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Ovule and female gametophyte in representatives of *Nymphaea* subgenus *Hydrocallis* and *Victoria* (Nymphaeaceae; Nymphaeoideae)

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ABSTRACT

Nymphaeaceae occupies an important phylogenetic position because of their placement as one of the basal angiosperms. From this perspective, morphological studies in the family are of great value to understanding plant phylogeny and evolution. Ovule development and female gametophyte in *Nymphaea amazonum*, *N. gardneriana* (subgenus *Hydrocallis*) and in *Victoria cruziana* were analyzed in order to provide further progress in characters of potential evolutionary interest. The ovules of all species are anatropous, bitegmic, distomic, weakly crasinucellate, and present an epistase. The female gametophyte is four-celled and corresponds to the Schisandra type, distinctive of the Nymphaeales. Comparisons among ovules of the subgenera of *Nymphaea* and others allied genera show differences with respect to micropyle conformation, thickness of nucellus and outer integument, and its degree of development on the raphal side. The studied species of *Nymphaea* share an annular outer integument and linear triad of megaspores. These results fill gaps in the current incomplete knowledge of character states especially within *Nymphaea*. In subgenus *Hydrocallis*, the ovules have an outer integument not markedly cup-shaped in contrast to subgenus *Nymphaea*, since the micropyle is closer to the funiculus as in *Nuphar*. The present observations suggest that the ovule morphology has diversified in *Nymphaea* and the characters studied clearly show no evidences to support the hypothesis of a monophyletic genus.

1. Introduction

In the current molecular phylogenetic tree, the order Nymphaeales constitutes one of the early diverging lineages of extant angiosperms (Qiu et al., 1999; Barkman et al., 2000; Zanis et al., 2002; Stefanović et al., 2004; Leebens-Mack et al., 2005; APG III, 2009). Phylogenetic analyses with consistent resolution have recognized the family Nymphaeaceae together with Cabombaceae as two clades comprising the order (Les et al., 1999). Results of phylogenetic analysis based on the nucleotide sequences of the chloroplast ITS2-4 region also support the taxonomic system of Nymphaeales which consists of these two families: Cabombaceae and Nymphaeaceae (Podoplelova and Ryzhakov, 2005). However, a more recent finding reveals that Hydatellaceae is the sister group to the Nymphaeales, and thus represents new implications for the evolution of the clade (Saarela et al., 2007).

Nymphaeaceae includes the subfamilies Nupharoideae (*Nuphar* Sm.), Barclayoideae (*Barclaya* Wall.) and Nymphaeoideae (*Euryale* Salisb., *Nymphaea* L., *Ondinea* Hartog, and *Victoria* Buc'hoz) distributed in tropical and temperate regions. *Nymphaea*, with approximately 50 species, is the major phenotypically diverse and widespread genus of the family. It was traditionally classified into five subgenera on the basis of morphological features and distribution (Conard, 1905): *Anecphya* (Casp.) Conard, *Brachyceras* (Casp.) Conard, *Lotos* (DC.) Conard, *Nymphaea*, and *Hydrocallis* (Planch.) Conard. The genus *Victoria* Lindl. comprises two South American species, *V. amazonica* (Poepp.) J.C. Sowerby and *V. cruziana* A.D. Orb.

A series of investigations on molecular systematic have elucidated phylogenetic relationships of the family at generic and specific levels, but between *Nymphaea* and the *Euryale-Victoria* clade the relationships are not fully resolved because they are strongly influenced by taxon sampling (Les et al., 1999; Padgett et al., 1999; Borsch et al., 2007, 2008; Löhne et al., 2007, 2008a, 2009; Dkhar et al., 2010; Borsch et al., 2011). The combined molecular analysis conducted by Borsch et al. (2008) suggests that *Nymphaea* is paraphyletic, with the subgenus *Nymphaea* being

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sister to a clade comprising the other four subgenera and the *Euryale-Victoria* clade. Furthermore, to date no clear phenotypic characters that support the monophyly of *Nymphaea* are known (Borsch et al., 2007). A morphological analysis revealed that liquid stigmatic fluid in first-day flowers is the only character state that has the best optimization on a tree assuming the monophyly of *Nymphaea* (Borsch et al., 2008). Consequently, the mentioned authors highlight the importance of examining both morphological and molecular characters to complete the comparative database as a crucial issue to clarify the relationships in the water lily family.

Over the last decade, embryological studies in basally diverging families, including the Nymphaeaceae, are one of the most important approaches in morphology because robust angiosperm phylogenies allow the reconstruction of the origin and early evolution of reproductive structures. Previous studies provide a patchy knowledge about ovule and female gametophyte in four subgenera of *Nymphaea* (Cook, 1902, 1906; Conard, 1905; Khanna, 1967; Batygina et al., 1980; Winter and Shamrov, 1991; Van Miegroet and Dujardin, 1992; Igersheim and Endress, 1998; Orban and Bouharmont, 1998; Dai and Zhou, 2010), but such information is still lacking in subgenus *Hydrocallis*. The monotypic genus *Euryale* and the two *Victoria* species were also embryologically studied, with illustrations based on line drawings (Khanna, 1964, 1967; Winter and Shamrov, 1991). Most of the above mentioned publications are currently known to have contradictory data on ontogenetic stages, regarding the number and arrangement of megaspores and the type of female gametophyte. With respect to these research topics, studies by Friedman and Williams (2003) distinguished a new type of female gametophyte development in *Nuphar*, and inferred the same mode of gametophyte formation for *Nymphaea* and *Victoria*. In addition, there has been much recent debate about variations of ovule morphology in the early divergent angiosperm lineages, so several hypotheses of ancestral conditions and early trends have been proposed (Igersheim and Endress, 1998; Yamada et al., 2001a, 2001b, 2003; Rudall et al., 2008; Endress, 2011). In some basal families, the outer integument is semiannular at initiation and hood-shaped at maturity, thus suggesting that anatropous ovules with a hood-shaped outer integument are primitive in angiosperms (Matsui et al., 1993; Umeda et al., 1994; Imaichi et al., 1995; Yamada et al., 2001a, 2001b, 2003). Accordingly, studies on morphology and development of the ovules within Nymphaeaceae may be of potential interest to evaluate evolutionary and phylogenetic implications. Yamada et al. (2001a) state that *Nymphaea* exhibit ovules with some derived character states, such as a cup-shaped outer integument, but *N. alba* was the only species of the genus examined, and thus the possible developmental variations among subgenera were not depicted, especially considering the still controversial relationships among subfamily Nymphaeoideae.

