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## DEVELOPMENTAL MORPHOLOGY OF OVULES AND SEEDS OF NYMPHAEALES<sup>1</sup>

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Ovule and seed development in six species of Nymphaeales was examined. In the Cabombaceae the two species studied resemble some extant basal angiosperms by having a hood-shaped outer integument. A micropyle–hilum complex results when the outer integument and derived testa are lacking between the micropyle and the funiculus, thus the hood-shaped appearance. In the Nymphaeaceae the outer integument is annular at an early stage and then cup-shaped though it is semiannular at initiation in *Nuphar japonicum* and *Nymphaea alba*. The micropyle and hilum are separated by an intervening testa. Developmental data on the formation of the outer integument, from semiannular to hood-shaped vs. from annular to cup-shaped, are useful for inferring the morphology of the outer integument from the relative position of the micropyle to the hilum in seed fossils. The oldest (early Cretaceous) probable nymphaealean seeds had the micropyle–hilum complex, suggesting that the hood-shaped outer integument may be primitive in the Nymphaeales. This needs to be tested by examination of this feature in other groups of basal angiosperms.

**Key words:** developmental morphology; evolution; integument; micropyle–hilum complex; ovules; Nymphaeales; seeds.

Bitegmic and anatropous ovules are common in basal angiosperms and are usually considered to be primitive in flowering plants as a whole (Stebbins, 1974; Bouman, 1984; Cronquist, 1988; Takhtajan, 1991; Johri, Ambegaokar, and Srivastava, 1992). Only a few authors regard anatropy as derivative and orthotropy as ancestral (Bocquet and Bersier, 1960). It has been suggested that the outer integuments are cup shaped and asymmetrical (Bouman, 1984). However, recent developmental and morphological studies have shown that in many basal angiosperms the outer integument is sharply asymmetric and hood shaped, that is, it is lacking on the concave (funicular) side of the ovule (Matsui, Imaichi, and Kato, 1993; Umeda, Imaichi, and Kato, 1994; Imaichi, Kato, and Okada, 1995; Endress and Igersheim, 1997, 1999; Igersheim and Endress, 1997, 1998; see also Johri, Ambegaokar, and Srivastava, 1992).

The origin of bitegmy has been variously interpreted in contrast to the agreement on the origin of the first (inner) integument by fusion of telomes (Herr, 1995). Crane (1985, 1986) and Doyle and Donoghue (1986, 1987) postulated that the outer integument is derived from the leafy lamina of a *Caytonia*-like ancestor, while the carpel enclosing ovules is derived by lateral expansion of its leaf axis. It is also hypothesized that the bitegmic ovules have been derived from unitegmic and orthotropous ovules of glossopterid-like gymnosperms by the enclosure of the unitegmic ovules by the ovuliferous leaf to form the outer integument (Stebbins, 1974; Dahlgren, 1983; Kato, 1990; Stewart and Rothwell, 1993; Umeda, Imaichi, and Kato, 1994; Imaichi, Kato, and Okada, 1995; Doyle, 1996). Kato (1991) also speculates that the anatropy is an extreme modification of the hyponastic curvature of leaves (ovuliferous leaf). However, the origin of the outer integument and the origin of anatropy are still uncertain, partly because the ovules

of primitive or basal angiosperms are not well understood even on a morphological level.

Recent molecular analyses (Mathews and Donoghue, 1999; Qiu et al., 1999; Soltis, Soltis, and Chase, 1999) suggest Amborellaceae are the sister group to all other angiosperms and the Nymphaeales are the next group to diverge in angiosperm phylogeny.

Nymphaeales are a monophyletic group composed of two families, Cabombaceae and Nymphaeaceae. Cabombaceae are composed of two genera, *Brasenia* and *Cabomba*, while Nymphaeaceae are composed of three subfamilies—Nupharoideae, including a single genus, *Nuphar*; Barclayoideae, including a single genus, *Barclaya*; and Nymphaeoidae, including four genera, *Euryale*, *Nymphaea*, *Ondinea*, and *Victoria* (Les and Schneider, 1995; Takhtajan, 1997; Les et al., 1999).

Ovules of Nymphaeales are uniformly bitegmic and crassinucellate but vary in their orientation from anatropy to orthotropy (Khanna, 1965, 1967; Moseley, 1972; Schneider, 1976, 1978; Williamson and Moseley, 1989; Johri, Ambegaokar, and Srivastava, 1992; Igersheim and Endress, 1998). Variations within a group may provide useful data for understanding the evolution of ovules in basal angiosperms. Collinson (1980) described the morphology and anatomy of the seeds of Nymphaeaceae *sensu lato* (s. l.) with special reference to the relative position of the micropyle to the hilum in the micropylar region, which was called a circular cap by Collinson.

Furthermore, Collinson (1980) compared seeds of living and fossil taxa of the Nymphaeales. The significance of comparative neo- and paleobotanical studies is increasingly obvious, because approaches combining fossil data with molecular data improve phylogenetic interpretations. Fossil records of delicate ovules are scarce as compared to seed fossils due to taphonomical bias (Holyoak, 1984). Therefore, combined data of seed fossils and extant angiosperm ovules are useful for determining the primitive characteristics of ovules.

The purposes of this study were to: (1) illustrate the variations of the outer integument in six species of Nymphaeales; (2) suggest evolutionary changes of ovule shapes relative to outer integument, and; (3) compare ovules and seeds of the order. In this study, the ovules and seeds of Cabombaceae,

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TABLE 1. Plants used in this study.

Family or subfamily (Takhtajan, 1997)	Species	Source	Voucher
Cabombaceae	<i>Cabomba caroliniana</i> A. Gray	Tamiyaji, Tamaki, Mie Pref., Japan	<i>Yamada 991001</i>
	<i>Brasenia schreberi</i> Gmel.	Yudani, Yokawa, Hyogo Pref., Japan	<i>Yamada 990701</i>
Nymphaeaceae Nupharoideae	<i>Nuphar japonicum</i> DC.	Botanical Garden, Nikko, University of Tokyo, Nikko, Tochigi Pref., Japan	<i>Yamada 990501</i>
Nymphaeaceae Nymphaeoidae	<i>Nymphaea alba</i> L.	Honsenji, Seto, Aichi Pref., Japan	<i>Yamada 990502</i>
	<i>Euryale ferox</i> Salisb.	Eigashima, Akashi, Hyogo Pref., Japan	<i>Yamada 990702</i>
	<i>Victoria cruziana</i> Orbign.	Karanambo, Guyana	<i>Yamada 990901</i>

Nupharoideae, and Nymphaeoidae except for *Ondinea* were examined. Those of Barclayoideae (*Barclaya longifolia* Wall.) and *Ondinea* were not examined because they have been well described by Schneider (1978) and Schneider and Ford (1978), respectively.

#### MATERIALS AND METHODS

Flowers and fruits of the Cabombaceae, Nupharoideae, and Nymphaeoidae (except for *Ondinea*) were collected in successive developmental stages and vouchers were prepared for the University of Tokyo herbarium (Table 1).

The materials were fixed in a mixture of 5% formalin, 5% acetic acid, and 50% ethyl alcohol (FAA), dehydrated in an ethyl alcohol series, and embedded in HistoResin Plus (glycol methacrylate, Leica, Heidelberg) or dehydrated in an acetone series and embedded in Spurr resin (TAAB, Berks, UK). They were cut into 2–3  $\mu\text{m}$  thick sections and stained with safranin, toluidine blue, and orange G (Jernsteadt et al., 1992).

