Comparative Ovule and Megagametophyte Development in Hydatellaceae and Water Lilies Reveal a Mosaic of Features Among the Earliest Angiosperms

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- **Background and Aims** The embryo sac, nucellus and integuments of the early-divergent angiosperms Hydatellaceae and other Nymphaeales are compared with those of other seed plants, in order to evaluate the evolutionary origin of these characters in the angiosperms.
- **Methods** Using light microscopy, ovule and embryo sac development are described in five (of 12) species of *Trithuria*, the sole genus of Hydatellaceae, and compared with those of Cabombaceae and Nymphaeaceae.
- **Key Results** The ovule of *Trithuria* is bitegmic and tenuinucellate, rather than bitegmic and crassinucellate as in most other Nymphaeales. The seed is operculate and possesses a perisperm that develops precociously, which are both key features of Nymphaeales. However, in the Indian species *T. konkanensis*, perisperm is relatively poorly developed by the time of fertilization. Perisperm cells in *Trithuria* become multinucleate during development, a feature observed also in other Nymphaeales. The outer integument is semi-annular ('hood-shaped'), as in Cabombaceae and some Nymphaeaceae, in contrast to the annular ('cap-shaped') outer integument of some other Nymphaeaceae (e.g. *Barclaya*) and *Amborella*. The megagametophyte in *Trithuria* is monosporic and four-nucleate; at the two-nucleate stage both nuclei occur in the micropylar domain. Double megagametophytes were frequently observed, probably developed from different megaspores of the same tetrad. Indirect, but strong evidence is presented for apomictic embryo development in *T. filamentosa*.
- **Conclusions** Most features of the ovule and embryo sac of *Trithuria* are consistent with a close relationship with other Nymphaeales, especially Cabombaceae. The frequent occurrence of double megagametophytes in the same ovule indicates a high degree of developmental flexibility, and could provide a clue to the evolutionary origin of the *Polygonum*-type of angiosperm embryo sac.

Key words: Embryo sac, megagametophyte, ovule, Hydatellaceae, *Trithuria*.

INTRODUCTION

Following more than a century of research since the momentous discovery of double fertilization in flowering plants (Nawaschin, 1898; Guignard, 1899*a, b*; reviewed by Batygina, 2006; Raghavan, 2006), the evolutionary origin of the angiosperm ovule and embryo sac remains enigmatic (Friedman, 2006; Rudall, 2006). Indeed, the various permutations of the relatively conservative number of cells and modules that constitute the embryo sac and its surrounding sporophytic tissues represent a compelling, Sudoku-like, puzzle. The high degree of organization in ovular tissues is controlled by a relatively large array of transcription factors (Skinner *et al.*, 2004), indicating strong developmental constraints governing their structure and arrangement.

Extensive pre-Cenozoic extinctions, especially in the angiosperm stem group, have rendered phylogenetic reconstruction in the basal angiosperm nodes highly sensitive to taxon sampling. The extant early-divergent groups, although species-poor, represent a high proportion of gross morphological diversity in angiosperms. Thus, the

discovery of a 'new' early-divergent angiosperm family, Hydatellaceae, recently transferred to the water-lilies (Nymphaeales) from the monocot clade Poales (Saarela *et al.*, 2007), allows us to re-evaluate morphological evolution among the extant members of the earliest angiosperm lineages (Rudall *et al.*, 2007; Sokoloff *et al.*, 2008*a, b*). Although Saarela *et al.* (2007) did not formally assign Hydatellaceae to an order, we follow the Angiosperm Phylogeny Website (Stevens, 2007), where Hydatellaceae is placed in Nymphaeales. Following essential detailed species-level studies resulting in placement of *Hydatella* in synonymy of *Trithuria* (Sokoloff *et al.*, 2008*a*), we present comparative developmental data on the ovule and megagametophyte of several species of Hydatellaceae and some of their closest allies among early-divergent angiosperms. Previous studies on embryology of Hydatellaceae compared them with Poales and other monocots rather than with Nymphaeales, due to the existing phylogenetic context. Hamann (1962, 1975, 1976, 1998) tentatively reported a five-celled female megagametophyte in Hydatellaceae (i.e. with antipodals absent or ephemeral), but did not examine early developmental stages. Gaikwad and Yadav (2003) briefly

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described development of both the male and female gametophytes in the sole Indian species, *T. konkanensis*.

The majority of angiosperms produce monosporic megagametophytes that result from mitoses of only one of four megaspores. The remaining three megaspores (normally the three closest to the micropyle) undergo programmed cell death (apoptosis). Current evidence suggests that bisporic and tetrasporic embryo sacs are derived conditions that have evolved multiple times in angiosperms, especially in relatively derived groups, but also in some early-divergent angiosperms such as Piperaceae (Williams and Friedman, 2004). The monosporic eight-nucleate/seven-celled *Polygonum*-type embryo sac, which characterizes more than 70% of flowering plant species that have been studied embryologically, consists of two similar mirror-image domains at the chalazal and micropylar poles (Favre-Duchartre, 1984; Williams and Friedman, 2004). The discovery of a monosporic four-nucleate/four-celled condition in some early-divergent angiosperms (Battaglia, 1986; Winter and Shamrov, 1991a, b; Batygina and Vasilyeva, 2002; Williams and Friedman, 2002, 2004; Friedman *et al.*, 2003) prompted the hypothesis that the four-nucleate condition is ancestral, and gave rise by duplication to the eight-nucleate condition (Friedman and Williams, 2003, 2004; Williams and Friedman, 2004). This feature is therefore critical in evaluating angiosperm evolution.