The aim of this work was to characterize the ontogenetic sequences of ovule and female gametophyte in two representative species of *Nymphaea* subgenus *Hydrocallis* (*N. amazonum* Mart. & Zucc. and *N. gardneriana* Planch.) and *V. cruziana*. This study provides significant characteristics of the ovules to evaluate relationships among the subfamilies of Nymphaeaceae and to understand the ovule evolution in Nymphaeales.

2. Materials and methods

Plant material of *N. amazonum* Mart. & Zucc., *N. gardneriana* Planch. and *V. cruziana* A.D. Orb. was collected from the province of Corrientes, Argentina. Herbarium material of *N. amazonum* (Zini et al., 6), *N. gardneriana* (Zini et al., 9) and *V. cruziana* (Zini et al., 13) are deposited at the Instituto de Botánica del Nordeste herbarium (CTES), Argentina. Floral buds and open flowers were fixed with formalin, acetic acid and alcohol (FAA).

For anatomical analysis, permanent slides were prepared by processing the fixed material and examined using light microscopy (LM). Samples of gynoecia were dehydrated with histological dehydrating BIOPUR® S.R.L. (Gonzalez and Cristóbal, 1997) and infiltrated in paraffin Histoplast® (Biopack, Buenos Aires, Argentina), according to Johansen (1940). The material was sectioned transversely and longitudinally (10–12 μm thickness) using a rotary microtome (Microm, Walldorf, Germany). Sections were stained in a safranin-astra blue combination (Luque et al., 1996) and mounted with synthetic Canada balsam (Biopur, Buenos Aires, Argentina). The serial sections were examined under a Leica DMLB2 (Leica, Wetzlar, Germany) light microscope equipped with a digital camera (Canon Power Shot S50 AIAF, Tokyo, Japan).

Ovules of *V. cruziana* were additionally examined using transmission electron microscopy (TEM) techniques for analyzing the nucellar epidermis in detail. Ovules were fixed in 1% glutaraldehyde, 4% formaldehyde in phosphate buffer (pH 7.2) for 2 h and post-fixed in 1.5% OsO_4 at 2 °C in the same buffer for 3 h. The materials were dehydrated using ascending graded series of acetone, and then embedded in Spurr resin. Ultrathin sections (750–900 nm) were made on a Reichert ultramicrotome and stained with uranyl acetate and lead citrate (O'Brien and McCully, 1981). The sections were examined using a JEOL 1200 EX II (JOEL USA, Inc., Peabody, MA).

For scanning electron microscopy (SEM) studies, dissected flowers were dehydrated using a graded series of ethanol solutions. The material was then critical-point dried with solvent-substituted liquid carbon dioxide and coated with gold-palladium. Micrographs were obtained with a JEOL 5800 LV scanning electron microscope operating at 20 kV.

3. Results

3.1. Ovule development

Ovules of *N. amazonum*, *N. gardneriana* and *V. cruziana* appear as slender protuberances on the inner surface of the ovary (Fig. 1A). By successive cell divisions in the funicle, these protuberances then become enlarged and curve towards the placentae. As the ovules bend, the inner integument is initiated by dermal periclinal cell divisions (Fig. 1B). Slightly later, in *Nymphaea* species the development of the outer integument is initiated in the dermal layer of the antiraphal side, but it is interrupted on the raphal side of ovule; therefore, it is semiannular (Fig. 1C and D). By contrast, in *V. cruziana* the outer integument is initiated by contribution of dermal and subdermal layers, and is completely annular from the moment of initiation (Fig. 3A–C).

Curvature of the ovules to an anatropous shape ends at the megaspore mother cell (MMC) stage (Fig. 1E–H and Fig. 3C and D). The inner integument is soon aligned at the same height with the outer integument (Fig. 1G). In both *Nymphaea* species, both integuments are two cells thick, whereas in *V. cruziana* the outer integument is over 12 cell layers thick and continues increasing in thickness throughout ovule growth. At this stage, many divisions of the funicular cells give rise to the aril development (Fig. 3C and D). By the time of the MMC meiosis, the outer integument overtops the inner one. When the MMC reaches the dyad stage, the outer integument is developed on the raphal side in *Nymphaea* species.