For scanning electron microscopic (SEM) observations, fresh materials were molded using epoxy resin (Replica SEM method; Williams, Vesk, and Mullins, 1987) or dehydrated in an ethyl alcohol series and t-butyl alcohol and freeze-dried. They were coated with platinum-palladium. Material was observed in a scanning electron microscope (JEOL JSM-820S) at 10–20 kV.

#### RESULTS

**Cabombaceae**—The ovules are anatropous (Figs. 7, 15), bitegmic, and crassinucellate in both genera (Figs. 4, 12). The inner integument is annular or ring-like even in an early stage (Figs. 2, 3) and consequently cup-shaped in both genera (Figs. 4–7, 12–15). The genera exhibit very similar patterns of ovule development.

At an early stage in *Brasenia schreberi* the ovular primordia curve inward before the initiation of integuments (Fig. 1). The outer integument initiates on the convex and lateral sides just below the inner integument, that is, it is semiannular (Figs. 2, 3). The outer integument ends at the lateral sides of the funiculus. After the ovule incurvation has finished, the outer integument is hood shaped and has no outgrowth on the concave side of the funiculus in both genera (Figs. 4, 5, 12, 13).

In mature ovules of both genera the micropyle is surrounded only by the inner integument, i.e., composed of the endostome (Figs. 7, 15). The inner integument is two cells thick except in the micropylar region where the epidermis divides periclinally and thickens to form an operculum before maturity (Figs. 6, 14). In *Cabomba caroliniana* the operculum is very thick in contrast to the other part of the inner integument (Fig. 14). The hood-shaped outer integument is three or four cells thick

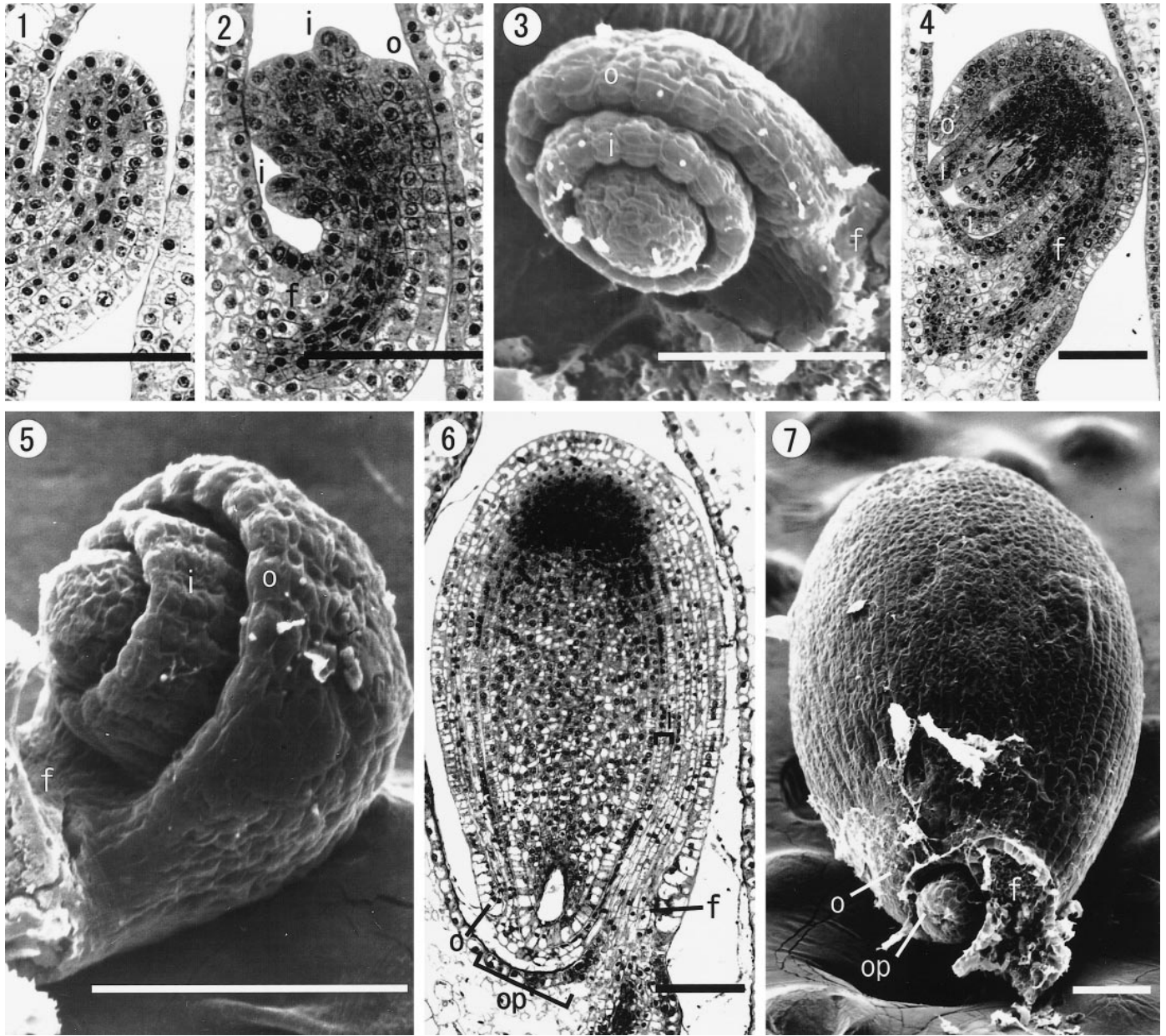
in *Brasenia schreberi* (Fig. 6) and two cells thick in *Cabomba caroliniana* (Fig. 14).

In postfertilized ovules of *Brasenia schreberi* the inner integument appears plicated (Figs. 8, 10; which is also the case with mature seeds of *Cabomba caroliniana*, Fig. 16). The outer epidermal cells of the outer integument enlarge without periclinial divisions and develop into a unilayered exotesta (Fig. 8). The exotesta thickens more than the other layers of the outer integument and the inner integument, and consequently the micropyle of immature seeds is apparently formed by the exostome and endostome (Figs. 8, 9). There is no testa (developed from the outer integument) between the hilum and the endostome, because no additional tissue forms between the two during seed maturation (Figs. 8, 9). Eventually the hilum, consisting mostly of vascular bundles in mature seeds, is adjacent to the endostome. We apply the term micropyle–hilum complex to this structure (Figs. 10, 11, 16, 17).

In mature seeds of both genera the exotesta is sclerified, so that the seeds are exotestal (Figs. 10, 16). In *Brasenia schreberi* the exotestal cells in the circular cap are rectangular in contrast to lobed cells of the surrounding region (Fig. 11). In *Cabomba caroliniana* the exotestal cells are smaller in the circular cap than in the surrounding region (Figs. 16, 17). In *Brasenia schreberi* the outer cell walls of the exotesta are very thick (~150–200  $\mu\text{m}$ ) and as thick as, or thicker than, its lumens (Fig. 10). In *Cabomba caroliniana* the cell walls of the exotesta are much thinner than in *Brasenia schreberi* (~20  $\mu\text{m}$ ; Fig. 16).

