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Repeated evolution of tricellular (and bicellular) pollen[1]

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- *Premise of study:* Male gametophytes of seed plants are sexually immature at the time they are dispersed as pollen, but approximately 30% of flowering plants have tricellular pollen containing fully formed sperm at anthesis. The classic study of Brewbaker (1967: *American Journal of Botany* 54: 1069–1083) provided a powerful confirmation of the long-standing hypothesis that tricellular pollen had many parallel and irreversible origins within angiosperms. We readdressed the main questions of that study with modern comparative phylogenetic methods.
- *Methods:* We used our own and more recent reports to greatly expand the Brewbaker data set. We modeled trait evolution for 2511 species on a time-calibrated angiosperm phylogeny using (1) Binary State Speciation and Extinction (BiSSE), which accounts for the effect of species diversification rates on character transition rates and, (2) the hidden rates model (HRM), which incorporates variation in transition rates across a phylogeny.
- *Key results:* Seventy percent of species had bicellular pollen. BiSSE found a 1.9-fold higher bicellular to tricellular transition rate than in the reverse direction, and bicellular lineages had a 1.8-fold higher diversification rate than tricellular lineages. HRM found heterogeneity in evolutionary rates, with bidirectional transition rates in three of four rate classes.
- *Conclusions:* The tricellular condition is not irreversible. Pollen cell numbers are maintained at intermediate frequencies because lower net diversification rates of tricellular lineages are counterbalanced by slower state shifts to the bicellular condition. That tricellular lineages diversify slowly and give rise to bicellular lineages slowly reflects a linkage between the evolution of sporophyte lifestyles and the developmental lability of male gametophytes.

Key words: cell cycle; constraint; diversification rate; Dollo's law; evolution of development; gametogenesis; heterochrony; parallelism; pollen germination; trade-off.

At sexual maturity, the male gametophyte of flowering plants is tricellular, consisting of a single somatic cell that forms a pollen tube that carries in its cytoplasm two free sperm cells. The first microscopic studies of angiosperm pollen anatomy found that male gametophytes were immature and binucleate at the time of pollen dispersal (Strasburger, 1877; Elfving, 1879). In such species, the generative nucleus undergoes mitosis to form two sperm nuclei after pollination, within the pollen tube cytoplasm. Hereafter, we refer to male gametic nuclei as generative cells and sperm cells, since it has since been shown that they are not free nuclei within the tube cell cytoplasm (Maheshwari, 1950; Knox, 1984a). The pollen tube itself was first observed in *Portulaca oleracea* (Amici, 1824), a species that later was found to form its two sperm cells before pollen dispersal (Cooper, 1935). It was soon apparent that species that formed their sperm late (bicellular pollen) were phylogenetically widespread, whereas those that formed sperm early (tricellular pollen) seemed to be restricted to aquatics, grasses, and some herbs (Elfving, 1879; Strasburger, 1884). Schürhoff (1926) proposed that tricellular pollen was independently derived numerous times and that the condition was irreversible. Subsequent surveys of many more species strengthened that hypothesis (Schnarf, 1939; Rudenko, 1959), but it was the massive study of J. L. Brewbaker (1967), published in *American Journal of Botany*, that seemed so elegantly and powerfully to settle the matter once and for all.

The landmark Brewbaker study stands as one of the first large-scale tests of an evolutionary developmental hypothesis using character mapping onto an explicit phylogenetic tree. The data came from his studies of 812 species and an additional 1096 species from the literature, representing 1219 genera. Based on the species-level data, 265 families were coded as having either bicellular, tricellular, or polymorphic pollen dispersal states. Coded families were then placed onto a bifurcating ordinal-level tree constructed from what was known of angiosperm relationships at the time. The resulting two-page phylogeny displayed a pattern in which families with tricellular pollen repeatedly appeared in nested (or potentially nested) positions in both monocots and dicots, whereas bicellular families were never clearly nested within tricellular groups. This distribution was consistent with Schürhoff's (1926) proposal of many unidirectional shifts to tricellular pollens in unrelated groups. The ancestral state of angiosperms was inferred as bicellular because woody magnoliales were assumed to have retained ancestral states and all had bicellular pollen.

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Based on Brewbaker (1957, 1967), a broad narrative emerged for why tricellular pollen has arisen so frequently, why it should be an irreversible state, and why, given repeated origins and irreversibility, it has not become even more common (about 30% of species were found to be tricellular). Tricellular pollen was thought to be favored by natural selection, but only in specialized niches. Tricellular pollen is difficult to germinate in vitro, especially in water, so it is advantageous in humid environments and in aquatics with submersed flowers. Though conditions for germination are more restrictive, on compatible stigmas, tricellular pollen germinates very rapidly, and hence is often seen in herbaceous annuals and weeds when rapid reproduction is favored (Stebbins, 1974, Hoekstra, 1983; Knox, 1984b; Heslop-Harrison, 1987; Torabinejad et al., 1998). However, tricellular pollen is terminally differentiated and is thought to be dispersed in a nutrient-poor condition, resulting in a short lifespan (Brewbaker, 1967). Hence, the competitive advantages of rapid germination are only useful when pollination is efficient (e.g., predictable and fast; Stebbins, 1974). Bicellular pollen is less differentiated, longer-lived, and often dormant at dispersal—traits that are advantageous in an inefficient pollination environment. But bicellular pollen is less competitive since germination is slower and more variable. Finally, constraints may have played a role in irreversibility, as implied by the repeated association of tricellular pollen with a novel set of integrated traits governing pollen–stigma interactions (Brewbaker, 1957; Brewbaker and Majumder, 1961; see also Heslop-Harrison and Shivanna, 1977). As such, the tricellular state becomes a complex trait that is much harder to reverse (Gould, 1970; Stearns, 1992).

We can look back now and appreciate the strikingly modern approach Brewbaker took to an essentially evolutionary developmental problem. His study preceded the realization of quantitative methods for the study of the evolution of organismal ontogenies and developmental processes by a decade (Gould, 1977; Alberch et al., 1979). The use of an angiosperm-wide, bifurcating tree also preceded the first large-scale morphological and molecular trees of the group (Doyle and Donoghue, 1986; Chase et al., 1993). Despite the lack of explicit comparative phylogenetic methods, the basic process of inference was essentially the same as that used in modern parsimony analyses, resulting in Brewbaker's (1967, p. 1069) carefully worded conclusion that, "in no instance must one infer the origin of binucleate taxa from trinucleate ancestors." Brewbaker also concluded that there were different rates of trait evolution in different parts of the tree. Ironically, the paper almost perfectly coincided with two developments that enabled quantitative analyses of character evolution: the birth of cladistics (Hennig, 1966) and the first demonstrations of neutral molecular variation (Hubby and Lewontin, 1966; see also Brewbaker et al., 1968).

In this study, we readdress the major questions of Brewbaker (1967). First, we greatly expanded the Brewbaker database, including some of our own reports from early-diverging angiosperm lineages. Second, we used a time-calibrated 31 749 species phylogeny of seed plants (Zanne et al., 2013). Time-calibration enabled us to use two recently developed comparative tools that improve the estimation of ancestral states of binary characters when (1) character states are associated with different diversification rates, as suggested by Maddison et al. (2007), or when (2) rates of character change are heterogeneous across the phylogenetic tree (Beaulieu et al., 2013). The finer resolution allowed by these analyses supports an asymmetrical but bidirectional pattern of transitions, as well as different rates of evolution within the tree. Finding evidence for bidirectional but asymmetrical transitions, we address the issue of whether there are biases inherent in development itself, or in interaction with the environment, and establish an explicit comparative framework for studying heterochronies that produce shifts in pollen cell number.