Despite advances in our understanding of megagametophyte evolution within angiosperms, the homologies of the nucellus and integuments with respect to those of other seed plants – and hence the origin of these characters in the angiosperms – require clarification. Thus, comparison of Hydatellaceae and other early-divergent angiosperms with other seed plants can help to inform debates on the evolutionary origin of flowering plants.

MATERIAL AND METHODS

Seven separate collections fixed in alcohol or formalin-acetic-alcohol (FAA) were examined, representing five (of 12) species of *Trithuria*, the sole genus of Hydatellaceae (Sokoloff *et al.*, 2008a). Each collection included numerous plants, each plant bearing numerous reproductive units: (1) *Trithuria submersa* Hook.f. (Doust 1123, Davis and Stevenson, South Australia 1998; voucher at MELU); (2) *Trithuria submersa* (Conran 961 and Rudall, near Bangham Conservation Park, South Australia 1998, in seasonally ephemeral swamp; voucher at ADU); (3) *Trithuria submersa* (HK: material grown at Kew from seeds collected by Tuckett at Mersa Road swamp, Western Australia 2006); (4) *Trithuria lanterna* D.A.Cooke (K: 47115; Dunlop 4740A, Northern Territory, Australia, 1978); (5) *Trithuria australis* (Diels) D.D. Sokoloff, Remizowa, T.D. Macfarl. & Rudall (Macfarlane 3357 and Hearn, Western Australia 2000, approx. 50 km E of Manjimup, 1999; vouchers at NSW and PERTH); (6) *T. konkanensis* Yadav & Janarth. (Yadav *s.n.*, near Kolhapur, India, 2006); and (7) *T. filamentosa* Rodway; only older stages available (K: 28269; de Malahide *s.n.*, Lake Dobson, Australia, 1966). Species of Cabombaceae examined for comparison were obtained

FIG. 1. *Trithuria submersa* (Doust 1123), embryo sacs at early developmental stages, orientated with stigmatic region towards top. (A) Outer integument just initiated; ovule orientated downwards. (B) Both integuments just initiated, slightly enlarged archesporial cell just visible, ovule still orientated downwards. (C) Both inner and outer integuments and archesporial cell visible, ovule starting to curve upwards. (D) Ovule curvature almost complete. a, archesporial; ii, inner integument; oi, outer integument. Scale bars = 10 μ m.

from the Kew Herbarium spirit collection: *Cabomba aquatica* Aubl. (K: 7405; Philcox 4636, Brazil), and *Brasenia schreberi* J.F. Gmel. (K: 15395; Drummond and Hemsley 4628, Uganda).

Fixed material was transferred to 70% ethanol prior to examination. For differential interference contrast microscopy (DIC), carpels were dissected on a microscope slide in a drop of a modified version of Herr's clearing fluid (lactic acid:chloral hydrate:phenol:clove oil:HistoClear, in proportions 2:2:2:2:1 by weight). For LM observations, material was embedded in paraplast using standard methods prior to sectioning at NYBG (for Fig. 1) and Moscow (for Fig. 9A), or embedded in Tecnovit for sectioning at the Royal Botanic Gardens, Kew (RBGK) using a Leica rotary microtome. Sections were stained in Toluidine Blue and mounted in DPX. All imaging was carried out at RBGK using a Leitz Diaplan photomicroscope fitted with a Leica DC500 digital camera. Some images were merged using the 'photomerge' option in Adobe Photoshop. Drawings were made using a camera lucida fitted onto a Leitz Diaplan microscope. For SEM investigation, material was dissected in 70% ethanol, then dehydrated through absolute ethanol and critical-point dried using a Balzers CPD 020 (BALTEC AG, Liechtenstein) at RBGK. Dried material was further

FIG. 2 *Trithuria lanterna*, DIC images of megasporogenesis, oriented with micropylar region towards top. (A) Megaspore mother cell (megasporecyte). (B) Megaspore mother cell at meiosis I. (C) Tetrad with three microphyllar megaspores degenerating. (D) Tetrad just before degeneration of three microphyllar megaspores. ii, inner integument; mmc, megaspore mother cell (megasporecyte); ms, meiotic spindle; ne, nucellus; oi, outer integument; p, tissue that will ultimately form perisperm. Numbered cells indicate four megaspores of tetrad. Scale bars = 10 μ m.

dissected and mounted onto specimen stubs using double-sided tape, coated with platinum using an Emitech K550 sputter coater (Emitech, Ashford, UK), and examined using a Hitachi cold field emission SEM S-4700-II (Hitachi High Technologies Corp., Tokyo, Japan) at RBGK.

RESULTS

Ovule development in Trithuria

The developing ovule apex (Figs 1–5) forms a hypodermal archesporial cell (Fig. 1). There is no premeiotic mitosis to form parietal tissue, so that the megaspore mother cell (megasporecyte) is formed directly from the archesporial cell (Figs 1–4); this represents the tenuinucellate condition. The outer integument is initiated at the archesporial stage, rapidly followed by initiation of an inner integument (Fig. 1). Differential growth causes the ovule to curve upwards so that the archesporial cell (and eventually the micropyle) is uppermost (Figs 1 and 3A). The outer integument grows only on the side opposite to the funicle (Fig. 1D), and is therefore semi-annular rather than annular. In the mature ovule and seed, the micropyle region is completely fused to the funicle (Fig. 6).

The single-layered micropylar region of the nucellus later divides periclinally to form extra cell layers at the micropyle; in some ovules these divisions occur at an early stage (e.g. Fig. 5B), while in others such divisions are absent at the stage when the embryo sac is already enlarged (Fig. 5F). At later stages, these cell divisions result in a small nucellar beak (Figs 7 and 8). Before fertilization, the micropylar region of the inner epidermis of the inner integument becomes tanniniferous and subsequently develops lignified wall thickenings, including a prominent thickening below the micropyle. In old ovules, some of the inner epidermal cells closest to the micropyle collapse and lose contact with their inner cell wall/cuticle, resulting in a cavity between the nucellar beak and the ‘operculum’ that is formed from this region of the inner integument, at least in *T. australis* (Fig. 8B, D). In many cases, a cuticular and/or cellulosic strand (probably derived from the united cell walls plus cuticles of adjacent margins of the inner integument) traverses the cavity longitudinally.