**Nupharoideae (*Nuphar japonicum*)**—The ovules are anatropous (Figs. 26, 27), bitegmic, and crassinucellate (Fig. 20). The inner integument is annular at an early stage when the ovules are weakly curved inward (Figs. 18, 19) and eventually cup shaped (Figs. 20–23, 26, 27). The outer integument initiates on the convex and lateral sides of the ovular primordia, and it is semiannular (Figs. 18, 19). The outer integument ends on the concave side near the lateral sides of the funiculus. At a little later stage the growth of the outer integument proceeds to the concave side of the funiculus but in the center on the concave side (Figs. 20, 21). As the ovules are further incurved, the development of the outer integument extends to the center on the concave side of the funiculus (Figs. 22, 23), so that it becomes cup shaped. The submarginal part of the outer integument on the concave side of the funiculus is four cells high (Fig. 24), the median part two cells high (Fig. 22), and the submedian part three cells high (Fig. 25). Thus, the outer in-





Figs. 1–7. Ovules of *Brasenia schreberi* at successive stages of development. 1. Median longitudinal section (MLS) of incurved ovular primordium. 2. MLS of young ovule with the inner and outer integuments initiating. 3. SEM micrograph of young ovule at a similar stage to that of Fig. 2. 4. MLS of immature anatrochous ovule. Note that there is no outer integument on the concave side. 5. SEM micrograph of immature ovule at a similar stage to that of Fig. 4. 6. MLS of mature ovule, showing no outer integument on the concave side. 7. SEM micrograph of mature ovule with the operculum projecting. Scale bars = 100  $\mu$ m.

Figure Abbreviations: a, aril; et, exotesta; f, funiculus; h, hilum; i, inner integument; m, micropyle; m-h, micropyle-hilum complex; o, outer integument; op, operculum; mt, mesotesta.

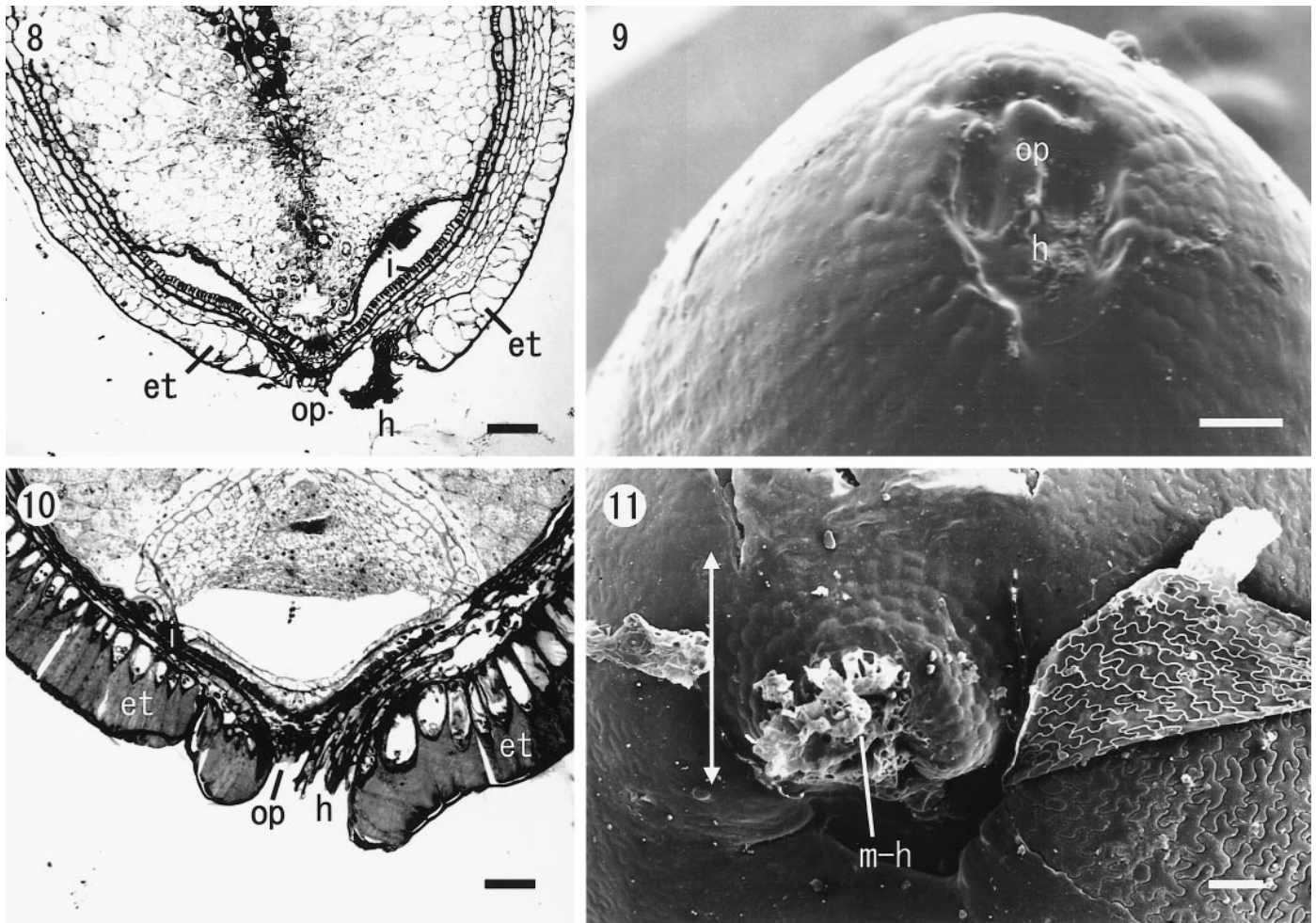
tegument is developed to the least extent in the center on the concave side of the funiculus.

In mature ovules the outer integument is five to eight cells thick (Fig. 26). The weaker development of the outer integument in the center on the concave side of the funiculus results in a depression of the outer integument on the concave side (Fig. 27). The micropyle is formed only by the endostome, the tip of the inner integument being exposed beyond the outer integument (Figs. 26, 27). The inner integument is two cells thick except in the micropylar region, which develops into an operculum (Fig. 26).

In postfertilized ovules the inner integument is plicated or crushed except for the operculum, while the outer epidermis of the outer integument develops into an exotesta (Figs. 28, 31). The micropyle is separated from the hilum by the exotesta (Figs. 29, 30). The circular cap becomes distinct in a surface view (Fig. 30).

In mature seeds the exotesta is sclerified, so that the seeds are exotestal (Fig. 34). The sclerified exotestal cells surround the endostome in a cross section, showing that the testa occurs between the hilum and the micropyle also in this stage (Fig. 32; see also Fig. 33 for a relative position of the micropyle to





Figs. 8–11. Seeds of *Brasenia schreberi* at different stages of development. 8. MLS of the micropylar part of immature seed. 9. SEM micrograph of immature seed at a similar stage to that of Fig. 8. Note that the operculum and hilum are surrounded by the exotesta. 10. MLS of the micropylar part of mature seed. 11. SEM micrograph of mature seed showing the micropyle–hilum complex. Double-headed arrow indicates the realm of the circular cap. Scale bars = 100  $\mu\text{m}$ .