MATERIALS AND METHODS

***The species and character states*—**Original data for 21 angiosperms from the ANA grade (Amborellales, Nymphaeales, Austrobaileyales), Chloranthales, and eumagnoliids were included for the following species: *Amborella trichopoda* Baill., *Nuphar advena* (Aiton) W.T.Aiton, *Nuphar polysepala* Engelm., *Nymphaea odorata* Aiton, *Cabomba caroliniana* A. Gray, *Brasenia schreberi* J.F. Gmel, *Trithuria bibracteata* D.A. Cooke, *Austrobaileya scandens* C.T. White, *Illicium floridanum* J. Ellis, *Illicium parviflorum* Michx., *Illicium anisatum* L., *Schisandra propinqua* var. *sinensis* (Oliv.) R.M.K. Saunders, *Hedyosmum brasiliense* Mart., *Ascarina lucida* Hook.f., *Sarcandra glabra* (Thunb.) Nakai, *Asimina triloba* Dunal, *Liriodendron tulipifera* L., *Magnolia grandiflora* L., *Pseudowintera colorata* (Raoul) Dandy, *Peperomia obtusifolia* (L.) A. Dietr., and *Macropiper excelsum* (G. Forst) Miq. Vouchers are deposited at TENN, and collection information is in Appendix S1 (see Supplemental Data with the online version of this article). For each population, pollen cell number was recorded for 100 pollen grains per flower from newly opened anthers on at least five individual plants (methods of Williams, 2012a).

The remaining data were almost entirely collected from the primary literature, comprising more than 630 reports on 4594 species. An attempt was made to represent as many families as possible. Family designations and diversity measures were from Stevens (2001). Secondary sources were used only when the primary source for a particular species could be verified (Schnarf, 1939; Maheshwari, 1950, Wunderlich, 1954; Davis, 1966, Yakovlev, 1981; Johri et al., 1992). Among primary sources, several covered large numbers of species within a group (Araceae [Grayum, 1986], Euphorbiaceae [Webster and Rupert, 1973; Webster et al., 1982], Lamiaceae [Leitner, 1942], Rubiaceae [Mathew and Philip, 1986; Puff, 1993], Saxifragaceae [Zhang and Gornall, 2011]), within a region (Japan [Shibata and Konishi, 1990], New Zealand [Gardner, 1975], or from local sources (Corriveau and Coleman, 1988; Saito et al., 2002; Torabinejad et al., 1998; Zhang et al., 2003).

***Character and diversification analyses*—**The time-calibrated seed plant phylogeny of Zanne et al. (2013) was used for all analyses. There were 1521 exact matches between species on the phylogeny and species in our pollen database. Near matches were found using Levenshtein similarity as calculated by the RecordLinkage R package (Borg and Sariyar, 2012), and 172 name corrections were made. For genera with one or more members of the genus in both the phylogeny and the pollen data set but no species matches, we selected one species from the genus at random from the pollen data set and one species from the genus at random from the phylogeny, deleted all other members of the genus, and assigned the generic name to both the surviving species ($N = 818$). Assuming monophyletic genera, this procedure allowed us to have a larger combined data set (2511 taxa) without making further assumptions about speciation times (as we would have had to make if we added two representatives from a genus to the tree).

For modeling binary-state character evolution, each species must be classified as either bicellular or tricellular. Yet, intraspecific variability was reported for many species, and there were conflicting reports for others. We accepted the conclusions of studies that evaluated prior conflicts (e.g., Schnarf, 1939; Brewbaker, 1967; Puff, 1993). Other conflicts were resolved by comparing the quality of support for each state, including the methodology used and whether the findings were substantiated with images or line drawings. For studies reporting intraspecific or even intraindividual variability, we assigned the most common state to that species, if one was reported. In sum, we discarded data from 10 species, changed two species from tri- to bicellular, 22 from variable to tricellular and 28 from variable to bicellular. Twenty-four species either had unresolvable conflicts or appeared to be truly variable and were removed from the BiSSE analysis (the entire database, plus changes and excluded taxa, is available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.8m7t2).

To estimate the effect of pollen state on diversification, we used the BiSSE approach (Maddison et al., 2007) as implemented in the program diversitree version 0.9-1 (FitzJohn, 2012). BiSSE returned likelihood estimates of transition rates between bi- and tricellular pollen as well as speciation and extinction rates for each state, though, due to general methodological issues estimating extinction rate (Rabosky, 2010), we only used net diversification rates. Diversitree can incorporate incomplete lineage sampling (FitzJohn et al., 2009) using given trait sampling frequencies. We assumed 264 000 angiosperms (Stevens, 2001) and the observed trait state frequencies from the 2511-taxon data set. To test robustness to the sampling frequency assumption, we re-estimated evolutionary rates over a variety of potentially true frequencies of bicellular pollen, ranging from 60 to 80%. Since we had >>300 taxa and each character state had a frequency >10%, the BiSSE analysis had sufficient power to distinguish differences in evolutionary rates of all six parameters (Davis et al., 2013). We ran the analysis four times, assuming a bicellular root, a tricellular root, equal probability of either state at the root, or the equilibrium frequency at the root.

BiSSE allows for both diversification rates and transition rates to be estimated, but it assumes that there is a constant transition rate from, say, bi- to tricellular over the entire tree. A different approach, the hidden rates model (HRM) carried out using corHMM (Beaulieu et al., 2013), assumes no differential diversification effect but does allow for different transition rates on different parts of the tree. It does this by allowing for a given observed state, such as bicellular, to be modeled as truly having a number of hidden states. For example, the bicellular to tricellular rate may be faster for aquatic plants than for terrestrial plants, and this variation can be incorporated automatically by the method. Models with a single rate category (the same as having no hidden states) up to five rate categories were compared by the size-corrected Akaike information criterion (AICc) (Akaike, 1973; Hurvich and Tsai, 1989). Joint estimates of states at nodes were made using the best-fitting model. On plotted reconstructions, edges were painted with the state at their descendant node only if the total relative likelihood for the node being any of the bicellular states or any of the tricellular states was greater than 95%. Models with the root state fixed as bicellular and models with the tricellular to bicellular transition rates set to zero were also evaluated.

RESULTS

A total of 2511 species were matched to the Zanne et al. (2013) phylogenetic tree. At the family level, 70.4% of angiosperm diversity was represented (319 families). A median of 33.3% of the generic diversity within families was covered (1822 genera). The unsampled families were not diverse: 50% were unigeneric, and the mean number of genera was 2.8.

All 21 species from the ANA grade, Chloranthales, and eumagnoliids that we observed had bicellular pollen (Fig. 1). In the data analyzed, 69.6% of species were bicellular. In the full database, 69.4% of species were bicellular.