Mature ovules of *Nymphaea* species are vascularized by a single vascular bundle which ends in the chalaza. The aril is morphologically more conspicuous in *N. gardneriana* than in *N. amazonum* (Fig. 2A–C). The ovules exhibit some peculiarities. A group of radially elongated nucellar cells with intercellular spaces between

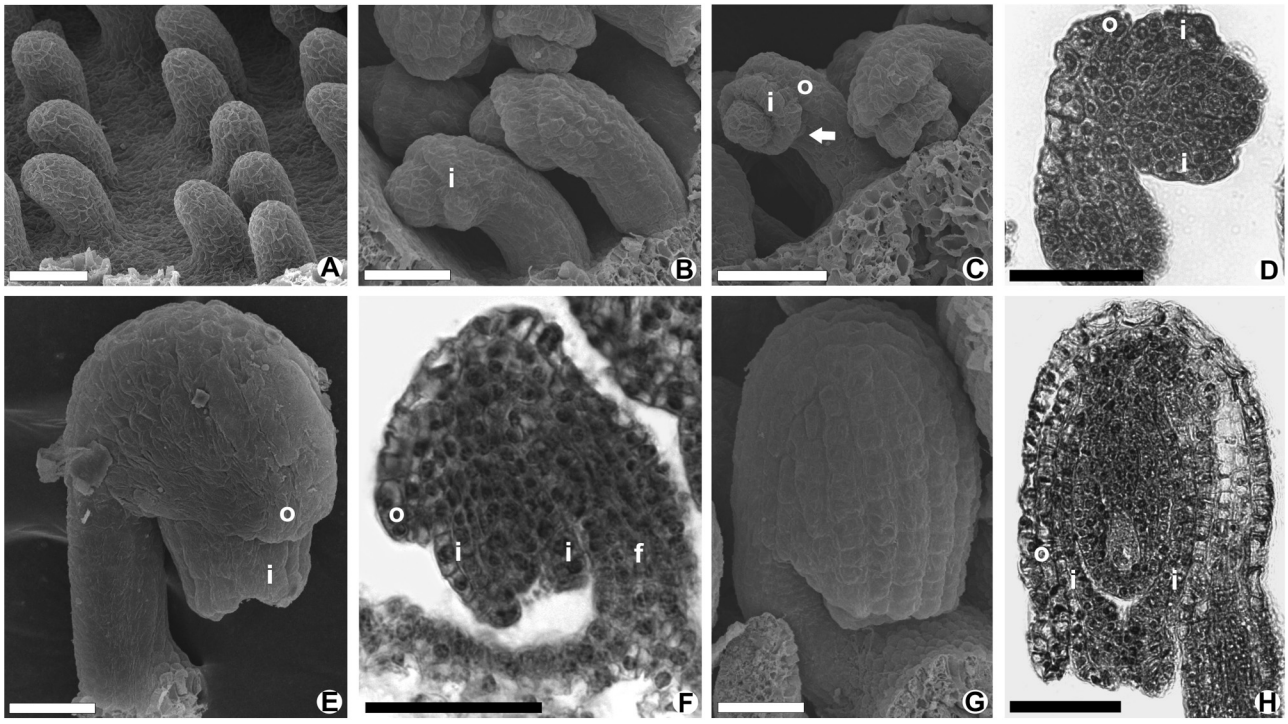

Fig. 1. SEM and LM (light microscopy) photomicrographs of ovules at successive stages of development in *N. amazonum* (B–E, G, H) and *N. gardneriana* (A and F). (A) Young ovules. (B–D) Young incurved ovules with the inner and outer integuments being initiated. Note in (C) that growth of the outer integument does not proceed on the raphal side of the ovule (arrow). (E and H) Anatropous ovules with both integuments in successive development at the stage of young megaspore mother cell. Scale bar = 50 μm (A–H) (f, funiculus; i, inner integument; o, outer integument).

them is distinguished in the chalazal region, in contact with the female gametophyte. These cells contain conspicuous nuclei and vacuoles at maturity (Fig. 2B). In addition, there is a cup-like region of cells at the chalaza, lying above the vascular bundle, with cells densely stained, without starch accumulation, and walls slightly thickened, which become well evident in post-fertilized ovules (Fig. 2D). At anthesis, the micropyle is delimited by the inner and outer integuments in *N. amazonum*, *N. gardneriana* (Fig. 2A and B) and *V. cruziana* (Fig. 3E). Other features observed in mature ovules of *V. cruziana* are: the vascular bundle branches in the chalaza (Fig. 3F); a group of chalazal cells characterized by a densely stained cytoplasm, which does not undergo major changes at least shortly after ovule fertilization (Fig. 3G). An epistase composed of the nucellar epidermal cells at the micropylar region, radially elongated, and with very conspicuous wall thickenings mostly on the inner tangential side of the cell is present in the three species. In *V. cruziana*, the cytoplasm of these cells is rich in mitochondria around the cell periphery, next to the wall, as well as in many plastids with starch grains, lipid bodies and endoplasmic reticulum (Fig. 4A and B). Dictyosomes and vesicles are very scarce. The cell walls are characterized by irregular ingrowths distributed on all the cell faces. Various plasmodesmata connect the adjacent nucellar cells (Fig. 4B).

The inner integument differentiates an operculum in all species, and the inner epidermis of this integument has cells that are densely stained with respect to the other cell layers of the integuments (Fig. 2B and Fig. 3E). The outer integument is developed on the raphal side of the ovule prior fertilization in *Nymphaea* species. However, it is thinner and shorter than the opposite side, being therefore asymmetrical in the micropylar region (Fig. 2C and E–G).