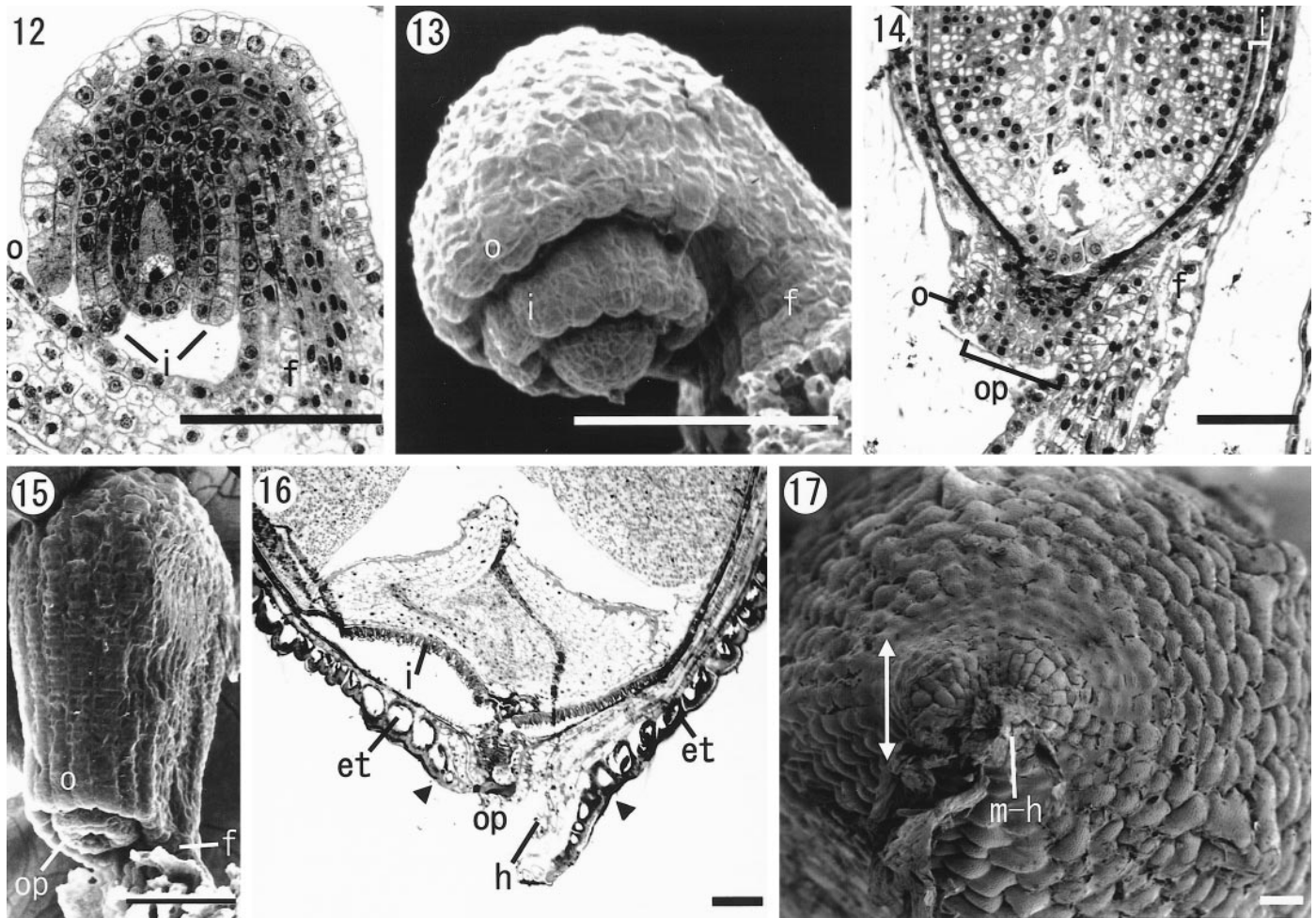
the hilum from a surface view). A longitudinal section shows that there are sclerified exotestal cells adjacent to parenchymatous cells of the hilum (Fig. 35). Thus, the hilum is adjacent to the micropyle but separated from it by the exotesta. The exotestal cells that compose the circular cap in the periphery of the micropyle are smaller than surrounding cells (Fig. 34).

**Nymphaeoidae**—The ovules are anatropous or hemianatropous (Figs. 38, 48, 56), bitegmic, and crassinucellate in the species examined (Figs. 36, 46, 56; see Williamson and Moseley [1989] for *Ondinea purpurea*). The inner integument is annular (Figs. 36, 37, 44, 45), then cup shaped and two cells thick except in the micropylar region where it thickens to form an operculum (Figs. 38, 46, 48, 56).

At an early stage, the outer integument is semiannular in *Nymphaea alba* and develops in a similar way to that of Nupharoideae (Figs. 36, 37). In *Euryale ferox* the outer integument is annular when the ovules are curved at nearly right angles to the funiculus (Figs. 44, 45). At a little later stage when the outer integument develops to almost the same height as the inner integument, the outer integument becomes cup shaped (Figs. 46, 47). The ovules become bent a little more and eventually are hemianatropous (Figs. 48, 49).

In mature ovules of all species examined the outer integument is asymmetrically cup shaped. The micropyle is formed by the endostome and exostome, although the exostome is loose (Figs. 38, 48, 56). Williamson and Moseley (1989) illustrated that *Ondinea purpurea* also has a cup-shaped outer integument. In *Nymphaea alba* the outer integument is four or five cells thick (Fig. 38). In *Euryale ferox* and *Victoria cruziana* it is very thick (~200–300  $\mu\text{m}$ ; Figs. 48, 56).

In postfertilized ovules of *Nymphaea alba* and *Euryale ferox* the inner integument is plicated or crushed except for the operculum (Figs. 40, 52), as in the mature seed of *Victoria cruziana* (Fig. 58). The outer epidermis of the outer integument grows into an exotesta (Figs. 42, 50, 52). In *Nymphaea alba* the parenchymatous subdermal layer of the outer integument does not thicken well during the seed maturation (Fig. 40, 42). In *Euryale ferox* the outer epidermis of the outer integument in the micropylar region enlarges so strongly that the circular cap becomes distinct (Figs. 50, 51). The parenchymatous cells of the outer integument (mesotesta), except in the micropylar region, divide both periclinally and anticlinally, so that the testa thickens. By contrast, the micropylar region remains relatively thin (Fig. 52) and is recognized more distinctly as a circular cap (Fig. 53).



Figs. 12–17. Ovules and seeds of *Cabomba caroliniana* at different stages of development. 12. MLS of young ovule. Note that there is no outer integument on the concave side. 13. SEM micrograph of young ovule at a similar stage to that of Fig. 12. 14. MLS of the micropylar region of mature ovule, showing well-developed operculum. 15. SEM micrograph of mature ovule. 16. MLS of the micropylar region of mature seed. Arrowheads indicate boundaries between the circular cap and the proximal exotesta. 17. SEM micrograph of mature seed showing the micropyle–hilum complex in the circular cap. Double-headed arrow indicates the realm of the circular cup. Scale bars = 100  $\mu$ m.

In mature seeds the hilum and the micropyle are separated by an intervening testa derived from the outer integument in all species examined (Figs. 43, 55, 59). It is the same as in *Ondinea purpurea* (Schneider and Ford, 1978). In *Nymphaea alba* the exotesta is sclerified and the parenchymatous cells of the testa are crushed, resulting in the exotestal seed. The exotestal cells are less sclerified and smaller in the micropylar region than in the surrounding region, so that the circular cap is recognizable (Fig. 42). In *Euryale ferox* the exotesta is sclerified and the mesotesta is very thick (850–1000  $\mu$ m) and also sclerified (Fig. 54). So the seeds are exomesotestal, although described to be exotestal by Collinson (1980). In *Victoria cruziana* the seeds are exotestal with the exotestal cells composed of very small lumens and thick cell walls. The layer of parenchymatous cells beneath the exotesta is very thick (150–200  $\mu$ m), and these cells are crushed except in the circular cap where they become sclerified (Fig. 58).