The BiSSE analysis assumes the true proportion of each of the binary states is known. Though we covered angiosperms broadly, our sample captured only 0.82% of their species diversity. Therefore, we performed the analysis over a range of possible true values centered on our empirical estimate of 69.6% species bicellular. As the true percentage of bicellular species was increased from 60 to 80%, the diversification rate of bicellular lineages was always higher than that of tricellular lineages; and the transition rate from the tricellular to the bicellular state was always lower than in the bicellular to tricellular direction (Fig. 2). At the empirical estimate, the diversification rate of bicellular lineages was 1.81 times higher than the tricellular rate ($r_2 = 0.0988$; $r_3 = 0.0545$); and the bicellular to tricellular transition rate was 1.92 times higher than the tricellular to bicellular transition rate ($q_{23} = 0.0073$; $q_{32} = 0.0038$, respectively). The magnitude of the asymmetry of diversification rates and of transition rates changed <1.3% under the three alternative

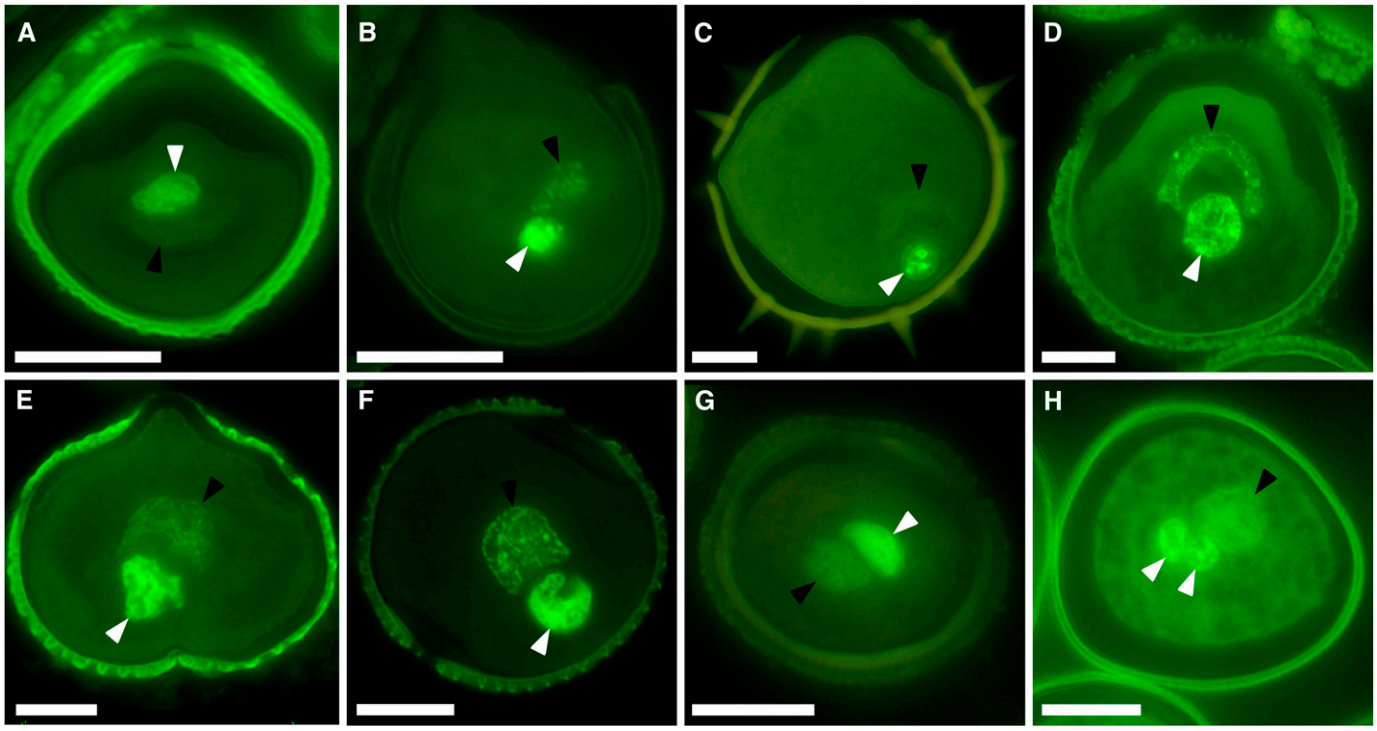

Fig. 1. Mature bicellular pollen of species in ancient angiosperm lineages. Tricellular pollen is shown in (H) for comparison. (A) *Amborella trichopoda* (Amborellales); (B) *Trithuria bibracteata* (Hydatellaceae; Nymphaeales); (C) *Nuphar advena* (Nymphaeaceae; Nymphaeales); (D) *Austrobaileya scandens* (Autrobaileyaceae; Austrobaileyales); (E, F) *Illicium anisatum*, *I. parviflorum* (Illiciaceae; Austrobaileyales); (G) *Hedyosmum brasiliense*, on stigma (Chloranthaceae; Chloranthales); (H) *Joinvillea plicata* (Joinvilleaceae; Poales). Black arrowheads, tube (vegetative) cell nuclei; white arrowheads, generative cell nuclei (or sperm cell nuclei in H). All are from glycol-methacrylate sections stained with DAPI. Scale bars = 10 μm.