3.2. Megasporogenesis and megagametogenesis

In *N. amazonum*, *N. gardneriana* and *V. cruziana*, a subepidermal archesporial cell is differentiated at the apex of the nucellus. It divides periclinally into a primary parietal cell and a single MMC (Fig. 5A). The MMC with relative larger volume and prominent nucleus is located below a parietal cell layer (Figs. 5B and 6A). Meiosis I of the MMC gives rise to a dyad, with the chalazal cell being larger than the micropylar one (Figs. 5C and 6B). Meiosis II occurs only in the chalazal cell, giving rise to a linear triad of megaspores (Figs. 5D, 5E and 6C). The nucleus of the micropylar dyad cell eventually divides to form two daughter nuclei in *V. cruziana*, but no cytokinesis occurs (Fig. 6D). The two upper megaspores degenerate, whereas the chalazal megaspore is functional (Figs. 5F, 6E and 6F). The first mitotic karyokinesis of the latter results in the formation of a two-nucleate embryo sac; the nucellar cells adjacent to the embryo sac start to degenerate. At this stage, both nuclei are located at the micropylar end, and there is no migration of either nuclei to opposite ends of the embryo sac (Figs. 5G and 6G). After that, the nuclei simultaneously undergo a second mitotic karyokinesis to produce the four-nucleate embryo sac (Fig. 5H). Following cytokinesis, the embryo sac differentiates into a four-celled female gametophyte composed of one egg cell, two synergids, and a large cell with one polar nucleus (Figs. 5I–M and 6H–J). At this stage, the epistase close to the egg apparatus in the *Nymphaea* species is slightly discernable (Fig. 5I) whereas in *V. cruziana* is well differentiated (Fig. 6H and I). The synergids appear to have dense cytoplasm and a prominent nucleus, at maturity they typically become wedge-shaped, and their cytoplasm present a vacuole polarized towards the chalazal side (Figs. 5I, 5J and 6H). The egg cell has a round contour, and generally has a vacuole situated at the micropylar pole (Fig. 5K). The cell that contains a single larger polar nucleus has numerous vacuoles and starch grains (Figs. 5L, 5M and 6J).

4. Discussion

Table 1 presents selected ovule features which are analyzed in the following discussion and contains the genera of Nymphaeales and species of *Nymphaea* investigated so far.

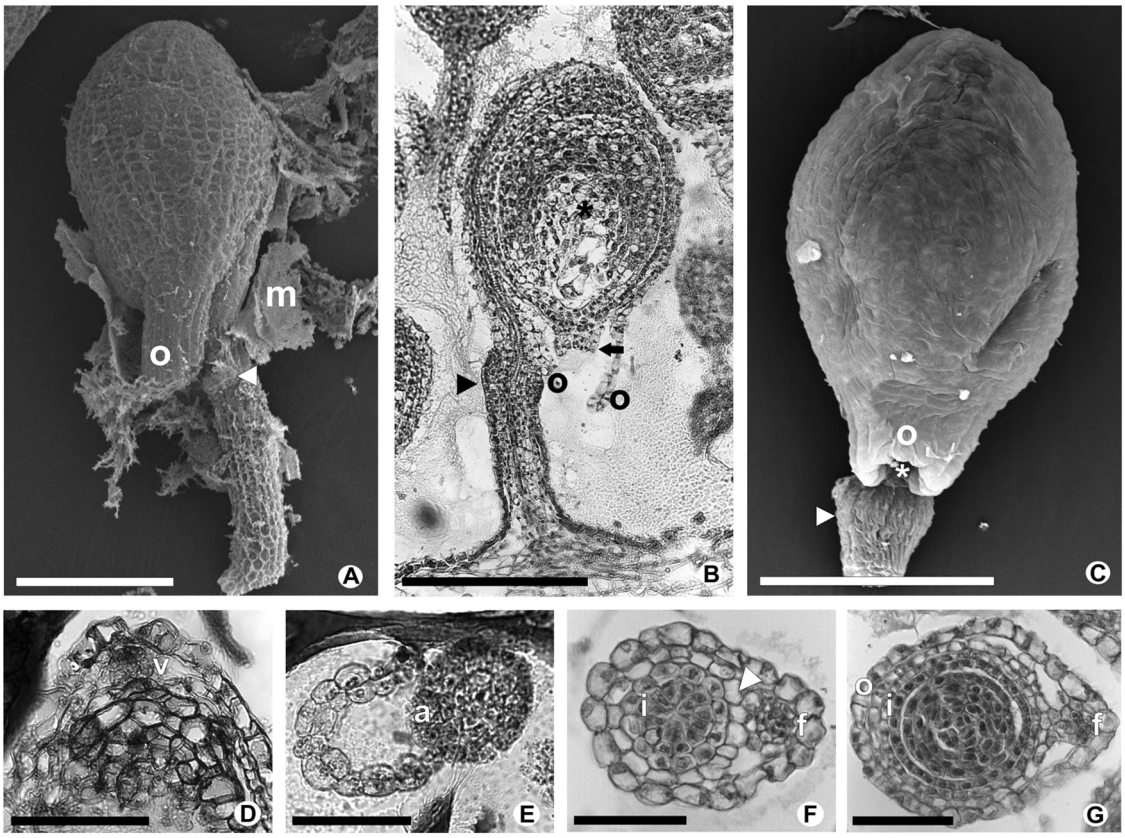

Fig. 2. SEM and LM photomicrographs of mature ovules of *N. amazonum* (A and B) and *N. gardneriana* (C–G). (A) Ovule showing zone of enlarged cells of outer integument and the small aril (arrowhead). (B) Median longitudinal section of mature ovule showing the distinctive cells that boundary the gametophyte (asterisk), the operculum (arrow), and aril (arrowhead). Note the lesser development outer integument on the raphal side. (C) Mature ovule of *N. gardneriana* showing the aril (arrowhead), the area of slower development of outer integument (arrowhead). (D) Detail of the chalaza in a post-fertilized ovule; location of the "podium" above the vascular bundle. (E–G) Three successive transverse sections of mature ovule: in (E), slightly below micropylar region showing aril and outer integument; in (F) at the micropyle with the arrowhead pointing to the inner epidermis of the outer integument on the raphal side, and in (G) at the upper nucellar region. Scale bar = 50 μm (D–G); scale bar = 200 μm (A–C) (a, aril; f, funiculus; i, inner integument; m, mucilage; o, outer integument; v, vascular bundle).