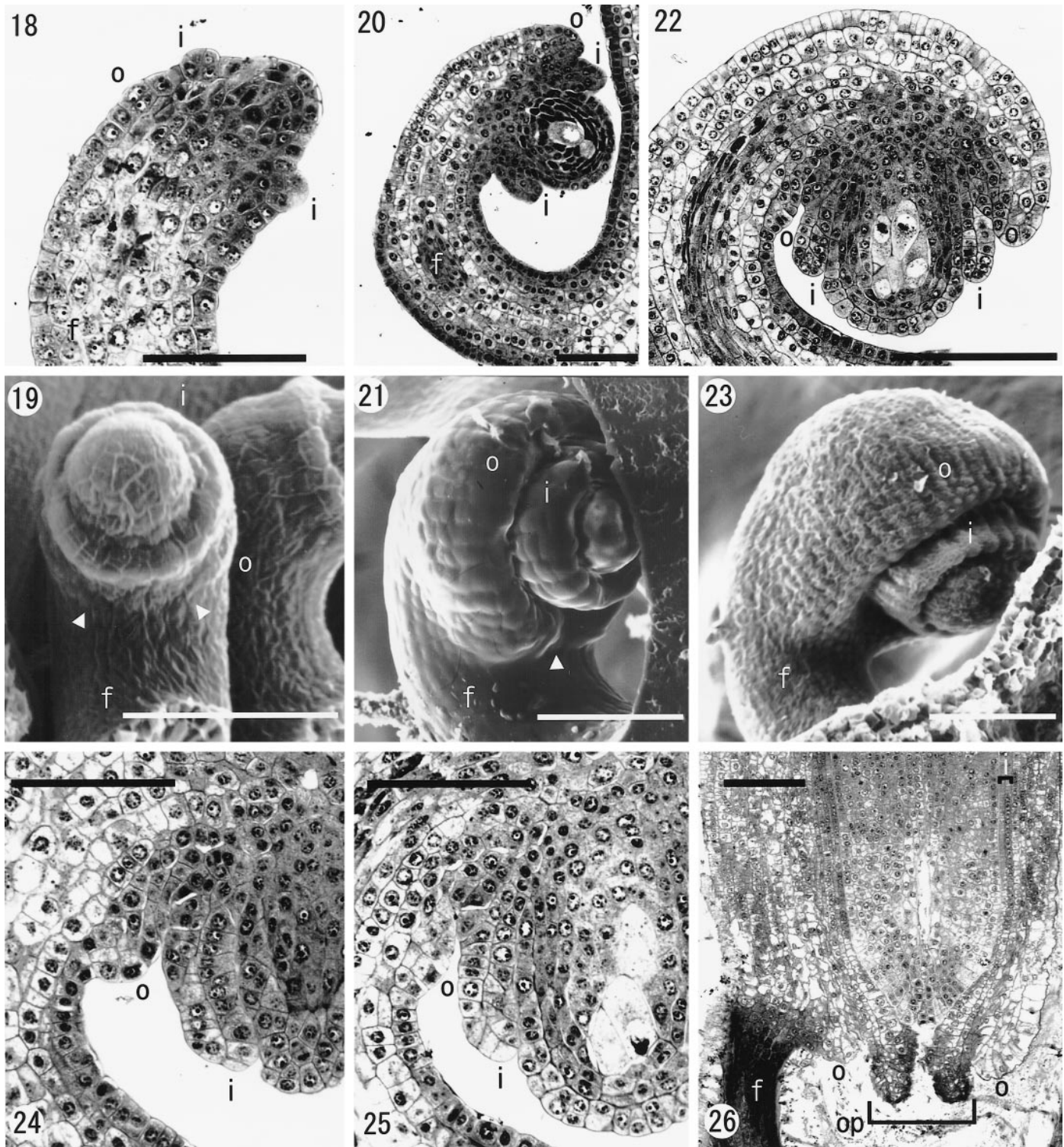
#### DISCUSSION

The ovules of Nymphaeales are classified into two groups with respect to outer integument morphology: one group (Ca-

bombaceae: *Brasenia schreberi* [Khanna, 1965; Richardson, 1969] and *Cabomba caroliniana*) has the hood-shaped outer integument. The other (Nymphaeaceae: *Nuphar japonicum*, *Nymphaea alba*, *Euryale ferox*, and *Victoria cruziana*, which is illustrated by Khanna [1967] and Schneider [1976]) has the cup-shaped outer integument. In *Brasenia* and *Cabomba* the hood-shaped outer integument develops from the young semiannular integument. The results of our developmental studies are in accord with Igersheim and Endress' (1998) descriptions of mature ovules for these genera except for *Nymphaea* and *Nuphar* in which Igersheim and Endress stressed intraspecific variations of ovules. Such variations were not detected in this study.

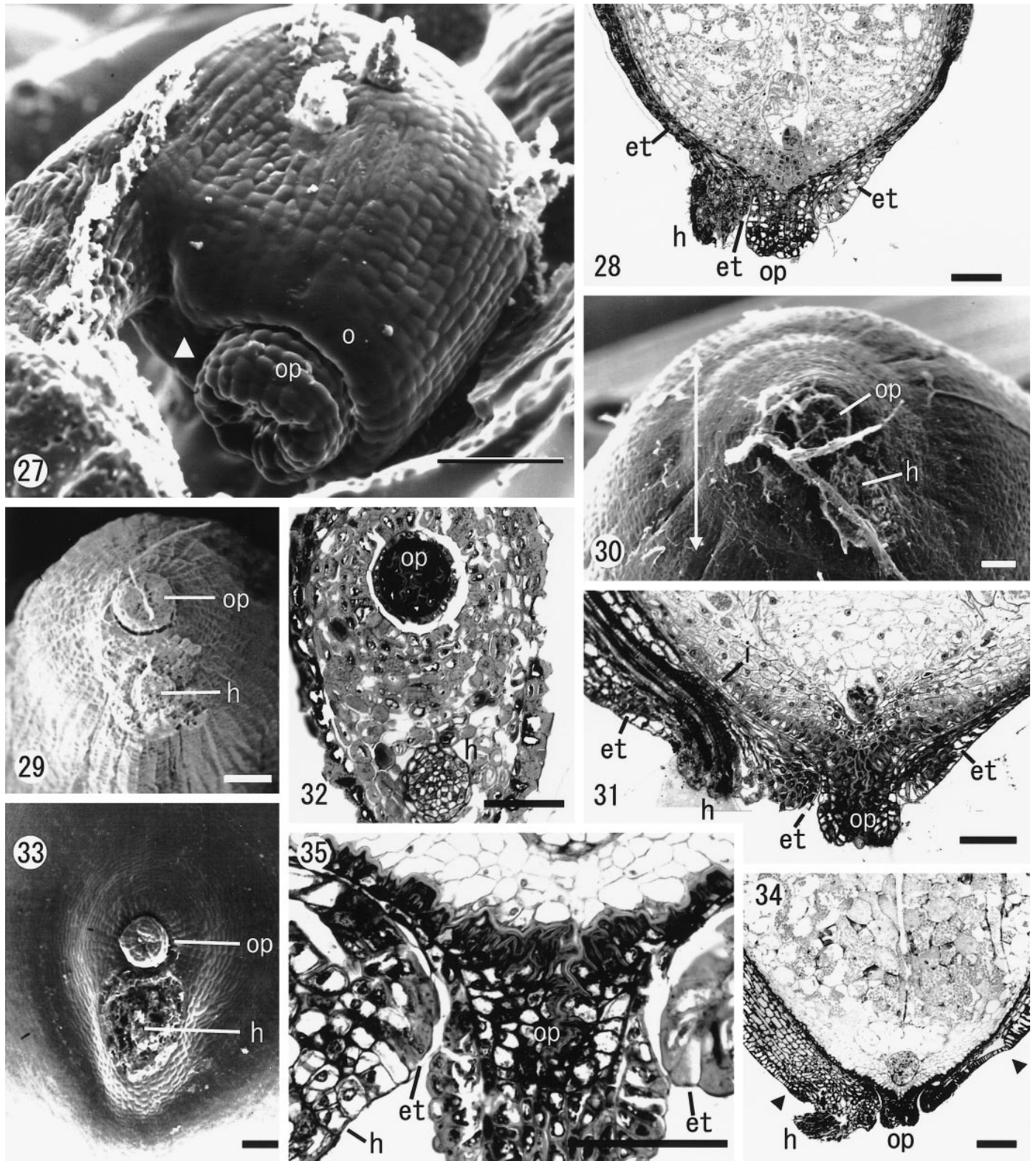
The outer integument of *Nuphar japonicum*, which is the sister group to all other Nymphaeaceae (Les et al., 1999), is developmentally intermediate between hood-shaped and cup-shaped forms. It is semiannular in a very early stage of development and cup shaped with a depression on the concave side in the mature ovule. Preliminary observations (data not shown) show that the outer integument is constantly cup shaped at maturity in *Nymphaea tetragona*, although the spe-