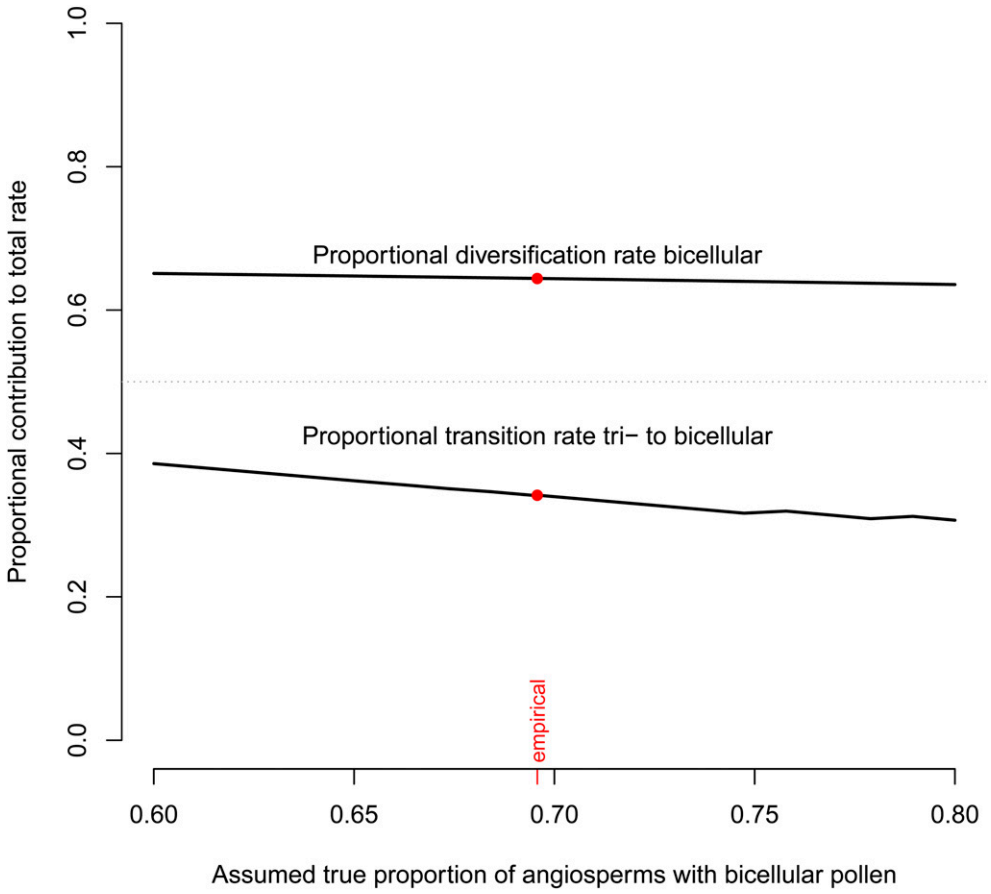

Fig. 2. Sensitivity analysis of BiSSE results (assuming equal probability of either state at the root). The *y*-axis represents the proportional contributions of the diversification rate of bicellular pollen, or the transition rate from tricellular to bicellular pollen, to their respective total rates: diversification proportion = $r_2/(r_2 + r_3)$ and transition proportion = $q_{32}/(q_{32}+q_{23})$. The *x*-axis indicates the range of potential values for the true proportion of bicellular pollen in angiosperms over which the estimates were done. For both measures, a value of 0.5 represents equal rates. At the values for our empirical estimates, shown in red, diversification proportion = 0.6441, and transition proportion = 0.3418.

assumptions about the root state (data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.8m7t2).

In the hidden rates analysis, the best-supported model was one of intermediate complexity, with four rate classes (Fig. 3; Appendix S2, see online Supplemental Data). Rate classes A and D are marked by high rates of character change, whereas classes B and C are slowly evolving classes (Fig. 3). The deepest internal branches of the tree are limited to the A and B rate classes, whereas rate C and D branches characterize derived clades and tip branches. Class A lineages are marked by an extremely high net transition rate from the tricellular to the bicellular state, whereas bicellular lineages in this rate class tend to persist for a relatively long time (shifts away from "bicellular-A" being rare). In contrast, tricellular-A lineages, once formed, quickly give rise to bicellular-A descendants, as reflected by the rarity of tricellular-A species (Fig. 3). Bicellular-A lineages are very common and widespread, but are absent from the asterid I and II clades (Fig. 3).

The asterid I and II clades are entirely in rate C with a few nested rate D clades. Class D is a fast rate class characterized by rapid switching back and forth between pollen dispersal states and a small transition bias in the bicellular to tricellular direction. Because transitions away from both states are high, the time spent in each state is very low, as indicated by the small size of both class D circles. Class D clades are not widespread—they occur only within derived positions in the asterid I clade, mainly in Gentianales and Solanales (Fig. 3).

The BiSSE and HRM analyses gave conflicting results about the ancestral state of angiosperms. Irrespective of the assumption about the root state, BiSSE always supported a tricellular ancestry at all deep nodes, including the ancestors of angiosperms, monocots, and the eumagnolid + eudicot clade (support values for both analyses are in online Appendix S3). However, the BiSSE analysis assumed a single tree-wide transition rate. The HRM analysis distinguished four rates and found more uncertainty at the base of the phylogeny: relative likelihoods slightly favored a tricellular ancestor for angiosperms (~0.55) and the eumagnoliid/eudicot clade (~0.60), and fairly strongly supported one for monocots (~0.85).

Given uncertainty of the ancestral state in the HRM analysis, we asked if fixing the root as bicellular (the Brewbaker prediction) would have an effect on evolutionary rates. That analysis strongly favored a class B rate over other classes for the fixed bicellular root, and the internal branches leading to the ancestors of Austrobaileyales, Chloranthales, Ceratophyllales, monocots, and the eudicot + eumagnoliid clade were more likely to be bicellular than tricellular (Fig. 4). But the ancestor of monocots without *Acorus* remained tricellular (relative likelihood

~0.65), and the ancestors of eudicots and eumagnoliids separately remained bicellular (Fig. 4). The magnitude and direction of transition rates between the eight rate/state categories were virtually identical in both the variable root and fixed root analyses (Figs. 3, 4). Thus, even when the ancestor of angiosperms was fixed as bicellular, transition rates in the tricellular to bicellular direction remained high (Fig. 4).

As a final test, we assumed the Brewbaker–Schürhoff unidirectional evolution hypothesis was true, allowing only bicellular to tricellular transitions. The log-likelihood of this constrained model dropped 79.08 log-units relative to the unconstrained HRM four-rate model, indicating a very poor fit to the data ($P < 1 \times 10^{-16}$).

DISCUSSION

The 1967 Brewbaker study represented an enormous leap in power for testing the already 40-yr-old unidirectional evolution hypothesis of Schürhoff (1926). The visual impact of the two-page angiosperm-wide tree with mapped character states (fig. 2 in Brewbaker, 1967) was so convincing that the hypothesis soon became known as the Brewbaker–Schürhoff law (Eg. Webster and Rupert, 1973; Gardner, 1976; Zhang and Gornall, 2011). Yet, Brewbaker (1967, p. 1074) recognized the limitations of the approach and was careful to report only that: "The distribution of binucleate and trinucleate angiosperms fully supports Schürhoff's proposal (1926) that the trinucleate condition is associated with an advanced or derived phylogenetic position." From a 2014 perspective, the major weaknesses of the Brewbaker analysis were that the phylogeny was at the ordinal level, the topology was determined qualitatively and lacked branch lengths, some orders were placed at nodes not tips, and character states were polarized at the family and order level, not the species level.

We tested the three main predictions that follow from the Brewbaker–Schürhoff hypothesis: bicellular pollen should be reconstructed as the ancestral state; tricellular pollen should arise repeatedly in derived clades from a bicellular ancestor; and there should be no secondarily derived bicellular species within tricellular clades. We also tested the Brewbaker prediction that some groups would show higher character transition rates than others. Finally, we asked if there was an association between diversification rates and trait states.

Are evolutionary shifts between pollen dispersal states unidirectional?—The BiSSE analysis found a high bicellular to tricellular transition rate within angiosperms, as predicted by the Brewbaker–Schürhoff hypothesis. However, the tricellular state was not irreversible, since the tricellular to bicellular transition rate was also substantial. When transition rates were allowed to vary in different parts of the tree (HRM analysis), two of the three highest transition rates were in the tricellular to bicellular direction. When we fixed the root as bicellular, transition rates were still bidirectional and of similar magnitude to those in the unfixed analysis. When we disallowed transitions in the tricellular to bicellular direction, model support fell dramatically. Evolution was clearly not unidirectional, but there was a strong bias in the predicted direction.

Why should transition rates be asymmetrical, and what forces govern the maintenance of both states at relatively intermediate frequencies? A new insight lies in the finding that the roughly 2-fold transition bias is accompanied by a roughly 2-fold diversification bias in the opposite direction. Bicellular lineages are diversifying faster than tricellular lineages, but they are also giving rise to tricellular lineages more often (via faster shifts to the tricellular state). Bicellular species are more common because diversification rates and transition rates do not counterbalance each other perfectly. The association between pollen dispersal state and net diversification rate supports a role for ecology in the pattern of variation, as discussed later.

Our analyses also support the observation that some parts of the tree had different evolutionary rates (Brewbaker, 1967). The HRM analysis distinguished four rate classes. The A and B classes characterize most of the deep internal branches of the tree. In these classes, lineages tend to cycle into the relatively persistent bicellular-A state, which characterizes much of the ANA grade, eumagnoliids, and rosids. Shifts to the slow C rate class are widespread as one moves tipward, and most tricellular lineages remain "stuck" in the persistent tricellular-C class.