4.1. Ovule development and morphology

In accordance with previous studies on *Nymphaea* species (Table 1), the outer integument in *N. amazonum* and *N. gardneriana* is semiannular (hood-shaped) at an early stage because growth on the raphal side is delayed, but it later becomes annular (cup-shaped) only after ovule curvature. Mature ovules in representatives of subgenus *Hydrocallis* have an outer integument on the antiraphe thicker and longer than on the raphe, in contrast to those of subgenus *Nymphaea* (*N. alba*) which have well-developed outer integument on both sides of the ovule (Yamada et al., 2001a). The developmental pattern in *Nuphar* is essentially the same, although there is a discrepancy concerning the time of outer integument becomes annular, at a very early stage (Yamada et al., 2001a) or after fertilization (Zhou and Fu, 2008).

In addition, the present results show that in *V. cruziana* the outer integument is annular from initiation which confirms previous reports in the genus (Table 1). The evolutionary trend postulated by Yamada et al. (2001a) for Nymphaeales is the annular outer integument of Nymphaeaceae as a derived condition from the semiannular one, by extension of the outer integument onto the funiculus. The plesiomorphic state occurs in the sister family Cabombaceae and in Hydatellaceae, but Yamada et al. (2001a) also pointed out that *Nuphar*, the basal genus of Nymphaeaceae, exhibits an intermediate type of outer integument, between hood-shaped and cup-shaped, which develops into a seed with the micropyle and hilum separated by a very narrow testa, in contrast to the other Nymphaeoideae studied. However, only a single species of *Nymphaea* had been examined. The results of this study show that *Nymphaea* subgenus *Hydrocallis* exhibits the same intermediate outer integument as that of *Nuphar*, therefore the micropyle and hilum in the seed are quite close to one another. *Hydrocallis* is considered a subgenus that retains some ancestral states, such as conspicuous carpellary appendages, psilate pollen, anthers embedded medially on the connective (Wiersema, 1987; Borsch et al., 2007), and also it appears that ovules with an intermediate type of outer integument. The small subgenus *Lotos* is phylogenetically related to *Hydrocallis* (Borsch et al., 2007), and both share the features mentioned by the authors. However, morphology of the outer integument differ between ovules of subgenus *Hydrocallis* and *Lotos* because *N. lotus* have a well-developed cup-shaped integument similar to *N. alba* (Zini, unpublished data). Comparisons across different taxa also reveal that the outer integument is predominantly two cell layers thick in species of *Nymphaea* (including *N. caerulea* and *N. prolifera*, Zini, unpublished data), as well as in *Barclaya*, whereas more cell layers thick has been described for species of subgenus *Nymphaea* and for *Nuphar* (Table 1). Evidently more species need to be evaluated for understanding its evolution within *Nymphaea*. A two-layered inner integument has regularly been found in Nymphaeales, indicating that it is relatively stable (Schneider, 1978; Galati, 1985; Williamson and Moseley, 1989; Igersheim and Endress, 1998; Rudall et al., 2008; Dai and Zhou, 2010). Moreover, this study supports that the trait represents a synapomorphy for the order (Saarela et al., 2007).

The micropyle delimited by both endostome and exostome in the two species of subgenus *Hydrocallis* is a character in