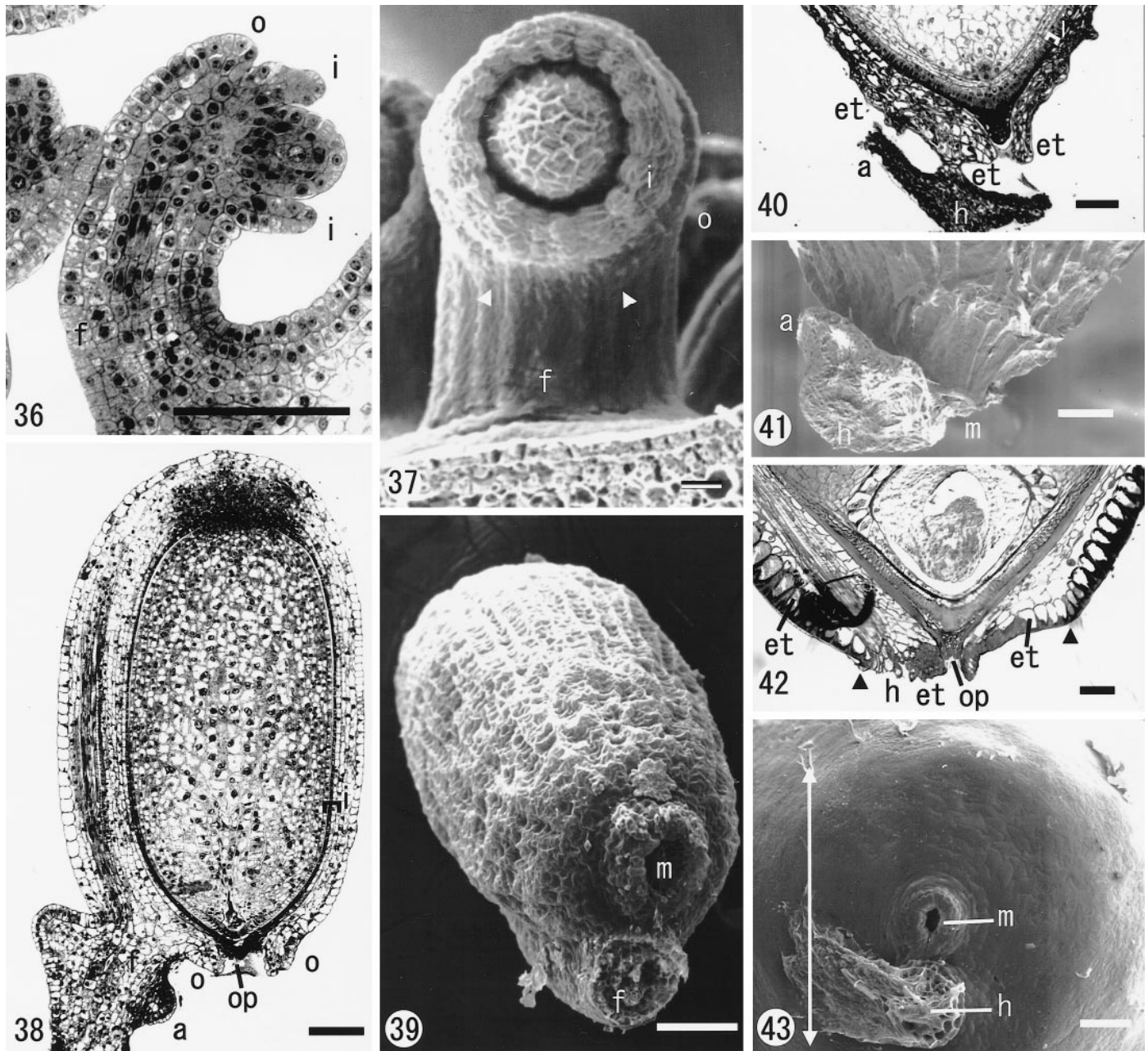
Figs. 18–26. Ovules of *Nuphar japonicum* at successive stages. 18. Nearly median longitudinal section of young ovule with the inner and outer integuments just initiating. 19. SEM micrograph of ovule at a little later stage than that of Fig. 18. Arrowheads indicate the ends of the semiannular outer integument. 20. Nearly median longitudinal section of young ovule at later stage than that of Fig. 18. Note that the outer integument does not grow on the concave side. 21. SEM micrograph of ovule at a similar stage to that of Fig. 20. Arrowhead indicates the place where there is no outgrowth of outer integument. 22. MLS of older ovule that has nearly finished incurvation. Note that the outer integument on the concave side is two cells high. 23. SEM micrograph of ovule at a similar stage to that of Fig. 22. 24. Sagittal longitudinal section through the marginal part of the same ovule as that of Fig. 22. Note the four-cell-high outer integument. 25. Submedian longitudinal section of the same ovule shown in Fig. 22. Note that the outer integument is three cells high. 26. Nearly median longitudinal section of the micropylar region of mature ovule, showing the developed outer integument on the concave side and the operculum. Scale bars = 100  $\mu$ m.





Figs. 27–35. Ovule and seeds of *Nuphar japonicum* at successive stages. 27. SEM micrograph of mature ovule. Arrowhead indicates depression on the concave side. 28. MLS of immature seed. Note thin exotesta between the micropyle (operculum) and hilum. 29. SEM micrograph of immature seed at a similar stage to that of Fig. 28. Note the micropyle (operculum) is separated from the hilum. 30. SEM micrograph of immature seed at a later stage than that of Fig. 29. Double-headed arrow indicates the realm of circular cap with the micropyle and hilum. 31. MLS of immature seed at a similar stage to that of Fig. 30. 32. Cross section through the micropylar region of mature seed. Note radial cell files round the operculum, separating the spongy-like hilum from the operculum. 33. SEM micrograph of mature seed. 34. MLS of mature seed. Arrowheads indicate the boundaries between the circular cap and the proximal exotesta. 35. Magnified micropylar region of Fig. 34, showing the exotesta between the micropyle (operculum) and the hilum. Scale bars = 100  $\mu$ m.