Gentianales was singled out by Brewbaker (1967, p. 1076) as being a particularly labile group, "in which one must suppose a high mutation rate of II to III, a high selective value of the III condition, or both." The HRM analysis also identified Gentianales (asterid I clade of Fig. 3) as having a fast evolutionary rate, with three shifts to rate class D, which is marked by high bidirectional transition rates. In Gentianales, only 7 of 45 transitions were in the predicted bicellular to tricellular direction, plus two more if one adds the Oleaceae clade that has been removed from Gentianales since 1967 (APG III, 2009 and Zanne et al., 2013).

The presence of different evolutionary rate categories could indicate correlation with one or more "hidden" traits: for example, species in a class with a fast rate of evolution between bicellular and tricellular pollen may have a fast evolutionary rate in general due to being annuals rather than perennials, being more prone to polyploidization, or having large population sizes allowing faster rates of adaptive change. Future work could examine these and other potential correlates to find which ones may be leading to rate differences. Another explanation could be that the rates of gain and loss of tricellular pollen themselves change gradually over the tree. This continuous heterogeneity can be dealt with in the HRM context only by putting these continuous rates into a discrete number of bins. If this is the case, then a large data set with even more power might allow even more hidden rate categories. Note that both these explanations may work in concert. At this point, the mechanism for why these rates vary is unknown, but the analysis provides strong evidence that heterogeneity in rates is important.

Developmental mechanisms for shifts in pollen dispersal states—Evolutionary shifts between pollen dispersal states involve an ancestor giving rise to a descendant with modified development, or heterochrony (Alberch et al., 1979; Reilly et al., 1997). Developmental transformations can occur via changes to developmental rate or to onset or offset timing, and these are determined by the interaction of pollen and anther development (Fig. 5). The pollen cell lineage is initiated by the anther when a sporogenous cell becomes a microsporocyte that undergoes meiosis to form four microspores (the process of microsporogenesis). The onset of microgametogenesis occurs when a microspore first functions independently (with gametophytic gene expression), either as a free cell or within a group of attached microspores (Fig. 5). Pollen dispersal state, the offset, is determined by the stage of microgametogenesis present at the time

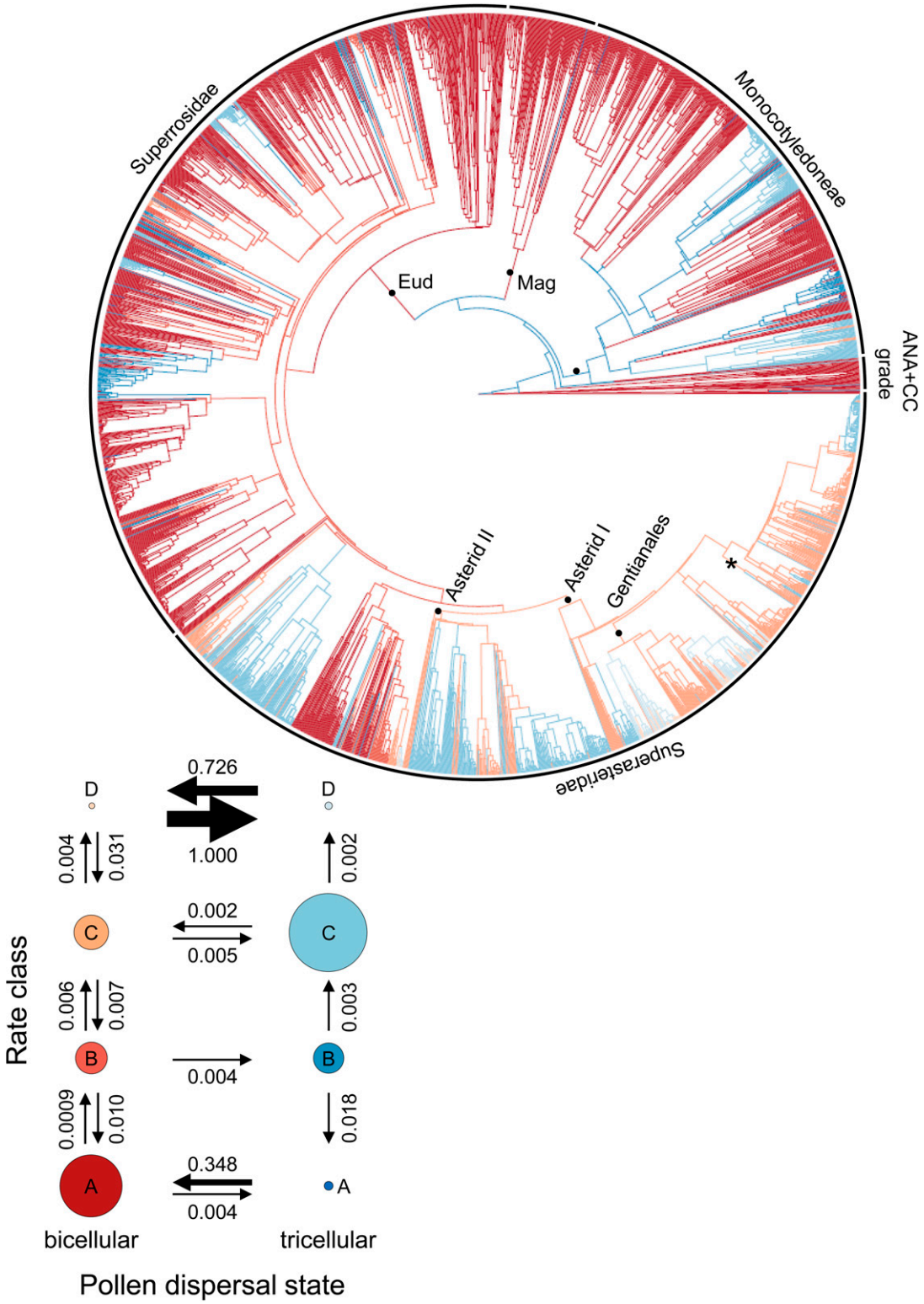

Fig. 3. Results of the hidden rates model with four rate classes. Time-calibrated angiosperm phylogeny comprising the 2511 species used in all character analyses is from Zanne et al. (2013). Reconstructed transitions between pollen dispersal states and four rate classes are indicated by changes in colors along internal branches of the tree. Branch colors correspond to eight possible combinations of rate class and pollen cell number. The size of each colored circle indicates the relative amount of time spent in that state/class combination and is determined solely by the total transition rate away from that combination. Transition rates between state/rate combinations are scaled to 1 and indicated by arrows of roughly proportional thicknesses. The maximum rate was $q = 1.0209$ (bicellular-D to tricellular-D). The ANA+CC grade topology is: (*Amborella*+Nymphaeales)(Austrobaileyales(Chloranthales(Ceratophyllales+rest). Asterisk, Oleaceae; Eud, eudicots; Mag, eumagnoliids.