Cuticular layers are present between the outer and inner integuments and between the inner integument and nucellus. In all species examined except *T. submersa*, the outer integument and carpel wall become closely pressed together.

FIG. 3. *Trithuria konkanensis*, developing ovules oriented with micropylar region towards top. (A–C) Megaspore mother cells. (A) Inner integument slightly longer than outer integument. (B, C) Outer integument closed at micropyle, inner integument surrounding lower part. (D, E) Megaspores undergoing meiosis. (F) Dyad. ii, inner integument; mmc, megaspore mother cell (megaspore); oi, outer integument. Scale bars = 10 μ m.

In *T. lanterna*, *T. konkanensis* and *T. australis*, a thick, firm cuticular layer lies between the carpel wall and the outer integument. In some paraplasm-embedded sections the cuticle is more-or-less wavy, so that it appears to be attached to the carpel wall in some places and to the ovule in others. However, this folding is an artefact caused by hygroscopic curvature of the cuticle. Folding is absent from all resin-embedded sections, and the cuticle fills the space between the ovary wall and the outer integument, which are tightly pressed together. We found no clear differences in cuticle structure between immediately pre-fertilized ovules and fruits of *T. lanterna*; when the fruit dehiscence, the cuticle remains associated with the seed. In mature fruits, this cuticle probably plays an important role in protecting the seed.

In material examined of *T. filamentosa*, the inner surface of the carpel wall and the outer surface of the outer integument each possess a cuticle, each one as thick as the single cuticle in other species. These two closely situated cuticles are distinguishable from each other as they stain in slightly different colours. In *T. submersa*, a distinct cuticle was absent from both the inner surface of the carpel and the outer surface of the outer integument, and also from the fruit.

Megasporogenesis and megagametogenesis

The megasporocyte stage occurs when the outer integument is already closed at the micropyle, but the inner integument is only half-grown (Figs 3B, C and 4A). Megasporocytes were the most frequent stage observed in young ovules of *T. konkanensis*, *T. lanterna* and *T. submersa*, indicating that this stage is more prolonged than later developmental stages. In *T. konkanensis*, *T. lanterna* and *T. submersa*, meiosis I was observed (Figs 2B, 3D, E and 4C), as were occasional dyads (Figs 3F, 4E and 5A) and several ovules containing a linear tetrad of megaspores (Figs 2C, D, 4F and 5D–F), in some of which at least two of the micropylar megaspores were degenerating (i.e. flattened and densely cytoplasmic). The chalazal megaspore is typically the largest megaspore in the tetrad, and the three micropylar megaspores are relatively small. However, in rare tetrads of *T. konkanensis*, megaspore size alternated so that (from the micropylar end) the second and fourth megaspore were larger than the other two (larger megaspores labelled 2 and 4 in Fig. 5D).

Among older ovules examined (Figs 7 and 8), we rarely observed non-cellularized two-nucleate stages (Fig. 8A), but frequently observed four-celled stages (Figs 7 and 8E, F). At the four-nucleate stage, the central cell nucleus is the largest and most prominent of the embryo sac nuclei. We observed the central cell nucleus either at the chalazal pole (Fig. 8E) or in the centre or to the side of the embryo sac (Fig. 8F), or occasionally (in *T. konkanensis*) in the micropylar sector.

In both *T. konkanensis* and the material of *T. submersa* grown at Kew, approx. 25 % of ovules were difficult to interpret because two or more female gametophytes were developing in the same ovule, with the result that the entire gametophytic part of the ovule appeared to be more cellularized, and to contain more nuclei, than would be expected if only one megagametophyte is developed (Figs 9, 10 and 11C–E).

Perisperm

In mature (non-fertilized) ovules, the chalazal region of the ovule forms a massive, starch-rich, non-vascularized tissue that we here term a perisperm. This term strictly applies to a nucellar storage tissue in a fertilized seed, but it is commonly also applied to pre-fertilized ovules (discussed in Rudall, 1997). Perisperm in *T. konkanensis* was much less extensive than in the other species examined here, at least in pre-fertilized ovules. Perisperm is initiated

FIG. 4. *Trithuria submersa* (HK), developing ovules oriented with micropylar region towards top. (A) Single megaspore mother cell (megaspore mother cell). (B) Later stage with elongated chalazal nucellus; two cells present (possibly abnormal). (C) Megaspore mother cell undergoing meiosis (meiotic spindle arrowed). (D) Young ovule in which the megaspore mother cell has recently divided along an axial plane (at right angles to a normal meiosis), resulting in two laterally adjacent nuclei (arrowed). (E) Young ovule with two axially adjacent cells (arrowed). (F) Possible tetrad stage, with four large nuclei in a single axial row (arrowheads), lacking clear cell walls between them, and the remains of degenerated cell (possibly a degenerated megaspore) at the micropylar end. (G) Two-nucleate stage with both nuclei (arrowed) in micropylar domain. (H) Stage with outer integument almost enclosing ovule; inner integument still relatively short. ii, inner integument; m, megaspore mother cell (megaspore mother cell); oi, outer integument. In E, G–I, inset squares indicate overlaid optical sections in appropriate positions (except for left hand box in G, in which position of synergids is indicated by arrow, over egg cell). Scale bars = 10 μ m.

shortly after meiosis, but develops mostly following the tetrad stage. Initially each perisperm cell contains a single large nucleus, but eventually the perisperm becomes more

highly organized. The outermost layer is relatively dense, and groups of central perisperm cells enlarge and become multinucleate even before fertilization. The nuclei clump

FIG. 5. *Trithuria konkanensis*, developing ovules oriented with micropylar region towards top. (A) Dyads. (B–F) Tetrads; chalazal megaspore larger than three micropylar megaspores in (B, C, E) alternating in (D), functional chalazal megaspore much larger in (F). fm, functional megaspore; ii, inner integument. Numbered cells indicate four megaspores of tetrad. Scale bars = 10 μ m.

together, and groups of nuclei often lie at the cell periphery, close to groups of nuclei in neighbouring cells. A region of tanniferous cells is present at the chalazal end.

