How many nuclei make an embryo sac in flowering plants?

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Summary
Research on early-divergent angiosperms, including *Amborella*, the putative sister to all other extant angiosperms, is increasingly used as a yardstick to infer the nature of the hypothetical ancestral angiosperm. Some traits are relatively diverse (and hence relatively labile) in this phylogenetic grade, compared with the more derived eudicot clade, in which developmental patterns have become increasingly canalized. One of the many mysteries surrounding the origin of the angiosperms is the evolutionary origin of the *Polygonum*-type embryo sac (monosporic, eight-nucleate and seven-celled) that occurs in the majority of flowering plants. Observations on the megagametophyte of *Amborella* are conflicting, but a recent report of a supernumerary synergid in this genus[1] raises the question of whether the *Polygonum*-type embryo sac is derived by duplication of a four-nucleate structure or by reduction from a multicellular structure. *BioEssays* **28:1067–1071, 2006.**
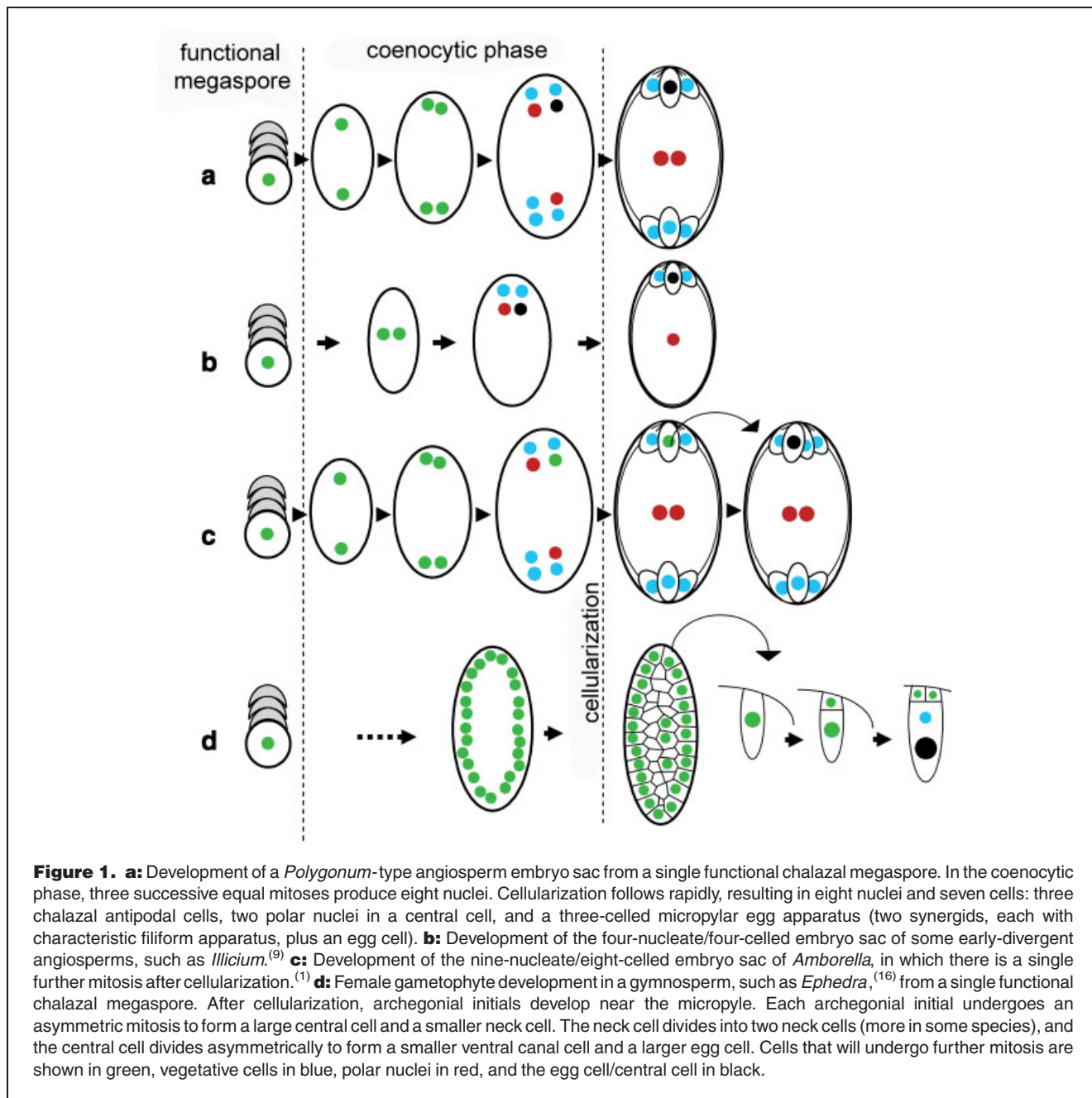**© 2006 Wiley Periodicals, Inc.**