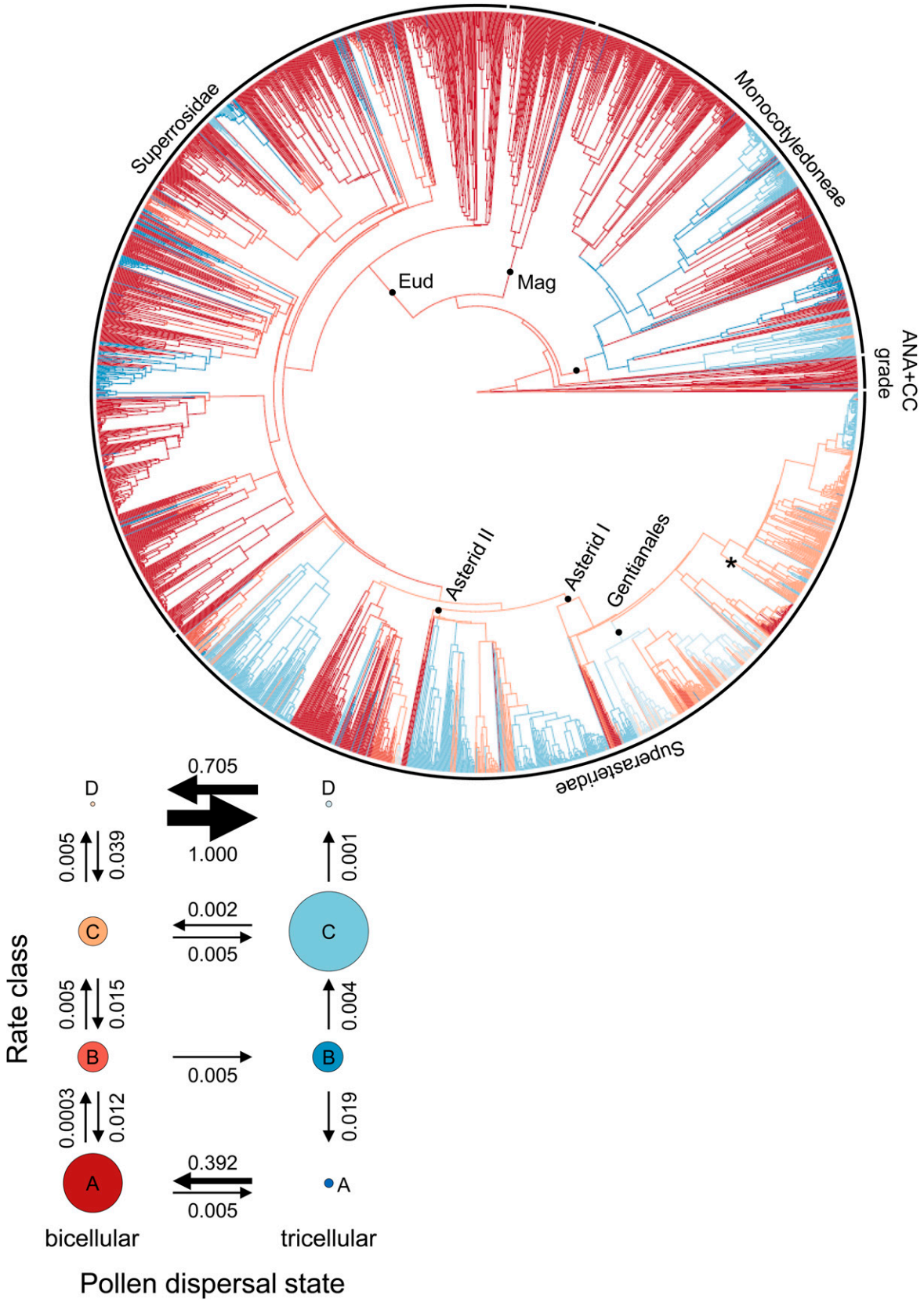

Fig. 4. Results of the hidden rates model with four rate classes and the ancestral state for pollen cell number fixed as bicellular. No assumption about the ancestral state of rate class was made. Transition rates are scaled to 1, and the maximum rate was $q = 0.9844$ (bicellular-D to tricellular-D). Phylogeny from Zanne et al. (2013).

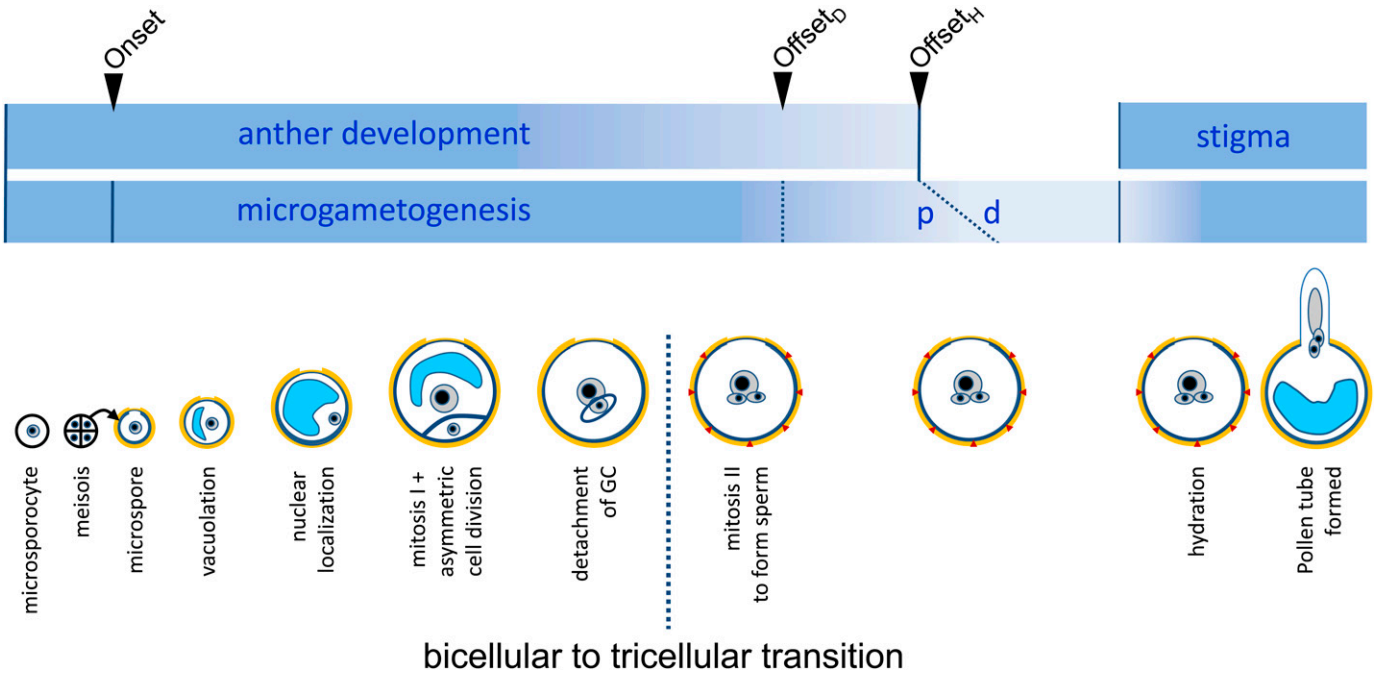

Fig. 5. Pollen developmental sequence and its interaction with anther development. For completeness, the figure illustrates the sequence of tricellular pollen dispersed in a dehydrated state (a relatively rare combination, e.g., in *Arabidopsis*). In most species, microgametogenesis continues after pollination until the species-specific terminal stage of the gametic cell cycle is reached. The stage of microgametogenesis present before dispersal can depend on whether pollen undergoes dehydration ($Offset_D$) or is dispersed in a relatively hydrated state ($Offset_H$). D, dispersal period; GC, generative cell; MS, microsporogenesis; P, presentation period.

of anther opening, or earlier, if development is arrested in the anther by pollen dehydration, as is common (Fig. 5).