Early embryo and endosperm development and pollen tube growth

In some post-anthetic reproductive units, all ovules contained a proembryo and a small amount of endosperm. At this stage, an abscission layer is formed at the top of the stalk of the developing one-seeded fruit (which is especially conspicuous in *T. filamentosa*) and fruits break off at this point.

In most species examined (*T. submersa*, *T. lanterna*, *T. konkanensis*), narrow pollen tubes were clearly visible

using SEM (Figs 12 and 13). Pollen tubes were always firmly attached to the stigmatic hairs, probably growing in the cell wall of the stigmatic hair cells. However, in *T. filamentosa* pollen tubes were entirely absent from stigmatic hairs attached to developing fruits (Fig. 12D). We also observed no pollen tubes in *T. australis*, but in this case developing fruits also were absent.

DISCUSSION

The megagametophyte of Trithuria is four-nucleate

Embryo sac development in *Trithuria* is monosporic and the megagametophyte is four-nucleate and four-celled at maturity. This pattern conforms to the *Schisandra*-type of Battaglia (1986) and Tobe *et al.* (2007), also termed the *Schisandra*-variation of the *Oenothera*-type (Batygina and Vasilyeva, 2002) and the *Nuphar*-sequence (Friedman and Williams, 2003). It is prevalent in two early-divergent angiosperm orders, Nymphaeales (Fig. 12) and Austrobaileyales (e.g. Friedman *et al.*, 2003; Tobe *et al.*, 2007).

The four-nucleate monosporic megagametophyte could be derived from an eight-nucleate one by reduction (i.e. by deletion of a single mitosis during early megagametogenesis). This might be expected in aquatic groups such as Hydatellaceae and other Nymphaeales, because aquatic plants are frequently associated with strong morphological reduction, high intraspecific variation and high plasticity (e.g. Arber, 1920; Bateman, 1996; Cook, 1999). Comparable four-nucleate embryo sacs occur in the anomalous aquatic family Podostemaceae (Maheshwari, 1950), although via a different developmental pathway. However, in contrast to an aquatic reduction hypothesis, a similar four-nucleate embryo-sac type occurs in the adjacent early-divergent clade, Austrobaileyales, which are terrestrial and woody. Furthermore, not all aquatic angiosperms possess this feature; *Ceratophyllum* has a typical *Polygonum*-type embryo sac, and *Nelumbo* has an increased number of antipodals (Batygina *et al.*, 1982).

Alternatively, the four-nucleate condition in the ANITA grade could have given rise by duplication to the eight-nucleate condition in other angiosperms, as proposed by Friedman and Williams (2003). Optimizations of female gametophyte ontogeny onto alternative angiosperm phylogenies (with *Amborella* placed either as sister to all other angiosperms, or sister to Nymphaeales) both result in the same equivocal plesiomorphic state for angiosperms, either four-nucleate or eight-nucleate. Friedman and Williams (2003) regarded the spatial arrangement of nuclei at the two-nucleate stage as crucial to this evolutionary transition, and further proposed an ‘early modification’ hypothesis. Thus, in the eight-nucleate type, nuclear migration along a cytoskeletal array results in a spatial arrangement at the two-nucleate stage with two distinct poles, which determines the presence of two mirror-image domains. By contrast, the four-nucleate type lacks such nuclear migration. Our observations of two-nucleate stages with both nuclei in the micropylar domain (Fig. 8A) reinforce the ‘early modification’ hypothesis.

FIG. 6. *Trithuria submersa* (A–C, Conran 961 and Rudall; D, McCallum Webster 640). SEM micropyle. (A) General view of ovule. (B) Enlarged micropylar region of the same ovule (micropyle obscured by a loose cell). (C) Enlarged micropylar region of another ovule. (D) Dry mature seed; arrowhead indicates elongate seed tip at micropylar end; this resembles a free funicle, but is derived from the outer integument fused with the funicle. fun, very short free region of funicle broken transversely when removing ovule from carpel; mic, micropyle. Scale bars = 50 μ m.

Double megagametophytes could provide a clue to the origin of the angiosperm embryo sac

Our frequent observations of two female gametophytes developing in the same ovule in *T. konkanensis* and *T. submersa* (Figs 9–11) are potentially significant because they indicate a high degree of developmental flexibility. These cases are comparable with similar flexibility in other early-divergent angiosperms and with normal development in some gymnosperms.