Introduction

Much of the evolutionary history of land plants can be summarized as increasing dominance in the life cycle of the diploid sporophyte generation over the haploid gametophyte generation.[2] This trend reached its peak in the flowering plants, in which the "male" microgametophyte is reduced to two or three cells, and the "female" megagametophyte to (most commonly) eight nuclei (Fig 1; Box 1: Glossary). Recent research on gametophytes of early-divergent angiosperms has challenged traditional concepts regarding the evolutionary origin of these structures.

Angiosperm gametophytes are highly organized and also remarkably conservative in their morphology, indicating that there are strong constraints governing their development. In female organs, several (typically three to five) nuclear divisions occur between the megaspore mother cell and the mature megagametophyte, including two meiotic phases that together result in four megaspores. The majority of angiosperms produce monosporic embryo sacs, in which three of the four megaspores suffer programmed cell death (apoptosis), and only one (usually the chalazal megaspore) subsequently

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undergoes mitotic divisions to produce a megagametophyte. Thus, polarity between chalaza and micropyle is established at an early stage. The *Polygonum*-type of megagametophyte (so-named because it was first described in *Polygonum*[3]) characterizes more than 70% of flowering plant species examined thus far, including the archetypal model organism *Arabidopsis*. In this type, the single functional megaspore undergoes three mitoses to produce an eight-nucleate structure, arranged in two four-nucleate groups at opposite poles of the embryo sac (Fig. 1a). One nucleus from each group migrates to the centre, and the other three become cellularized, resulting in three antipodal cells at the chalazal pole and a three-celled egg apparatus (two synergids and an egg cell) at the micropylar pole. Thus, the coenocytic phase is followed very rapidly by a cellular phase,[4,5] and the resulting seven-celled (eight-nucleate) structure is highly modular, consisting of two mirror-image domains.[6] The two central nuclei fuse to form a diploid polar nucleus that ultimately fuses with a secondary sperm cell to form a triploid endosperm.
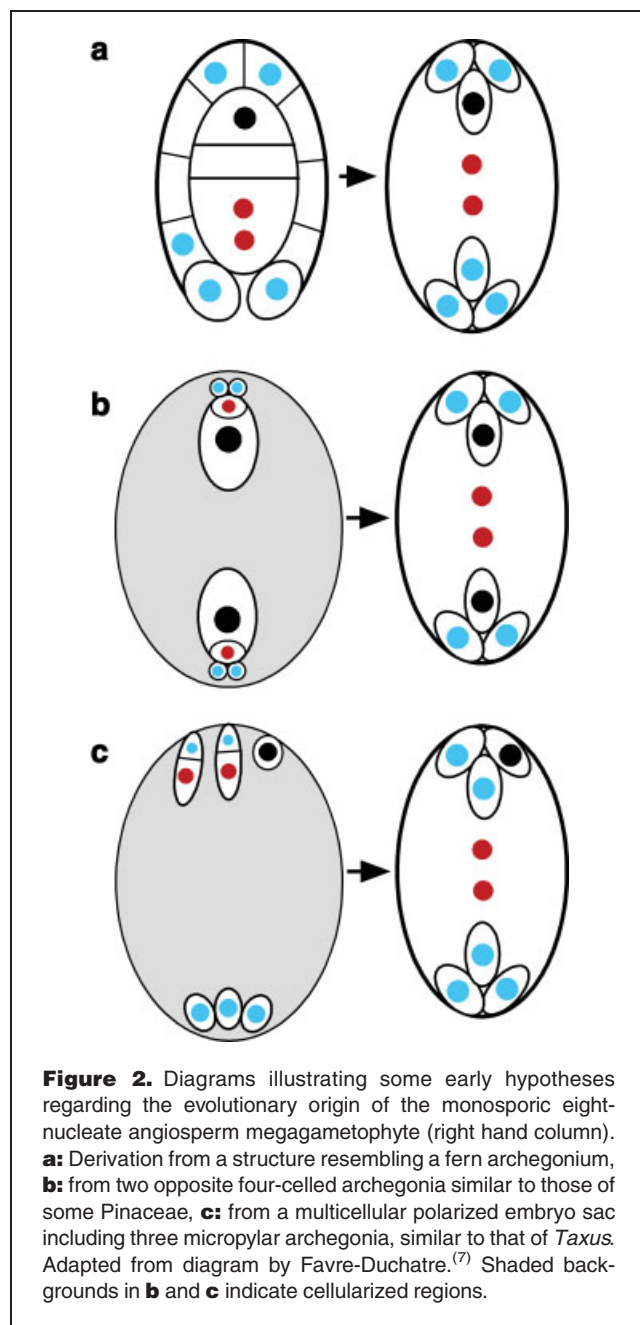