Microgametogenesis ultimately involves the formation of a two-celled gametic lineage (two sperm) within a single-celled somatic lineage (the tube cell). At the time of fertilization, sperm can be in the G_1, S, or G_2 stages of the cell cycle, and are synchronized with the egg cell cycle (Friedman, 1999; Tian et al., 2005). At the time of pollen dispersal, all possible cell cycle stages—from a generative cell in the G_1 phase to two sperm cells in the G_2 phase—have been reported in at least some species. Bicellular pollen is often dispersed with the generative cell in late G_2 or prophase of mitosis (Maheshwari, 1950; Brewbaker, 1967), and hence is already near the tricellular condition (Fig. 5).

Species-specific variation in the amount of time available for microgametogenesis before pollen dispersal depends on variation in the duration of anther maturation and presentation and whether dormancy occurs via dehydration of the pollen (Figs. 5, 6). For example, there is a relatively short window for microgametogenesis in species with pollen that undergoes programmed dehydration within a rapidly maturing anther. Conversely, when anther development is greatly prolonged and pollen is dispersed in a relatively hydrated state, there is more time for microgametogenesis to proceed to a later stage before dispersal (Fig. 6).

A sufficiently large change in the amount of time available for microgametogenesis in the anther can result in an evolutionary transition in pollen cell number (Table 1). For example, bicellular pollen can arise from a tricellular ancestor by shifts to earlier offset timing, either by precocious anther maturation or by the gain of pollen dehydration from an ancestrally hydrated dispersal state (Table 1; Fig. 5). Since active dehydration has a genetic basis and is a largely gametophytically controlled process (Ingram and Bartels, 1996; Taylor and Hepler, 1997; Hoekstra, 2005; Franchi et al., 2011), gains of dormancy might be more difficult than losses.

Heterochrony due to altered onset of microgametogenesis must be caused by a change in timing or duration of development prior to the microspore stage that is independent of offset timing. The duration of male meiosis among plant species of similar ploidy at similar temperatures does vary considerably (Bennett, 1977), and even closely related species can have extreme differences due to dormant periods during microsporogenesis (e.g., Endress, 1977). Variation in the dynamics of wall formation and dissolution during tetrad development (Blackmore

Duration of anther development	Degree of pollen dormancy: Strongly dehydrated	Degree of pollen dormancy: Weakly dehydrated
Short	Very short	Short
Long	Long	Very long

Fig. 6. The amount of time available for microgametogenesis within the anther, as determined by the timing of anther maturation and degree of pollen dormancy.

TABLE 1. Potential heterochronies underlying evolutionary transitions between pollen cell numbers. The type of heterochrony involved depends on the direction and magnitude of change in the amount of time available for microgametogenesis within the anther.

Shift in duration of development in anther	Bicellular to tricellular transition	Tricellular to bicellular transition
Increase	Earlier onset (predisplacement)	Strong deceleration
Increase	Later offset (hypermorphosis)	Strong deceleration
No change	Acceleration	Deceleration
Decrease	Strong acceleration	Later onset (postdisplacement)
Decrease	Strong acceleration	Earlier offset (hypomorphosis, or progenesis)

and Crane, 1998) might also affect onset timing. Such variation is only important if it in turn affects the duration of microgametogenesis. Minter and Lord (1983) found that both meiosis and anther dehiscence occurred 2 d earlier in cleistogamous flowers relative to chasmogamous flowers on the same plant. Since the window for microgametogenesis within the anther was unchanged, pollen remained bicellular.

Shifts in developmental rate are another source of transitions in pollen cell number (Table 1). Changes in developmental rate are particularly likely when the duration of development becomes shortened and yet the developmental sequence undergoes extension, or vice versa. We know of no studies that explicitly compare the timing of pollen developmental sequences within a group of closely related taxa, as would be necessary to infer specific mechanistic changes underlying a transition from one pollen dispersal state to another. However, Eady et al. (1994) reported that both tricellular pollen of *Arabidopsis* and bicellular pollen of *Nicotiana* took 8 d to progress from the microspore stage to anther opening under greenhouse conditions. Since both have dehydrated pollen (Johnson and McCormick, 2001; Hrubá et al., 2005), development of tricellular pollen in *Arabidopsis* appears to be accelerated relative to bicellular pollen in *Nicotiana* (Fig. 5).

Extending the narrative: Developmental and ecological origins of tricellular and secondarily derived bicellular pollens—A major conclusion of the Brewbaker paper, and the implied basis for the hypothesis that repeated and irreversible evolution of tricellular pollen is an angiosperm-wide phenomenon, was that bicellular pollen is plesiomorphic. No study has found otherwise. Yet, our results were ambiguous on the issue of ancestral state. The BiSSE analysis strongly supported a tricellular ancestry, but it assumed a single angiosperm-wide transition rate in each direction. In the more likely case that transition rates vary across the tree (the HRM analysis), there was uncertainty around the root state. There are other reasons to question the possibility of a tricellular origin. First, in most recent molecular phylogenetic analyses, eumagnoliids diverge below monocots, *Ceratophyllum,* and eudicots (Soltis et al., 2011), whereas in the tree of Zanne et al. (2013) eumagnoliids are sister to eudicots and *Ceratophyllum* is sister to the monocot + eumagnoliid + eudicot clade (Fig. 3). The topology of Zanne et al. increases support for tricellular ancestry because of the presence of tricellular pollen in *Ceratophyllum,* the ancient monocot lines, Araceae and Alismatales, and in Nymphaeaceae (sister to *Amborella* in the analysis). These are largely herbaceous aquatics, groups in which tricellular pollen is thought to be selectively advantageous over bicellular pollen. If so, then shifts to tricellular pollen may have followed the parallel origins of herbaceous growth forms and/or aquatic habits in early angiosperm history (Doyle, 2012).

We did not include any gymnosperm outgroups in our analyses because our primary focus was on estimating evolutionary rates within angiosperms. Since rates were robust to assumptions about the root state, the effect of adding gymnosperms would only have added noise to rate estimates, as the rates would be those that maximized the likelihood of the gymnosperm and angiosperm data, rather than just the angiosperms. All gymnosperms disperse their pollen in a sexually immature state before sperm are formed (Friedman, 1999; Fernando et al., 2010). On that basis, the immature condition of gymnosperm male gametophytes is homologous to the bicellular state in most woody and herbaceous perennials of the ANA grade, Chloranthales, and eumagnoliids and supports a bicellular ancestral state for angiosperms.

Tricellular pollen is associated with uniquely angiosperm lifestyles (within extant seed plants)—herbs, herbaceous aquatics, annuals, and plants with exceptionally brief reproductive cycles (Stebbins, 1992; Rowe and Paul-Victor, 2012; Williams, 2012b). Shifts into ecological situations that require shorter life cycles often involve reducing plant size and shortening reproduction (Guerrant, 1988; Snell and Aarssen, 2005). These types of transitions reduce the time for development in the anther, making it more difficult to evolve the tricellular condition, a more advanced stage of development that requires more, not less developmental time. Thus, substantial acceleration of development is a likely arbiter of most transitions to the tricellular state (Table 1). A loss of pollen dormancy can provide an extension of development, if ecology allows, and tricellular pollen often has high water content (Towill and Walters, 2000; Aylor et al., 2005; but see Nepi et al., 2001).