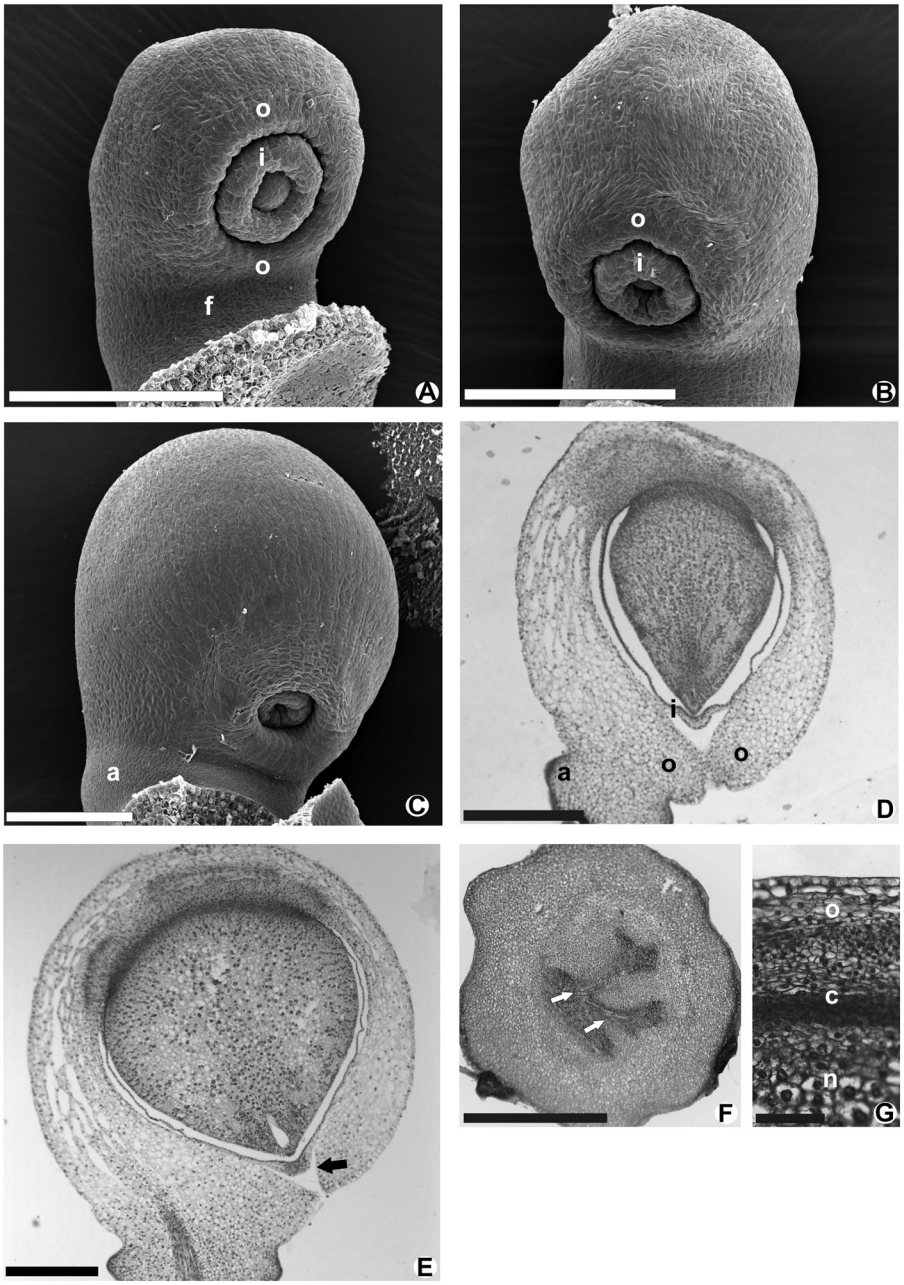

Fig. 3. SEM and LM photomicrographs of ovules at different stages in *Victoria cruziana*. (A–C) Three successive stages of young ovules incurving with the annular integuments developing. (D) Longitudinal median section of ovule at meiosis time. (E) Mature ovule; the arrow points to the operculum. (F) Transverse section of the ovule at the chalaza, showing the vasculature branching (arrows). (G) Detail of the ovule showing the chalazal zone of densely cytoplasmic cells. Scale bar = 100 μm (G); scale bar = 200 μm (A–D); scale bar = 500 μm (E, F, H) (a, aril; c, cells of the chalaza; f, funiculus; i, inner integument; n, nucellus; o, outer integument).

common with others species of the subgenera *Anecphya*, *Brachyceras* (*N. caerulea*, Zini, unpublished data), *Hydrocallis* (*N. prolifera*, Zini, unpublished data), *Lotos* and *Nymphaea*. The micropyle is also distomic in *Euryale* and *Victoria*. By contrast, in *Barclaya*, *Nuphar*, *N. tetragona* (subgenus *Nymphaea*) and in Cabombaceae the micropyle is endostomic (Table 1). According to Yamada et al. (2001a), the wide occurrence of endostomic ovules in basal angiosperms suggests its plesiomorphy. However, within Hydatellaceae (Rudall et al., 2008) and the Nymphaeaceae studied so far, both integuments frequently form the micropyle.

Nucellus thickness varies greatly in angiosperms, but is relatively stable in larger groups (Endress, 2011). Following the current classification by Endress (2011) the nucellus of *N. amazonum*, *N. gardneriana* and *V. cruziana* correspond to the weakly crassinucellar type, characterized by the presence of a single parietal cell layer between the MMC and nucellar epidermis. The same condition is also present in the closest family Cabombaceae (Galati, 1985; Endress, 2011). Many other species of Nymphaeaceae have been described as having a weakly crassinucellar nucellus (Conard, 1905; Orban and Bouharmont, 1998; Khanna, 1964), but this type does not seem to occur in some species of *Nymphaea* (Winter and Shamrov, 1991) and *Nuphar* (Zhou and Fu, 2008), in which the MMC was observed in layers of more than one cell in depth within the nucellus. In Hydatellaceae, the condition is markedly different (Rudall et al., 2008). A pattern of nucellus thickness for Nymphaeales following Endress's classification is still inconclusive.

Among the special modifications in ovule tissues, the epistase consists of apical cells of the nucellar epidermis with a marked