Occasional development of more than one functional megaspore from a multicellular archesporium is common in angiosperms (Maheshwari, 1950), but multiple gametophytes derived from a single megasporocyte are unusual. One example is the rosid *Phellodendron amurense*, in which all four megaspores of the same tetrad start to develop embryo sacs, but typically only one, or rarely two, develop to maturity (Starshova and Solntseva, 1973;

Poddubnaya-Arnoldi, 1976). We consider it possible that the paired gametophytes in ovules of *Trithuria* did not develop from separate archesporial cells within the nucellus, because we never observed separate enlarging archesporial cells. Instead, we observed cases of (1) possible insertion of a mitosis before meiosis; and (2) development of gametophytes from more than one of the four megaspores in a tetrad. Rare double megasporocytes in *T. submersa* (Fig. 4B, D) could be cases in which the megasporocyte had undergone mitosis, because, in angiosperms, meiosis I is invariably axially polarized with a transverse cell plate, although meiosis II is sometimes at right angles to this (e.g. Bouman, 1984). However, we cannot exclude the possibilities that these are either (a) rare cases of tetrahedral tetrads, or (b) multiple archesporial cells. The unusual tetrad in Figure 5D, in which two alternate megaspores are larger than the other two, could be a case

FIG. 7. *Trithuria australis*, embryo sac at four-nucleate stage (DIC), orientated with micropyle towards top. n, nucellus, p, perisperm. Scale bar = 10 μ m.

in which both larger megaspores would form embryo sacs. It compares closely with similar sporadic cases illustrated in *Nuphar lutea* (Nymphaeaceae) by Batygina and Vasilyeva (2002).

Gaikwad and Yadav (2003) reported a tetrasporic *Adoxa*-type of development in *T. konkanensis*; in this type none of the four megaspores degenerate, and each contributes to the component organization of the mature megagametophyte. Our results contradict this interpretation because in the *Adoxa*-type (as in all other tetrasporic and bisporic types) the four megaspores form a coenocyte, and cellularization occurs only after subsequent mitoses (Maheshwari, 1950). This is not the case in *Trithuria*, in which we (and Gaikwad and Yadav, 2003) consistently found a linear cellularized tetrad of megaspores, sometimes with the micropylar ones degenerating. Based on stronger comparative data and evidence from tetrad morphology, we reinterpret the structures observed by Gaikwad and Yadav (2003) as two adjacent embryo sacs rather than as a single tetrasporic eight-nucleate embryo sac, although the majority of ovules of *T. konkanensis* contain a single four-nucleate embryo sac.

In ovules of *Trithuria* with double gametophytes, the chalazal one is usually (but not always) the larger of the two, although the distinction between them is not always clear (Figs 9–11). In many ovules of *T. konkanensis*, the smaller (micropylar) gametophyte contains two prominent cells that superficially resemble synergids, so that the pair of four-nucleate embryo sacs resemble a single (possibly six-nucleate) one. Interestingly, similar cases of paired embryo sacs are common among other early-divergent angiosperms such as Nymphaeaceae (Cook, 1906; 1909) and Schisandraceae (Friedman *et al.*, 2003; Williams and Friedman, 2004). In *Kadsura* (Schisandraceae), one of the two gametophytes is frequently binucleate, and passage of nuclei sometimes occurs between the two embryo sacs (Friedman *et al.*, 2003). In similar cases in *Nymphaea*, one embryo sac is ‘always absorbed by the other’ (Cook, 1906, page 378). Otherwise, such compound embryo sacs

are rare in other angiosperms; Maheshwari (1950) cited some examples, but these records apparently differ from *Trithuria* in that each embryo sac is reportedly derived from a different cell in a multicellular archesporium. Batygina *et al.* (1982) also reported cases of multiple embryo sacs developed from a multicellular archesporium in *Ceratophyllum*. By contrast, gametophytic doubling in *Trithuria* apparently occurs during megasporogenesis.

Given the frequency of compound embryo sacs in early-divergent angiosperms, it is possible that species of these ancient lineages are relatively tolerant of major gametophytic teratologies. Such a high degree of plasticity is surprising given the apparently rigorous genetic constraints and otherwise conservative organization of the angiosperm embryo sac (Skinner *et al.*, 2004). A different type of mutation presumably stabilized into the nine-nucleate embryo sac reported in *Amborella* (Friedman, 2006), which has no obvious homologue among other angiosperms.

Exploring the enigmatic evolutionary origin of the angiosperm embryo sac requires comparison with the condition in gymnosperms, particularly extant species, because the data on fossil gymnosperms are relatively sparse. Admittedly, such comparisons are highly speculative given the vast lacuna that separates extant gymnosperms from extant angiosperms (e.g. Bateman *et al.*, 2006); extreme phylogenetic divergence makes comparative data on the organization of megagametophytes of extinct seed plants limited and individual stages difficult to homologize. In most extant gymnosperms a variable number of archegonial initials develop at the micropylar end of a polarized female gametophyte during megagametogenesis (Biswas and Johri, 1997). A gymnosperm archegonial initial is therefore not homologous with an angiosperm archesporial cell (reviewed by Favre-Duchartre, 1984).

The origin of the *Polygonum*-type embryo sac was probably of major adaptive significance in angiosperms, because it allowed stabilization of triple fusion and triploid endosperm formation. Could the *Polygonum*-type embryo sac be derived from fusion of two gametophytes, as we have observed in *Trithuria*? Several authors have plausibly postulated derivation of the angiosperm megagametophyte by fusion of two or more gymnosperm archegonia within the same megagametophyte, assuming reduction of sterile tissue of the gymnosperm megagametophyte (reviewed by Favre-Duchartre, 1984; Rudall, 2006). Such evolutionary transitions are normally thought to have occurred before the origin of the angiosperms, but lability in early-divergent angiosperms could indicate similar evolutionary ‘tinkering’ prior to canalization of a highly structured organization. The ‘duplication’ hypothesis that the four-nucleate condition is ancestral in angiosperms, and gave rise by duplication to the eight-nucleate condition (Friedman and Williams, 2003, 2004; Williams and Friedman, 2004), provides no clue to this, because the ancestral condition is unknown. Alternatively, a pair of four-celled embryo sacs could represent a step towards an eight-nucleate *Polygonum*-type gametophyte. One counter-argument to this is that the innermost (chalazal) embryo sac has the same polarity as the supernumerary one in early-divergent angiosperms, including *Trithuria*, i.e., with the egg cell