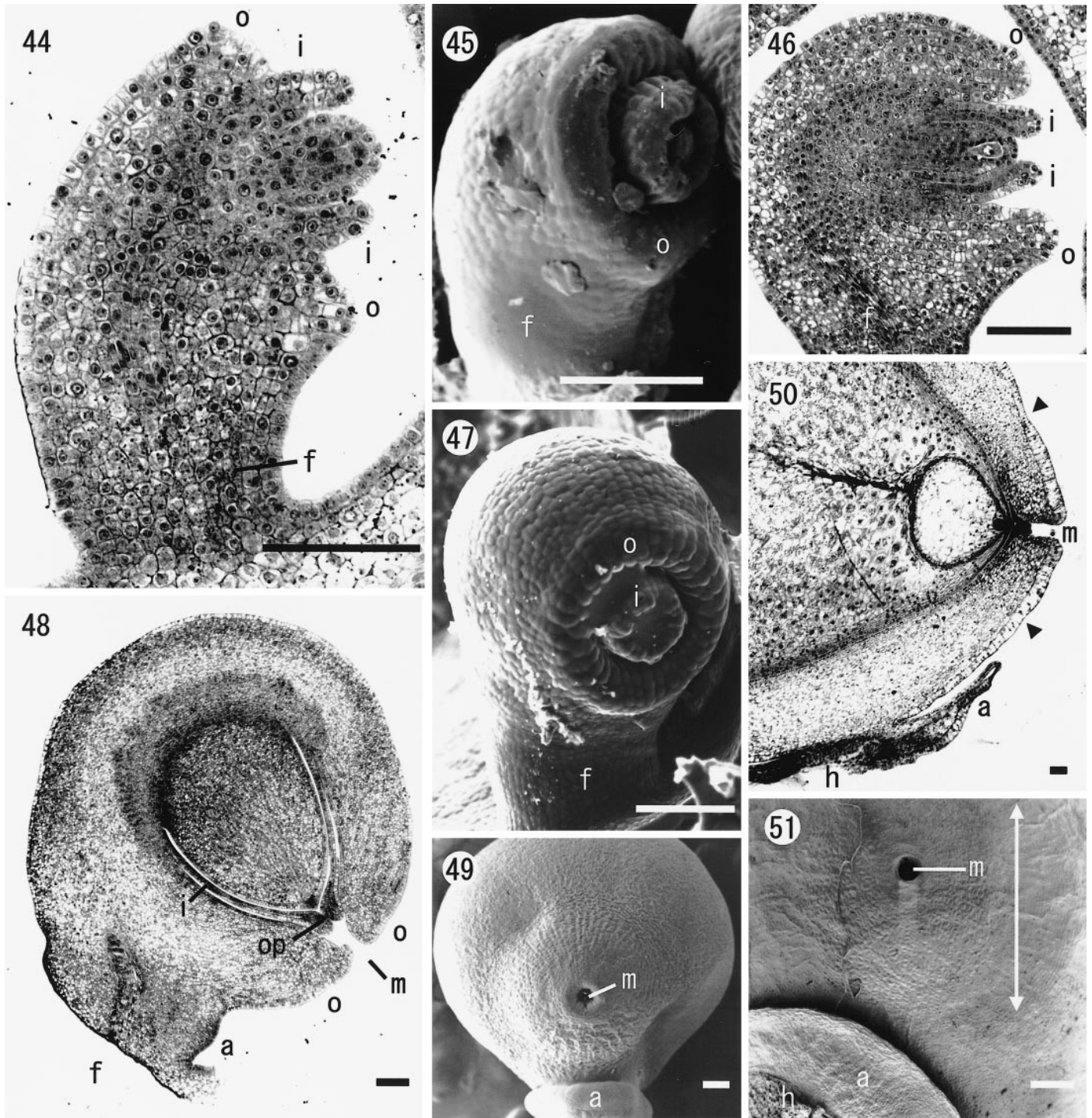
Figs. 36–43. Ovules and seeds of *Nymphaea alba* at different stages. **36.** MLS of young ovule with the outer integument not yet initiating on the concave side. **37.** SEM micrograph of young ovule at a similar stage to that of Fig. 36. Arrowheads indicate the ends of the semiannular outer integument. **38.** MLS of mature ovule with the outer integument on the concave and convex sides. **39.** SEM micrograph of mature ovule with the aril removed. **40.** MLS of immature seed. **41.** SEM micrograph of immature seed at a similar stage to that of Fig. 40. **42.** MLS of mature seed with the aril removed. Note the existence of testa between the micropyle and the hilum. Arrowheads indicate the boundaries between the circular cap and the proximal exotesta. **43.** SEM micrograph of mature seed. Double-headed arrow indicates the realm of the circular cap including the micropyle and hilum. Scale bars = 100  $\mu$ m.

cies was described by Igersheim and Endress (1998) to have a hood-shaped outer integument in some ovules and the cup-shaped in the others. Our observations show that in *Nymphaea alba* the mature outer integument is cup shaped though it is semiannular at a very early stage of development (but later annular). In addition, many other species of *Nymphaea* are illustrated to have a cup-shaped outer integument at maturity, such as *N. stellata* (Khanna, 1967) and *N. gigantea* (Batygina,

Kravtsova, and Shamorov, 1980). This study and previous reports indicate *Nymphaea* has a cup-shaped outer integument.

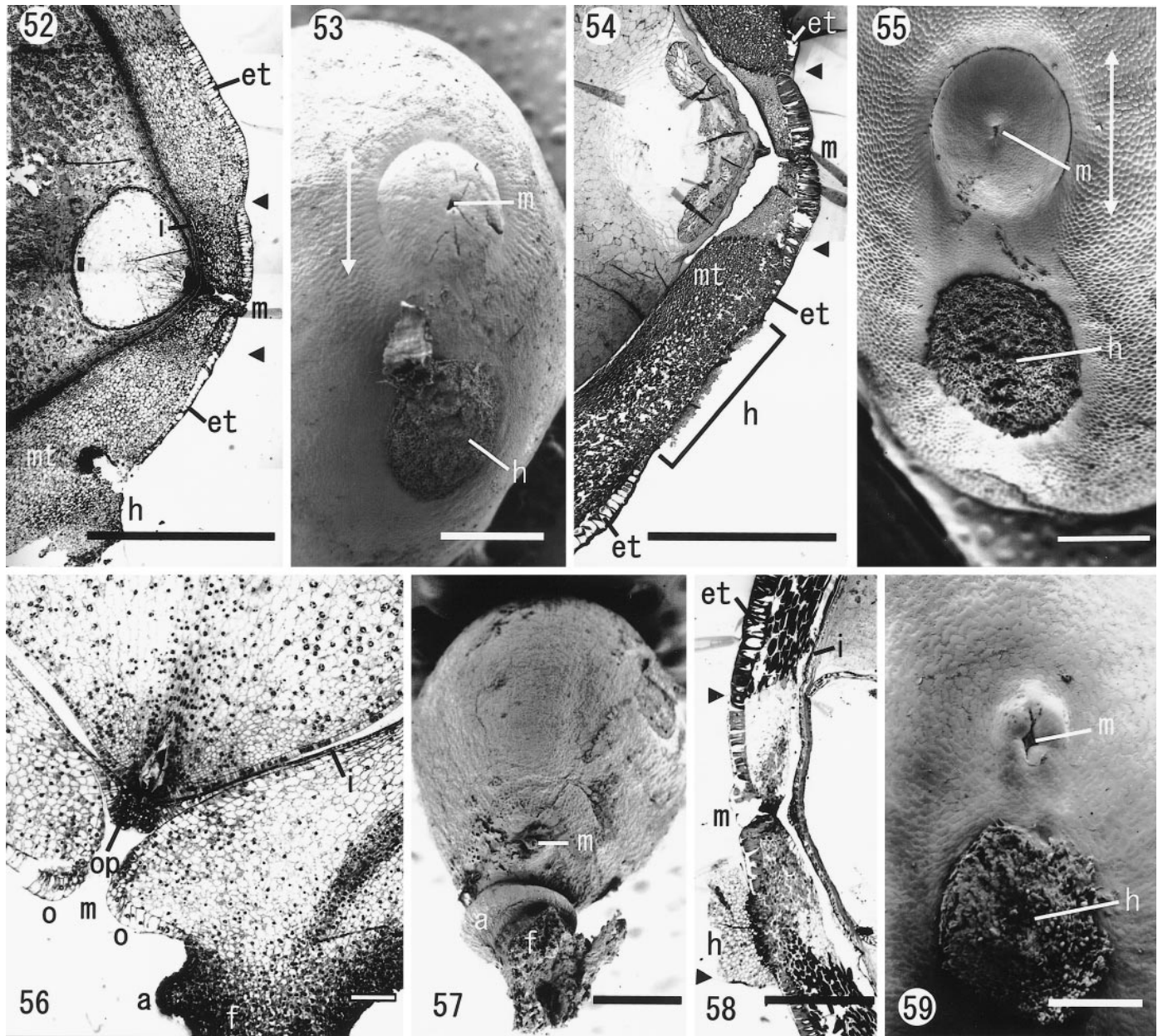
Collinson (1980) pointed out differences among the nymphaealean seeds with regard to the relative position of the micropyle to hilum, but evidence for the developmental relationship between these seed characters and the relevant characters of ovules was not available. In seeds of Cabombaceae in which the micropyle–hilum complex develops from ovules with a