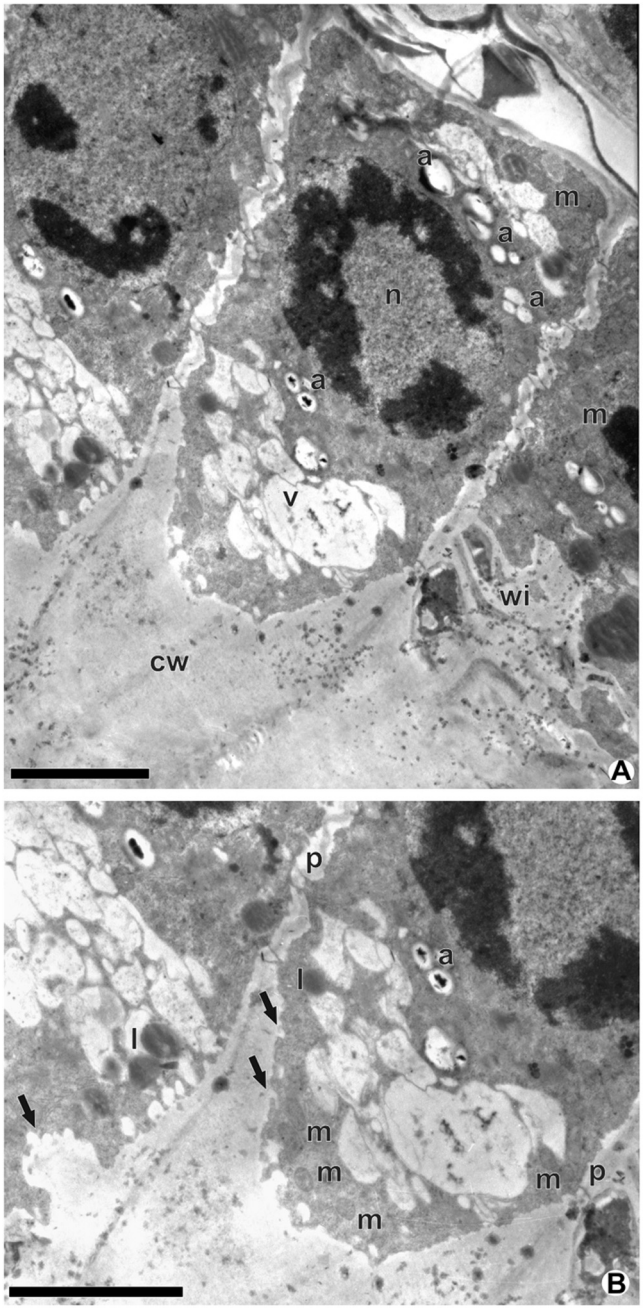

Fig. 4. TEM photomicrographs of the epistase in mature ovule of *V. cruziana*. (A) An entire cell and a part of its adjacent cells showing the conspicuous thickening on the inner tangential wall and the wall ingrowths. (B) Detail of Fig. 3A indicating organelles and the cell wall invaginations (arrows). Note that many mitochondria are present near the ingrowths. Scale bar = 400 μm (a, amyloplast; cw, cell wall; l, lipid bodies; m, mitochondria; n, nucleus; p, plasmodesmata; wi, wall ingrowths).

radial elongation that become somewhat thickened or suberized. The cells occasionally undergo one or more periclinal divisions to form the so called nucellar cap (Maheshwari, 1950). An epistase is present in *Euryale*, *Victoria* (Khanna, 1964, 1967; Winter and Shamrov, 1991; present paper), as well as in species of *Nymphaea* subgenus *Nymphaea*, *Lotos* (Cook, 1902, 1906; Winter and Shamrov, 1991), and now also in *Hydrocallis*, thus suggesting that it is characteristic of the family. Moreover, the epistase would represent a phenotypic trait that supports the close relationship between the Nymphaeaceae and Cabombaceae (Galati, unpublished).

The epistase has received little attention and, interestingly, ultrastructural examination in *V. cruziana* revealed some aspects related to their possible function. In the basic organization of the reproductive tissue of higher plants, the occurrence of cytoplasmic discontinuities between sporophyte and female gametophyte leads to special problems in transport (Gunning and Pate, 1969). Transfer cells are plant cells with secondary wall ingrowths. They are considered to play a central role in nutrient distribution by facilitating high rates of transport for apo-/symplasmic solute exchange (Offler et al., 2003). The presence of numerous wall ingrowths in the cells forming the epistase indicates transfer cell specialization, and therefore is probably involved in nutrient transport into the adjacent cells. Several studies have described transfer sites in ovular tissues, such as integuments and nucellar cells that surround the female gametophyte and embryo (Jensen, 1965; Schultz and Jensen, 1968; Gunning and Pate, 1969; Peterson et al., 1979; Tilton, 1980; Cass et al., 1986; Sumner and van Caeseele, 1990; Olesen and Bruun, 1990; Johansson and Walles, 1993; Galati et al., 2005). Particularly, transfer cells in the nucellar cap were associated with a secretory activity related to guidance of pollen tubes towards the female gametophyte (Tilton, 1980; Tilton and Lersten, 1981; Bruun and Olesen, 1989). Accordingly, the cytological characteristics of the epistase of *V. cruziana* indicating a very active metabolism are consistent with the interpretation postulated by those authors.

4.2. Megasporogenesis and female gametophyte development

Linear triad of megaspores is a character consistent among the studied species of *Nymphaea* (Table 1). In *V. cruziana*, the development of linear or T-shaped tetrads was not observed, in contrast to findings of Khanna (1967) for this species and in *Euryale ferox* by Khanna (1964). However, the present study shows that the micropylar cell of the dyad often undergoes a karyokinetic event but no cytokinesis occurs. Megaspore number and arrangement among the Nymphaeales show some degree of developmental plasticity. In addition, the suppression of karyokinesis or cytokinesis in the nonfunctional dyad as found in *Cabomba*, *Nuphar*, *Nymphaea* and *Victoria* might be interpreted as independent transitions to a reduction from tetrads to triads of megaspores.

The female gametophyte of the studied species develops from the functional chalazal megaspore and is consistently four-celled. This mode of gametophyte formation prevails among Nymphaeales and Austrobaileyales (Friedman and Williams, 2004; Tobe et al., 2007; Rudall et al., 2008), although historically it has been recognized under different denominations, such as a variant of Oenothera type (Galati, 1985; Orban and Bouharmont, 1998), Schisandra type (Battaglia, 1986), Schisandra variant of the Oenothera type (Batygina and Vasilyeva, 2002) or Nuphar type (Friedman and Williams, 2003). Differences between the Oenothera and Schisandra types were later addressed by Tobe et al. (2007), specifically the position of the functional megaspore in both types (micropylar in Oenothera type and chalazal in Schisandra type) and the occurrence within the basal angiosperms. However, the characterization of the Oenothera type made by Maheshwari (1950) is unclear because it has been described that frequently the micropylar, sometimes the chalazal or both megaspores of the same tetrad may simultaneously give rise to two independent embryo sacs. Ovules with compound embryo sacs within early-divergent angiosperms were also detected (Cook, 1902, 1906; Batygina and Vasilyeva, 2002; Rudall et al., 2008) but their presence is scarce, and in those cases, the female gametophytes are derived from chalazal and not micropylar megaspores. Therefore, in accordance with Tobe et al. (2007), the Schisandra type should be recognized as a distinctive pattern of gametophyte formation characteristic for Nymphaeales and Austrobaileyales.