FIG. 8. Ovules oriented with micropyle towards top. (A–D) *T. australis*, (E, F) *T. submersa*. (A) Two-nucleate stage, with both nuclei in micropylar domain. Optical section overlayed in actual position. (B–D) Old unfertilized ovules. Inner integument partially degraded at micropyle in B, D, leaving remains of cuticle. (E) Four-nucleate stage, with micropyle formed by both integuments; nucellus 2–3 cells thick at micropyle. Optical section overlayed in actual position, showing central cell nucleus at the chalazal pole. (F) Four-nucleate stage, with micropyle formed by both integuments; nucellus 2–3 cells thick at micropyle. Optical section with central cell overlayed in actual position (to one side of centre), and synergids placed to one side of egg cell. c, central cell nucleus; e, egg; mi, micropyle; p, perisperm; s, synergid. Scale bar = 10 μ m.

and synergids at the micropylar side. The ‘fusion’ hypothesis would imply reorientation of the innermost embryo sac during evolution (not during ontogeny), so that its egg apparatus transforms into synergids. Such a reorientation has been described in *Nuphar lutea*, in which Winter and Shamrov (1991a) reported four (of more than 100) ovules containing developing embryo sacs with inverted polarity. Assuming that morphological evolution results from changes in developmental pathways, the fusion hypothesis does not necessarily contradict the duplication hypothesis, because both hypotheses imply insertion of an additional mitosis after megaspore formation.

Such highly speculative hypotheses will become testable when more is known about the developmental genetics of embryo sac organization. The fusion hypothesis differs

from formation of bisporic and tetrasporic embryo sacs, in which a single embryo sac develops from two or four megaspores. In theory, formation of a bisporic female gametophyte would appear to be the easiest way to combine two four-celled embryo sacs, but bisporic embryo sacs are extremely rare among primitive angiosperms (although rarity does not in itself negate the argument). Interestingly, the most common types of bi- and tetrasporic embryo sacs are superficially extremely similar to monosporic *Polygonum*-type female gametophytes (i.e. with two synergids and three antipodals). All of these eight-nucleate types are non-homologous by developmental origin via different cell lineages, but show a high degree of similarity, which cannot be explained solely by similarity of function.

FIG. 9. *Trithuria konkanensis*. (A–D) Different abnormal ovules in which two embryo sacs are developing, orientated with micropyle uppermost. 1, smaller micropylar embryo sac; 2, larger chalazal embryo sac; r, cellular remains. Scale bars = 10 μ m.

Nucellus and integument development

Regarding the ovular (i.e. sporophytic) tissues that surround the embryo sac, the ovule/seed in *Trithuria* resembles that of other Nymphaeales in possessing a perisperm and operculum, but differs in some aspects of the integuments and nucellus.

Most current evidence suggests that two conditions are ancestral within angiosperms: (1) the presence of two integuments (bitegmy), and (2) the crassinucellate condition – the presence of micropylar parietal tissue that (crucially) is derived from the archesporium. These conditions are prevalent among early-divergent angiosperms (Winship Taylor, 1991; Herr, 1995, 2000; Endress and Igersheim, 2000; Shamrov, 2000), but there have been numerous shifts within angiosperms to both unitegmy and the tenuinucellate condition

(in which a hypodermal archesporial cell gives rise directly to the megasporocyte). For example, a major shift to unitegmy occurred at the base of the asterid eudicot clade (e.g. Young and Watson, 1970; Philipson, 1974; Albach *et al.*, 2001), although both ontogenetic data (on a phylogenetically broad range of taxa) and developmental-genetic data (on *Arabidopsis*) have indicated that not all transitions to unitegmy are homologous (Bouman and Calis, 1977; Endress and Igersheim, 2000; Skinner *et al.*, 2004; McAbee *et al.*, 2006).

In contrast to most other authors, Shamrov (1998a) distinguished three rather than two ovule types by nucellar morphology: crassinucellate, medionucellate and tenuinucellate. He defined the tenuinucellate condition by a combination of characters, such as (1) the nucellus consists of a single dermal layer, and (2) the nucellus normally degenerates prior to fertilization. Since *Trithuria* does not fit the second criterion, its ovule would be medionucellate in Shamrov's classification.

The shift to unitegmy has frequently been correlated with other evolutionary transitions, especially from the crassinucellate to the tenuinucellate condition (e.g. Albach *et al.*, 2001). However, this is not the case in *Trithuria*, in which ovules are bitegmic and tenuinucellate, compared with bitegmic/crassinucellate in all other Nymphaeales (Cook, 1906; Schneider, 1978; Batygina *et al.*, 1980). The tenuinucellate condition in angiosperms is normally regarded as the derived state (e.g. Herr, 1995), and in *Trithuria* it is presumably a result of reduction, although subsequent divisions in the micropylar nucellus result in formation of a short nucellar beak. Such apparently minor differences in the ontogenetic derivation of micropylar nucellar tissue (whether from the archesporium or the nucellus) may appear trivial when applied within the angiosperms, but gains more significance in comparison with other seed plants, because in crassinucellate ovules the nucellus can be interpreted as a sporangiophore–sporangium complex (Bouman, 1984; Herr, 1995), in contrast to the tenuinucellate condition.

