fitness were selectively favored as a consequence of increases in the inclusive fitness of the altruist and beneficiary embryos (Friedman 1995).

**Biological significance of double fertilization in Ephedra and Gnetum**

Double fertilization and the production of a zygote and associated endosperm within a seed are ubiquitous among angiosperms. Through its role in the acquisition of nutrients from the maternal sporophyte and the subsequent contribution of these reserves to the more slowly developing embryo, endosperm serves as an essential and critical link in the life cycle of flowering plants.

What is less clear now that regular double fertilization events have been documented in Ephedra and Gnetum, is what role these events may play in the reproductive biology of the Gnetales. The biological significance of double fertilization in Gnetales may be explained in one of only three ways: (1) Production of supernumerary embryos from second fertilization events may be a developmental accident of history that has always been biologically meaningless (a "spandrel," sensu Gould and Lewontin [1979]). (2) Double-fertilization events that do not yield an endosperm may represent a former exaptation or adaptation for which the original biological function has been lost (double fertilization in Ephedra and Gnetum is currently biologically meaningless). (3) Double fertilization in Gnetales may represent an exaptation or adaptation, in which the supernumerary embryo has current biological significance.

It is tempting to speculate that the production of supernumerary embryos from second fertilization events in Gnetales may be neutral, since all embryos in excess of one eventually terminate development and are likely to be broken down and consumed by the single successful embryo within a seed. In Ephedra and Gnetum, the female gametophyte still functions as the sole embryo-nourishing tissue. Thus, the retention and expression of double fertilization in Ephedra and Gnetum may simply reflect a historically contingent or developmentally constrained feature of reproduction among extant members of the Gnetales; in essence, an artifact of chance innovation in the common ancestors of angiosperms and Gnetales and subsequent preservation through time. If double fertilization in Gnetales does not function in successful reproduction, the issue of whether this pattern of double fertilization has ever played a biologically significant role in reproduction (was once an exaptation or adaptation) or has always been a process without "meaning" cannot now be resolved.

However, the fact that double fertilization, which yields a supernumerary zygote, has been retained, at
the very minimum, from the divergence of Ephedra and Gnetum (if double fertilization is homologous among Gnetales, but not with angiosperms), and most likely, since at least the time of divergence from the common ancestor of Gnetales and angiosperms in the Late Triassic (fig. 22; Doyle and Donoghue 1993) indicates that the production of supernumerary embryos from a second fertilization event may be biologically significant among members of the Gnetales. While supernumerary fertilization products in Ephedra and Gnetum express typical embryo developmental patterns from morphological, anatomical, and ultrastructural perspectives, extra embryos derived from second fertilization events may, at the physiological/behavioral level assist in the development of normal embryos. Double fertilization in Ephedra and Gnetum does not produce a structurally recognizable "endosperm." Nevertheless, endosperm behavior (i.e., an altruistic developmental program that assists the associated or committed embryo derived from double fertilization) may actually be expressed in members of the Gnetales with double fertilization (Ephedra and Gnetum). Indeed, evolutionary historical and inclusive fitness analyses predict that the original acquisition of embryo-nourishing behavior could have first evolved in a supernumerary embryo prior to the divergence of the angiosperm stem lineage from its sister group, which includes the Gnetales (Friedman 1995).

Given the ancient origins and continued expression of double fertilization in extant Gnetales, as well as a complete absence of knowledge of the specific biological role(s) of the second fertilization product in Gnetales, we must be open to the possibility that an entity that results from a fertilization process and initiates embryo development may in fact have acquired the essential characteristics of an embryo-nourishing endosperm in primordial form. Perhaps the age-old concept of "endosperm" as an embryo-nourishing tissue that is unique to angiosperms will need to be reexamined as more is learned of the significance of double fertilization in Gnetales.

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