There have been several hypotheses regarding the evolutionary origin of the monosporic eight-nucleate angiosperm megagametophyte. These include derivation from a structure resembling a fern archegonium (Fig. 2a), from two opposite four-celled archegonia similar to those of some gymnosperms (Figs 1d, 2b), from a multicellular polarized embryo sac including three micropylar archegonia, similar to that of *Taxus* (Fig. 2c), or from a female gametophyte similar to that of *Gnetum*.[7] Over the past decade, molecular-based phylogenies of flowering plants have prompted numerous revised assessments of character evolution. Friedman and co-workers[6,8,9] hypothesized that an ancestral four-nucleate/four-celled condition, in which the polar nucleus is haploid, gave rise to an eight-nucleate/seven-celled condition by duplication and insertion of a cytoskeletal apparatus that separates the two domains. This hypothesis was fueled by the discovery of a four-nucleate/four-celled condition in some putatively early-divergent angiosperms such as *Illicium mexicanum* (Fig. 1b).[9] Their hypothesis resembles that of some earlier workers who noted the similarity between the chalazal and micropylar domains, and hence postulated their derivation from equivalent structures (Fig. 2b),[7] though earlier hypotheses tended to adopt a "bottom-up" approach, focusing on comparison with various gymnosperm groups, rather than a "top-down", angiosperm-centred view.[10]

Figure 1. a: Development of a *Polygonum*-type angiosperm embryo sac from a single functional chalazal megaspore. In the coenocytic phase, three successive equal mitoses produce eight nuclei. Cellularization follows rapidly, resulting in eight nuclei and seven cells: three chalazal antipodal cells, two polar nuclei in a central cell, and a three-celled micropylar egg apparatus (two synergids, each with characteristic filiform apparatus, plus an egg cell). **b:** Development of the four-nucleate/four-celled embryo sac of some early-divergent angiosperms, such as *Illicium*.[9] **c:** Development of the nine-nucleate/eight-celled embryo sac of *Amborella*, in which there is a single further mitosis after cellularization.[1] **d:** Female gametophyte development in a gymnosperm, such as *Ephedra*,[16] from a single functional chalazal megaspore. After cellularization, archegonial initials develop near the micropyle. Each archegonial initial undergoes an asymmetric mitosis to form a large central cell and a smaller neck cell. The neck cell divides into two neck cells (more in some species), and the central cell divides asymmetrically to form a smaller ventral canal cell and a larger egg cell. Cells that will undergo further mitosis are shown in green, vegetative cells in blue, polar nuclei in red, and the egg cell/central cell in black.