In *Trithuria* and other Nymphaeales (e.g. *Cabomba* and *Brasenia*: Fig. 14; *Barclaya*: Schneider, 1978), perisperm (a nutritive tissue derived from the chalazal region of the nucellus) develops precociously, well before fertilization; chalazal mitoses commence at the tetrad stage in *Trithuria*, and continue up to fertilization, when the perisperm is filled with starch. Precocious perisperm development is not uniform in all *Trithuria* species examined, and is least prominent in the Indian species, *T. konkanensis* – an important feature distinguishing *T. konkanensis* from its morphologically closest species, the Northern Australian *T. lanterna*. Perisperm is rare in eudicots, but relatively common in early-divergent angiosperms such as Piperaceae (e.g. Johnson, 1900, 1902), Saururaceae (Raju, 1961), Cabombaceae and Nymphaeaceae (Seaton, 1908; Schneider, 1978; Batygina *et al.*, 1980; Schneider *et al.*, 2003). Perisperm is also a feature of some monocots, including Poales (Rudall, 1990, 1997), the order in which Hydatellaceae were formerly placed.

The multinucleate perisperm in *Trithuria* is unusual, although a similar condition may occur in another early-

FIG. 10. *Trithuria submersa* (HK), abnormal embryo sacs orientated with micropyle uppermost. (A) Two or more axially adjacent embryo sacs containing a variable number of nuclei. Superimposed optical section outlined in white. (B) Two more-or-less laterally adjacent embryo sacs, each with 2–4 nuclei (drawn in Fig. 11E). (C, D) Optical sections through a single ovule showing two axially adjacent embryo sacs, each with approx. 2 nuclei. (E, F) Optical sections through a single ovule showing two or more axially adjacent embryo sacs, each with approx. 2 nuclei (drawn in Fig. 11D). Embryo sacs numbered. n, nucellus; p, perisperm; r, cellular remains. Scale bars = 10 μ m.

divergent angiosperm family, Piperaceae (Johnson, 1900, 1902). Batygina *et al.* (1980) reported binucleate nucellar cells in some Nymphaeaceae (*Euryale*, *Nuphar* and *Barclaya*). Maheshwari (1950) cited some monocots (*Hedychium* and *Pandanus*) in which the nucellar nuclei wander from cell to cell and collect together in groups, sometimes entering the embryo sac. Species of the eudicot family Podostemaceae, which is not closely related to Hydatellaceae but shares an ecophysiological preference for aquatic habitats, form a nucellar ‘pseudo-embryo sac’, in which the chalazal nucellar cells enlarge and eventually disorganize to form a large cavity, associated with endosperm suppression (Maheshwari,

1950; Masand and Kapil, 1966). Conversely, we found no counterpart in *Trithuria* for the haustorium that grows into the perisperm after fertilization in some of its close relatives in Nymphaeales, such as *Cabomba*, *Brasenia* and *Nymphaea* (e.g. Cook, 1906; Khanna, 1964, 1965; Schneider, 1978; Schneider and Jeter, 1982). This will be further explored in studies of later embryo development and early seed germination.

A semi-annular (sometimes termed ‘hood-shaped’) outer integument, which we found in *Trithuria*, has also been reported in Cabombaceae (*Brasenia* and *Cabomba*; e.g. Batygina *et al.*, 1982; Igersheim and Endress, 1998). In contrast, *Amborella* has an annular (‘cap-shaped’) outer

FIG. 11. Drawings of embryo sacs of *Trithuria*. (A) *T. submersa*, two-nucleate embryo sac. (B) *T. submersa*, four-celled embryo sac. (C) *T. konkanensis*, pair of two-nucleate embryo sacs shown in Fig. 9A. (D) *T. submersa*, pair of embryo sacs shown in Fig. 10(E, F). (E) *T. submersa*, pair of embryo sacs shown in Fig. 10(B). c, central cell nucleus; ea, egg apparatus; r, cellular remains. Numbers indicate different embryo sacs. Scale bars = 10 μ m.

integument (Endress and Igersheim, 1997). Both conditions, with intermediates, occur in Nymphaeaceae (Shamrov and Winter, 1991; Winter and Shamrov, 1991a, b; Igersheim and Endress, 1998; Yamada *et al.*, 2001); a completely annular outer integument is present in *Barclaya*. Yamada *et al.* (2001) suggested that the semi-annular outer integument is the plesiomorphic condition, both in Nymphaeales and angiosperms. Given placement of *Trithuria* as sister to other Nymphaeales (Saarela *et al.*, 2007), our data conform with this conclusion, at least with respect to Nymphaeales. Yamada *et al.* (2001) also noted that the micropyle is composed of both integuments in most Nymphaeaceae (*Euryale*, *Nymphaea*, *Ondinea* and *Victoria*), but composed of the inner integument alone (i.e. the endostomic condition) in *Brasenia*, *Cabomba* and *Nuphar*, as also in many other basal angiosperm lineages (e.g. *Amborella*; Endress and Igersheim, 1997; Igersheim and Endress, 1998). Since *Nuphar* is putative sister to all other Nymphaeaceae (Les *et al.*, 1999), they postulated that the endostomic condition is primitive, both in Nymphaeales and angiosperms. However, in *Trithuria* both integuments form the micropyle; the tissues of the two integuments are closely pressed to each other and to the funicle to form a single pathway (Fig. 6C, D). Parsimonious optimization suggests that this is a unique condition for *Trithuria*. During ovule ontogeny in *Trithuria*, the curiously retarded development of the inner integument relative to the outer (i.e. acropetal development) is also an unusual feature; in other Nymphaeales growth of the outer integument never quite catches up with the inner integument (Fig. 12; see also images in Batygina *et al.*, 1982; Igersheim and Endress, 1998; Yamada *et al.*, 2001), although the micropylar side of the seed is ultimately very similar between Cabombaceae and Hydatellaceae. Endress (1996) pointed out that integument initiation is distinctly acropetal in Gnetales, compared with distinctly basipetal, or almost simultaneous, in angiosperms.