Secondarily derived bicellular pollen may have evolved from tricellular ancestors during shifts away from rapid life cycles or from time-limited reproduction. Ecological shifts into less predictable pollination environments are also thought to favor bicellular pollen. Evolving a slower rate or shorter window of development are possible pathways to the bicellular state. But if tricellular pollen commonly originates by accelerated development in species that have an extremely short reproductive phase, then there are fewer options for "reverting" to the bicellular state. In such cases, the window for anther development may already be near a minimal limit, and therefore a shift to the earlier developmental stage of bicellularity is less likely to occur via further reduction in developmental time than by deceleration of development (Table 1). A gain of pollen dormancy from an ancestor with hydrated, tricellular pollen could cause precocious developmental arrest at the bicellular stage, but the machinery necessary for pollen dehydration may have already been lost.

There were at least 30 transitions from tricellular to bicellular pollen in our most conservative analysis (Table 2). Secondarily derived bicellular pollens may have features that indicate their lack of homology to other bicellular pollens. Gardner (1976) found that the derived bicellular pollen of *Triglochin striatum* within the aquatic Alismatales (Table 2) displayed a germination and growth physiology more typical of tricellular pollen.

TABLE 2. Taxa with secondarily derived bicellular pollen inferred from the hidden rates model four-rate analysis (unfixed root). Each row represents a minimum of one origin of a secondarily bicellular species or clade that had at least 95% support for a tricellular branch leading to a node whose descendants include those species (Appendix S3A).

Origin	Order	Family	Secondarily derived bicellular taxa
1	Alismatales	Araceae	*Alocasia*
2			*Anchomanes*
3			*Arisaema dracontium* + *A. jacquemontii* + *A. sikokianum* + *A. serratum* + *A. tortuosum* + *A. triphyllum*
4			*Anubias*
5			*Asterostigma lividum* + *A. riedelianum* + *Taccarum weddellianum* + *Synandrospadix vermitoxicus* + *Dieffenbachia seguine*
6			*Calla palustris*
7			*Chlorospatha*
8		Hydrocharitaceae	*Ottelia alismoides* + *O. acuminata*
9		Juncaginaceae	*Triglochin striata* + *T. maritima* + *T. palustris*
10	Asterales	Asteraceae	*Bidens*
11			*Lecocarpus*
12			*Syneilesis aconitifolia*
13	Brassicales	Brassicaceae	*Armoracia rusticana*
14			*Cardamine flexuosa*
15	Caryophyllales	Droseraceae	*Drosera filiformis*
16		Halophytaceae	*Halophytum ameghinoi*
17		Nyctaginaceae	*Bougainvillea*
18		Polygonaceae	*Rumex vesicarius*
19	Fabales	Polygalaceae	*Securidaca longepedunculata*
20	Lamiales	Lamiaceae	*Perovskia abrotanoides* + *P. atriplicifolia*
21	Malpighiales	Euphorbiaceae	*Euphorbia millii*
22			*Euphorbia dregeana* + *E. obesa*
23			*Euphorbia terracina*
24			*Hura*
25			*Neoguillauminia cleopatra*
26			*Sebastiania*
27	Poales	Cyperaceae	*Fimbristylis dichotoma*
28		Flagellariaceae	*Flagellaria indica*
29		Restionaceae	*Chondropetalum hookerianum*
30	*Unplaced*	Boraginaceae	*Trichodesma*

The meaning of intraspecific variation—The study of embryology has long been undertaken with an eye to the systematic value of early developmental traits. Pollen cell number is one such trait that has often served to distinguish higher-level relationships, especially as an indicator of membership in one of the many apomorphic tricellular clades (Palser, 1975; Tobe, 1989). The finding of variation in pollen cell number below the genus level is usually noteworthy, and Brewbaker accepted only 10 cases of variation within the same genus and discounted variation within species. Yet there continue to be conflicting studies on single species; and single studies that report intraspecific variation, some at the flower or anther level (Dnyansagar and Cooper, 1960; Sampson, 1969; Grayum, 1986; Lora et al., 2009).

Our analyses required that we eliminate conflicts and intraspecific variation from the data set. This was a difficult task, because there were many reports of bicellular pollen with tricellular variants, and vice versa, and some in which the researcher found both states to be common (Sampson, 1969; Foreman, 1984; Grayum, 1986; Lora et al., 2009; see also Johri et al., 1992). Some of this variation might reflect artifacts of collection (Maheshwari, 1950; Brewbaker, 1967). But we suspect that much of it is real and that the perceived stability of pollen cell number has tended to reinforce a typological approach to assessing the pollen dispersal state of a species. It is worth considering why pollen should lack variation. Most pollen undergoes dehydration and dormancy, and the timing of that process is probably regulated in concert with the gametic cell cycle, such that pollen cell number at anthesis is a stable outcome. In angiosperms, pollen with high water content has turned out to be more common than expected (Hoekstra and Bruinsma, 1978; Nepi et al., 2001), and such pollen may have less control over pausing the gametic cell cycle (Franchi et al., 2011).

We found at least 24 species with more than minor variants in pollen cell number, and these were phylogenetically diverse (data available on Dryad). All but four of the variable species are from genera or families that are also polymorphic at the intrageneric or intrafamilial levels, respectively, suggesting that the different rates of evolution found in the HRM analyses extend to the intraspecific level. The exception that proves the rule is the work of Lora et al. (2009, 2012) on temperature- and phenology-dependent variation in *Annona cherimola.* This species is in the almost completely bicellular eumagnoliid clade (Fig. 3), but it seems to have become variable under domestication. There is much left to discover about what traits or combinations of traits mediate the appearance of developmental plasticity in species and whether such plasticity is a precursor to higher level variability.

Conclusions—In this study, we found a strong bicellular to tricellular transition bias that was independent of the ancestral state of pollen cell number and differential diversification rates. Heterochronies underlie transitions between pollen dispersal states and developmental biases may contribute to the transition asymmetry. For example, pollen dehydration may be easier to lose than to gain, favoring evolution of extended development and the tricellular condition. Bias is also expected when considering organismal history. Relative to bicellular pollen, tricellular pollen is often found in species with extreme, time-constrained

lifestyles. Evolutionary rates can be slower when character extremes are approached, such as in seed morphologies (Sims, 2013). In the case of pollen, extreme lifestyles may have been consequent on evolutionary trade-offs in gametophyte biology (faster development for reduced longevity) resulting in fewer options for reversing development. Character correlations are also stronger in tricellular pollen due to the more specialized nature of their pollen–stigma interactions (Brewbaker, 1967) and because tricellular pollen is nearer the terminal fertilization state at dispersal, which is contingent on a greater number of associated changes in cell biology (Twell, 2011; Berger and Twell, 2011). That tricellular lineages diversify slowly and give rise to bicellular lineages slowly suggests that both ecology and development have been involved in reducing their evolutionary rates. More explicit comparative studies may help disentangle the relative importance of selection versus constraints in producing evolutionary patterns in pollen dispersal states.

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