New evidence has now prompted Friedman[1] to present an alternative hypothesis. Applying an impressive array of imaging techniques to a large sample size, derived from both wild-source and cultivated material of *Amborella trichopoda*, he has reported a novel variation of the *Polygonum*-type megagametophyte with nine nuclei and eight cells, including three synergid cells that are easily identified by their characteristic filiform apparatus. The "extra" synergid reportedly results from a further mitosis in one of the three nuclei that remain at the chalazal pole, at the eight-nucleate stage after cellularization (Fig. 1c). Two factors make Friedman's discovery potentially highly significant: (1) megagametophyte typology is long-established,[11] and new discoveries are rare, and (2) in many recent molecular analyses *Amborella* is placed in a phylogenetically critical position, as sister to all other extant flowering plants. Consequently, this genus is increasingly viewed as the most critical taxon in attempts to infer the nature of the hypothetical ancestral angiosperm.[12,13]

Figure 2. Diagrams illustrating some early hypotheses regarding the evolutionary origin of the monosporic eight-nucleate angiosperm megagametophyte (right hand column). **a:** Derivation from a structure resembling a fern archegonium, **b:** from two opposite four-celled archegonia similar to those of some Pinaceae, **c:** from a multicellular polarized embryo sac including three micropylar archegonia, similar to that of *Taxus*. Adapted from diagram by Favre-Duchatre.[7] Shaded backgrounds in **b** and **c** indicate cellularized regions.

Developmental lability in early-divergent angiosperms

This new finding suggests that more comparative data are needed on key developmental traits in the six main groups of basally divergent extant angiosperms: *Amborella*, Nymphaeales, Austrobaileyales (Austrobaileyaceae plus Schisandraceae plus Trimeniaceae), Chloranthaceae, magnoliids (including Piperales/Aristolochiaceae and Laurales) and *Ceratophyllum*. Several traits have been shown to be relatively diverse (and hence relatively labile) in this phyloge-

netic grade, perhaps reflecting evolutionary experimentation occurring in closely related lineages for a relatively brief but critical time in flowering-plant evolution. This is particularly the case among reproductive features that can be correlated with the early evolution of specific insect-pollination syndromes, such as the plasmodial tapetum, a specialized cell layer surrounding the microsporangium.[14] Microspore development, and hence pollen aperture types, also appear labile in early-divergent extant angiosperms when compared with the more-derived eudicot clade, in which less variable (and therefore more recognizable) patterns have become increasingly canalized within lineages.[15]

However, most of these features represent iterative patterns of evolution, in contrast to the nine-nucleate embryo sac reported in *Amborella*, which has no obvious homologue among angiosperms. Friedman's[1] interesting suggestion of possible homology between the egg mother cell of *Amborella* (which forms the egg plus the supernumerary synergid) and the central cell of a gymnosperm (Fig. 1d) merits further exploration. In most gymnosperms, each archegonial initial undergoes an asymmetric mitosis to form a large central cell and a smaller neck cell.[16] The central cell then divides asymmetrically to form a smaller ventral canal cell and a larger egg cell; the neck cell produces a short row of cells that facilitate entry of the male gamete, and thus have a similar function to the synergids of angiosperms. (Note that the "central cells" of angiosperm and gymnosperm megagametophytes are not homologous: see Box 1.)