FIG. 12. Surfaces of stigmatic hairs (SEM). (A) *T. konkanensis*, left side of part of stigmatic hair with two pollen tubes. (B) *T. lanterna*, part of stigmatic hair with two pollen tubes, one still attached to a pollen grain. (C) *T. lanterna*, part of stigmatic hair with pollen tubes. (D) *T. filamentosa*, reproductive unit with developing fruits (containing embryos); three bracts removed and one bract remaining (behind). Most fruits have abscised at the tops of the stalks, but those remaining have stigmatic hairs with no traces of pollen tubes, indicating that the plant is apomictic.

Scale bars: (A, C) = 30 μ m, (B) = 20 μ m, (D) = 500 μ m.

In general, miniaturization of all parts, including the ovule, makes nucellar structure much less complex in Hydatellaceae than in Nymphaeaceae. For example, Shamrov (1998b) described a complex nucellar morphology in *Nuphar* (Nymphaeaceae); in addition to the nucellar cap and parietal tissue, he distinguished structures that he termed a postament, podium and hypostase. The postament, a column of tissue between the chalazal side of the ovule and the embryo sac, possesses longitudinally elongated and densely cytoplasmic cells, some of which degenerate when the embryo sac enlarges towards the chalazal side of the ovule, where a haustorium forms. The postament is completely destroyed by the globular stage

FIG. 13. Surfaces of stigmatic hairs (SEM). (A) *T. submersa*, (B) *T. lanterna*. Stigmas with growing pollen tubes, ovary down. In each figure one pollen tube is coloured red, while others are not coloured, but are indicated by arrowheads. Among four stigmatic hairs in A, one is mature and several pollen grains are visible on its upper (collapsed) part; the other hairs are at various earlier stages of development. Note that the coloured pollen tube in A grows across the youngest stigmatic hair. Scale bars: (A) = 100 μm , (B) = 50 μm .

of embryo development. The podium is a densely cytoplasmic cup-like region in the chalazal part of the nucellus. Both podium and postament cells lack starch accumulation before fertilization. After fertilization, most podium cells accumulate tannins and their walls become lignified. Shamrov (1998b) also distinguished a disk-shaped group of cells in the chalazal region, just below the podium, which he termed a hypostase, although it is clearly non-

homologous with the hypostase of some other early-divergent angiosperms, such as *Acorus* (Rudall and Furness, 1997). Most hypostase cells are thin-walled and accumulate starch, although some central hypostase cells also accumulate tannins and develop lignified walls. According to Shamrov's terminology, we can distinguish in *Trithuria* only a very small, central portion of both hypostase and podium below the perisperm. The postament is absent, corresponding to the absence of a well-developed chalazal haustorium in the embryo sac of *Trithuria*.

Our preliminary data on the presence of cuticles between the ovule/seed and the carpel wall/pericarp indicate that this character has taxonomic potential in Hydatellaceae. Hamann *et al.* (1979) described a single cuticle between the seed and the pericarp in *T. filamentosa*, but used dry herbarium material rather than alcohol-fixed specimens. This character is probably also of adaptive significance. Both precocious cuticle formation and precocious perisperm formation could facilitate rapid fruit development, which is important for short-lived annuals.

Some Hydatellaceae could be apomictic

Previous authors (e.g. Hamann, 1998) have suggested that apomixis (agamospermy) could occur in the New Zealand species *Trithuria inconspicua*, because males are extremely rare, and plants consist mostly of female individuals, although embryos develop and fertile seeds are produced (Hamann, 1976). Asexual reproduction is a frequent strategy of aquatic plants (e.g. Les, 1988). However, pollen-tube growth is common in some other species of *Trithuria*. For example, Gaikwad and Yadav (2003) reported pollen germination on stigmatic hairs of *T. konkanensis*, and we have observed pollen-tube growth on *T. lanterna*, *T. konkanensis* and *T. submersa* (Figs 12A–C and 13). By contrast, pollen tubes were entirely absent from our material of *T. filamentosa* (Fig. 12D), even though embryos were present in the fruits. Thus, we hypothesize that *T. filamentosa* is also apomictic, at least in material examined here.

CONCLUSIONS

Several relatively unusual features of the *Trithuria* ovule, notably the four-nucleate embryo sac, copious perisperm and seed operculum, are consistent with a close relationship with Nymphaeales. Furthermore, the hood-shaped outer integument supports a close affinity with Cabombaceae. In light of the sister-group relationship demonstrated by Saarela *et al.* (2007) using molecular data, this high degree of morphological similarity supports the suggestion that Hydatellaceae should be included within Nymphaeales in future classifications (Stevens, 2007). The ovule of *Trithuria* is tenuinucellate, rather than crassinucellate as in most Nymphaeales, perhaps reflecting the high degree of morphological reduction in Hydatellaceae.

The frequent occurrence of double megagametophytes in the same ovule, especially in *Trithuria*, but also in other early-divergent angiosperms, indicates considerable developmental flexibility, and could provide a clue to the

FIG. 14. Cabombaceae ovules oriented with micropyle uppermost. (A) *Cabomba aquatica*, megaspore mother cell stage with inner integument almost closed at micropyle. (B, C) *C. aquatica*, tetrad stage with chalazal functional megaspore larger than the other three megaspores and inner integument forming micropyle. (D) *C. aquatica*. (E–H) *Brasenia schreberi*, different four-nucleate stages. c, central cell; e, egg cell; fm, functional megaspore; n, nucellus; ii, inner integument; oi, outer integument; p, perisperm; r, remains of nucellar cell. Arrowheads indicate positions of other embryo sac nuclei. Scale bars = 10 µm.

evolutionary origin of the *Polygonum*-type of the angiosperm embryo sac, and hence the enigmatic transition from the gymnosperm to the angiosperm condition.

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