Figs. 44–51. Ovules and seeds of *Euryale ferox* at successive stages. 44. MLS of a little incurvating young ovule. Note the existence of the outer integument on the concave side. 45. SEM micrograph of ovule at a similar stage to that of Fig. 44, showing the annular outer integument. 46. MLS of ovule at a later stage than that of Fig. 44. Note the well-developed outer integument on the concave side. 47. SEM micrograph of ovule at a similar stage to that of Fig. 46. 48. MLS of mature hemianatropous ovule. 49. SEM micrograph of mature ovule. 50. MLS of immature seed. Arrowheads indicate the boundaries between the circular cap and the proximal exotesta. 51. SEM micrograph of immature seed at a similar stage to that of Fig. 50. Double-headed arrow indicates the realm of circular cap. Scale bars = 100  $\mu$ m.





Figs. 52–59. Seeds of *Euryale ferox* and ovules and seeds of *Victoria cruziana* at different stages. Arrowheads indicate the boundaries between the circular cap and the proximal exotesta. Double-headed arrows indicate the realm of the circular cap. Figs. 52–55. Seeds of *Euryale ferox*. 52. MLS of immature seed at a later stage than that of Fig. 48, showing the existence of the exotesta between the micropyle and the hilum. 53. SEM micrograph of immature seed at a similar stage to that of Fig. 52. 54. MLS of mature seed. Note the more sclerified mesotesta than that of Fig. 52. 55. SEM micrograph of mature seed with a distinct circular cap. Figs. 56–59. Ovules and seeds of *Victoria cruziana*. 56. MLS of mature ovule. 57. SEM micrograph of mature ovule. 58. MLS of mature seed with the micropyle and the hilum separated. 59. SEM micrograph of mature seed. Note that the circular cap is not distinct. Scale bars = 1 mm in Figs. 52–55; 500  $\mu$ m in Figs. 56–59.

hood-shaped outer integument, the absence of the outer integument between the funiculus and the micropyle throughout development results in the lack of the testa in that region. In contrast, in members of the Nymphaeoidae in which the outer integument is cup shaped, the hilum is separated from the micropyle by the testa, which developed from the outer integument intervening between the funiculus and the micropyle. In *Nuphar* with an intermediate type of outer integument, the micropyle and hilum are separated by a very narrow testa.

A similar manner of development from the ovules with the

hood-shaped outer integument to the seeds with the micropyle–hilum complex is suggested here for the Annonaceae by comparing the ovules (Imaichi, Kato, and Okada, 1995) with the seeds (Corner, 1949, 1976). It is the case with the Hernandiaceae, Lauraceae, and Winteraceae (Corner, 1976; Heo and Tobe, 1995; Endress and Igersheim, 1997; Igersheim and Endress, 1997). These four families are basal to eudicots (Qiu et al., 1999; Soltis, Soltis, and Chase, 1999).

The above developmental data indicate that the morphology of the outer integument is able to be inferred from the seeds.

The oldest probable nymphaealean seeds from the Lower Cretaceous (Barremian to Aptian?) of Portugal (Friis, Pedersen, and Crane, 1999) have the micropyle-hilum complex, suggesting that ovules with the outer integument hood-shaped already existed in early Cretaceous. Therefore it seems possible that the hood-shaped outer integument is primitive in the Nymphaeales, as has also been argued for other basal angiosperms (Matsui, Imaichi, and Kato, 1993; Umeda, Imaichi, and Kato, 1994; Imaichi, Kato, and Okada, 1995).

Considering together the fossil data and the phylogenetic relationship of the Nymphaeales (Les et al., 1999), an evolutionary trend is suggested with the anatropous ovules with the hood-shaped outer integument evolving into the anatropous ovules with the cup-shaped outer integument via an intermediate type of ovule, as seen in *Nuphar*, and further into the orthotropous ovules of *Barclaya* with the cup-shaped outer integument (Schneider, 1978).

Seeds of Nymphaeales are characterized by the circular cap at the micropylar end (Collinson, 1980). This structure is useful to identify the fossil seeds of the Nymphaeales (Collinson, 1980; Friis, Pedersen, and Crane, 1999). The spatial relationships between the circular cap and the micropyle and hilum were argued by Collinson (1980). The circular cap was defined from its appearance. We noted the circular cap is associated with the histology of the exotestal cells and in some species mesotestal cells, and both kinds of cells are smaller and less sclerified than those in surrounding seed coat. We also observed that in *Euryale ferox* the circular cup does not include the hilum, its area being smaller than circumscribed by Collinson (1980).

There is a difference in micropyle structure among the Nymphaeales. The micropyle is endostomic or composed of the inner integument in *Brasenia*, *Cabomba* (Cabombaceae), *Nuphar* (Nupharoideae), and *Barclaya* (Barclayoideae; see Igersheim and Endress, 1998), whereas it is both endostomic and exostomic or composed of the inner and outer integuments in *Euryale*, *Nymphaea*, *Ondinea* (Williamson and Moseley, 1989), and *Victoria* (Nymphaeoidae). A phylogenetic tree (Les et al., 1999) suggests that the endostomic micropyles are ancestral to the double endo- and exostomic ones in the Nymphaeales. Many other basal angiosperms including the basal-most *Amborella* also have endostomic micropyles (Johri, Ambegaokar, and Srivastava, 1992; Imaichi, Kato, and Okada, 1995; Endress and Igersheim, 1997; Igersheim and Endress, 1997, 1998), suggesting the primitiveness of endostomy.

In *Arabidopsis thaliana*, a model plant of molecular genetics, the outer integument initiates first on the convex side of the ovules and its development is repressed on the concave side, resulting in an asymmetrically cup-shaped outer integument similar to that of *Nuphar* and *Nymphaea* (Gaiser, Robinson-Beers, and Gasser, 1995; Villanueva et al., 1999). This configuration is considered to be regulated in the way in which the expression of *INNER NO OUTER (INO)* gene is repressed by *SUPERMAN (SUP)* gene on the concave side of the funiculus (Villanueva et al., 1999). Mutants obtained exhibit a variety of ovules of seed plants. For example, the *sup* mutant has orthotropous ovules like those of *Barclaya*, and the unintegmic orthotropous ovules of the *ino* mutant apparently resemble gymnospermous ovules, implying that the *INO* gene is a critical component in the evolution of the outer integument (Gasser, Broadhvest, and Hauser, 1998). As suggested by some workers (Gasser, Broadhvest, and Hauser, 1998; Villanueva et al., 1999), analysis of patterns of expression of such genes in

primitive angiosperms will help us to understand the process of ovule evolution. Primitive families or orders that have intergeneric variations of the outer integument morphology and plenty of fossil records, like the Nymphaeales, may be suitable for the analysis.

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