Friedman[1] proposed that the supernumerary synergid in *Amborella* could be the homologue of the ventral canal cell in gymnosperms. One problem with this interpretation is that it is difficult to homologize earlier developmental stages of the two structures (Fig. 1). In the majority of gymnosperms, female gametophyte development is monosporic (as in angiosperms), and the chalazal megapore is most commonly the functional one. However, in gymnosperms, the subsequent coenocytic phase involved numerous mitoses, producing many nuclei (up to 8000 in *Ginkgo*), followed by cellularization (Fig. 1d). A variable number of archegonial initials develop at the micropylar end of this polarized embryo sac. This pattern is consistent with Friedman's report that the "extra" mitosis in *Amborella* occurs after cellularization (Fig. 1c). However, the homologies of the other embryo sac nuclei, especially the antipodals, remain ambiguous. Furthermore, there are other problematic factors; for example, the "extra" mitosis in *Amborella* appears symmetrical, whereas the putatively homologous gymnosperm mitosis is normally highly asymmetric.[16] Asymmetric mitoses, including the primary mitosis of the microspore and the first division of the zygote,[17,18] play a critical role in production of specialized cell types, but their role in the megagametophyte has not hitherto been fully explored.

What the papers say

Cellular proliferation

Friedman's[1] observations contradict an earlier well-documented report on *Amborella trichopoda* by Tobe and coworkers.[19] On the basis of a comparable sample size, they described an eight-nucleate *Polygonum*-type megagametophyte with the "normal" complement of two synergids. Furthermore, Tobe's group[19] reported ephemeral antipodals, whereas in Friedman's material the three antipodal cells are reportedly persistent. Such discrepancy cannot simply be dismissed as erroneous, clearly requiring more detailed explanation. For example, teratological cases of supernumerary synergids and egg cells have been reported in other angiosperms, albeit rarely.[20] However, the extensive sampling and consistency of observation seem to exclude the possibility of teratology here. Furthermore, both of the *Amborella* studies report a binucleate central cell. Thus, Friedman's observations cannot be explained by recruitment of an extra synergid from one of the other component nuclei of an eight-nucleate structure, which could result from disrupted timing of one of the mitoses. Is it possible that both of the described conditions are maintained within *Amborella trichopoda*? Assuming that they are ultimately genetically controlled, could *Amborella* maintain such an allelic polymorphism among (or within) local populations? This seems unlikely in a lineage as ancient and geographically isolated as *Amborella*, in which developmental constraints should by now be well established. (Flexibility within lineages should not be confused with "lability"[1,21] among them).

Reports of supernumerary nuclei are common in angiosperm embryo sacs, but normally occur in the central or (most commonly) chalazal domains.[4,5,20] For example, in tetrasporic embryo sacs such as the *Peperomia* and *Drusa* types, up to four mitoses result in a final complement of up to 16 nuclei. However, in the mature megagametophyte, the egg apparatus normally remains three-celled even in tetrasporic types, except in *Peperomia*, in which the egg apparatus is sometimes two-celled within a 16-nucleate megagametophyte.[11] In tetrasporic embryo sacs, the supernumerary nuclei are expressed either as antipodal or central cells, or as peripheral cells of unspecified nature, but not as synergid or egg cells. Furthermore, they are the direct result of a developmental switch that occurs much earlier in ontogeny: in tetrasporic embryo sacs, both cell-wall formation and cell death fail to occur at the tetrad stage, resulting in a tetranucleate coenomegaspore.

Perhaps a closer analogy of the *Amborella* case occurs in embryo sacs that conform in developmental pattern to the "typical" monosporic *Polygonum*-type (Fig. 1a) except in the behaviour of the antipodal cells. Records of fixed (rather than teratological) supernumerary nuclei are most common in antipodal cells, which play only an indirect role in the reproductive process. Antipodals of different species can undergo programmed cell death, failure of cell-wall formation,

polyploidization or proliferation. For example, up to 12 antipodal cells have been reported in some Gentianaceae, and up to 300 in some Poaceae,[11] occasionally with a variable number of nuclei. Such proliferation has been compared with the prothallial tissue of some gymnosperms.[7] The similarity in behaviour between the egg apparatus of *Amborella* and the antipodals of other species seems to support earlier hypotheses[6,8,9] of evolutionary homology between the antipodals and the egg apparatus. Furthermore, supernumerary synergids have been reported in mutants of maize and *Arabidopsis*.[22,23] Maize *indeterminate gametophyte1* (*ig*) mutants combine a variable number of extra micropylar mitoses either before or after cellularization with a reduced number of chalazal mitoses, indicating a possible late-stage polarity switch.

Conclusions and outlook

Developmental–genetic studies of model organisms have begun to improve our understanding of megagametophyte formation in angiosperms,[22–24] but future research on these mechanisms in seed plants would benefit from a more explicitly phylogenetic approach. However, attempts to reconstruct the evolution of the characteristic features of flowering plants (i.e. to distinguish angiosperms from other seed plants) remain hampered by problems of outgroup comparison, largely due to vast gaps reflecting pre-Cenozoic extinctions. Even in the modern era of molecular systematics, the phylogenetic context for flowering plants is by no means resolved. Sequence-based molecular analyses of seed plants are character-rich but restricted to a severely depleted range of extant lineages. They produce hypotheses of relationship that contrast strongly with results of morphological analyses that include fossil taxa.[10]

Comparative data on the organization of megagametophytes of extinct seed plants are relatively limited.[25] Furthermore, in common with data from extant taxa, they provide conflicting evidence. For example, among fossil Carboniferous seed plants, the most common ovule condition includes three micropylar archegonia, as in some extant conifers, implying that the angiosperm embryo sac could have been derived from a similar structure (Fig. 2c). By contrast, ovules of the late Permian gymnosperm *Glossopteris*, which represents a likely angiosperm stem-group, possessed two enlarged synergid-like neck cells.[26] In my view, these cells resemble the neck cells of the extant genus *Cycas*,[27] perhaps indicating that they gave rise directly to the synergids from a single archegonium, as in the hypothetical example in Fig. 2b. Thus, further constructs remain to be explored in the enigmatic transition from the gymnosperm to the angiosperm condition. Friedman's suggestion regarding the possible evolutionary significance of a supernumerary synergid in *Amborella*[1] is stimulating and potentially paradigmatic, but inevitably raises as many questions as it answers.

Glossary

antipodal cell (in angiosperm embryo sac): one of (typically) three pair of cells located at chalazal end, opposite micropyle.

archegonium: egg-producing organ within mega-gametophyte (term applied to gymnosperms and all land plants except angiosperms).

central cell (in gymnosperm archegonium): cell that will divide to form ventral canal cell and egg cell. In an angiosperm embryo sac, the central cell is the one that contains the polar nuclei.

chalaza: region of ovule or seed where nucellus and integuments merge, located opposite micropyle.

egg cell: haploid "female" gamete that will fuse with haploid "male" sperm to produce a diploid zygote.

endosperm: seed storage tissue in angiosperms (usually triploid), normally formed by fusion of one haploid sperm cell with diploid nucleus resulting from fusion of two haploid polar nuclei.

gametophyte: haploid stage of plant life cycle.

megagametophyte ("female" gametophyte): mature embryo sac, most commonly consisting of seven cells and eight nuclei (two synergid cells, an egg cell, three antipodal cells, two polar nuclei).

megaspore: female haploid cell resulting from meiosis; usually one of four (or two), of which only one is functional.

microgametophyte ("male" gametophyte): cellular component of mature pollen grain, in angiosperms consisting of a vegetative cell and a generative cell.

micropyle: opening at one end of ovule, usually surrounded by integuments.

neck cell (in a gymnosperm archegonium): one of a short row of cells at micropyle.

sporophyte: diploid stage of plant life cycle.

synergid (in angiosperm embryo sac): one of (typically) a pair of cells located at micropylar end, characterized by wall thickenings (filiform apparatus).

ventral canal cell (in gymnosperm archegonium): small cell located near micropyle.

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