Based on our results and previous reports, we conclude that the ovule characters variable in the family are: ovule type, with only

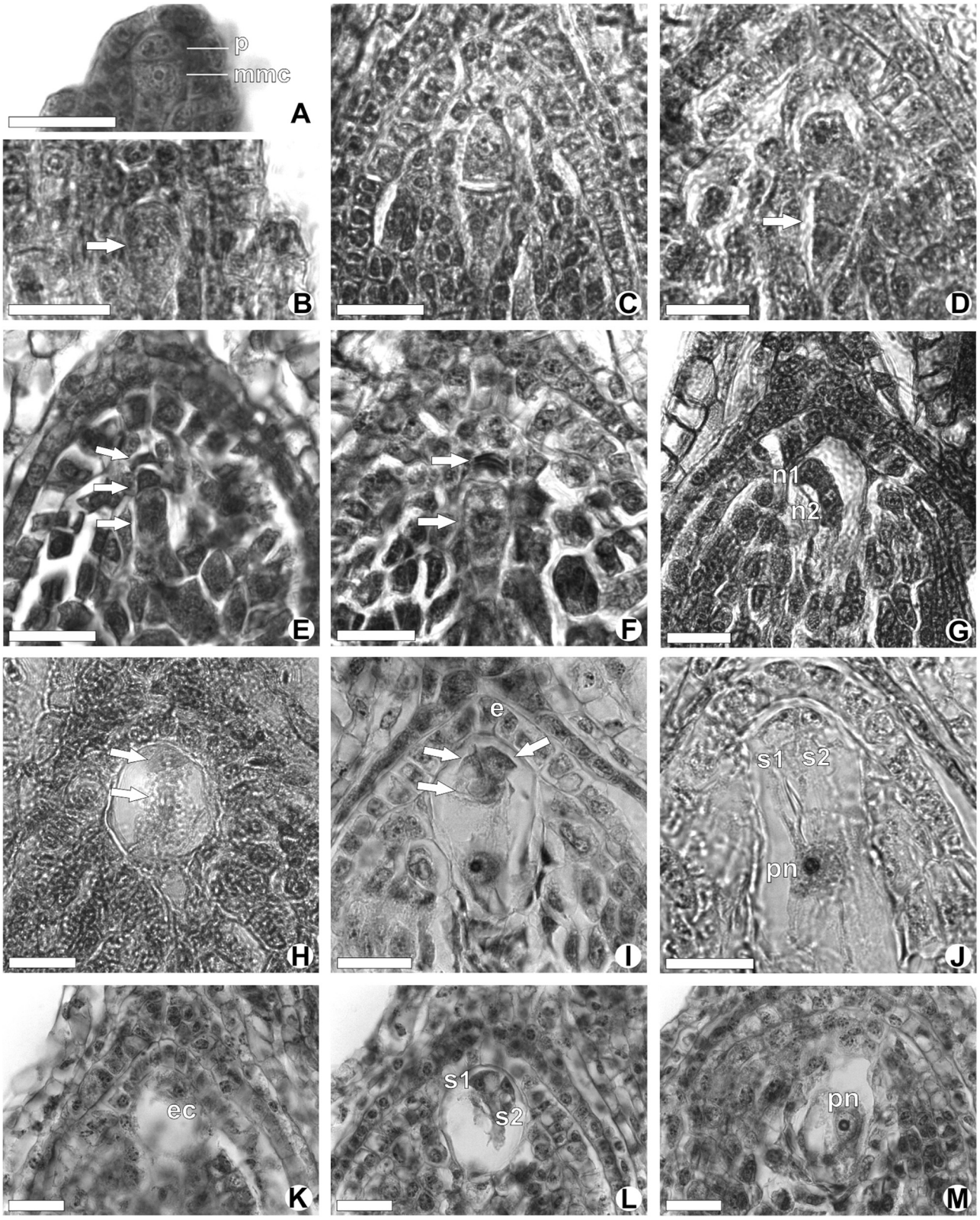

Fig. 5. LM photomicrographs of megasporogenesis and female gametophyte development in *N. amazonum* (C, F–H, K–M) and *N. gardneriana* (A, B, D, E, I, J). (A) Detail of the ovule apex showing periclinal division of the archesporial cell. (B) Detail of single megaspore mother cell (arrow) developing below one hypodermal cell layer. (C) Dyad stage of the megaspore mother cell. (D) Meiosis II of the chalazal dyad cell (arrow). (E) Triad of megaspores. The two upper arrows points to micropylar megaspores while the inner one point to the functional megaspore. (F) Triad with degenerating megaspores (upper arrow), and the well-developed chalazal megaspore (lower arrow). (G) Embryo sac at two nucleate stage. (H) The two nuclei at anaphase of the second mitosis. (I) Young 4-celled female gametophyte, the arrows indicate the cells of the egg apparatus. (J) Detail of female gametophyte showing the two mature synergids and the polar nucleus. (K–M) Detail of three successive longitudinal sections of the ovule showing the 4-celled female gametophyte of *N. amazonum*. Scale bar = 20 μm (e, epistase; ec, egg cell; mmc, megaspore mother cell; n1, nucleus 1; n2, nucleus 2; p, parietal cell; pn, polar nucleus; s1, synergid 1; s2, synergid 2).

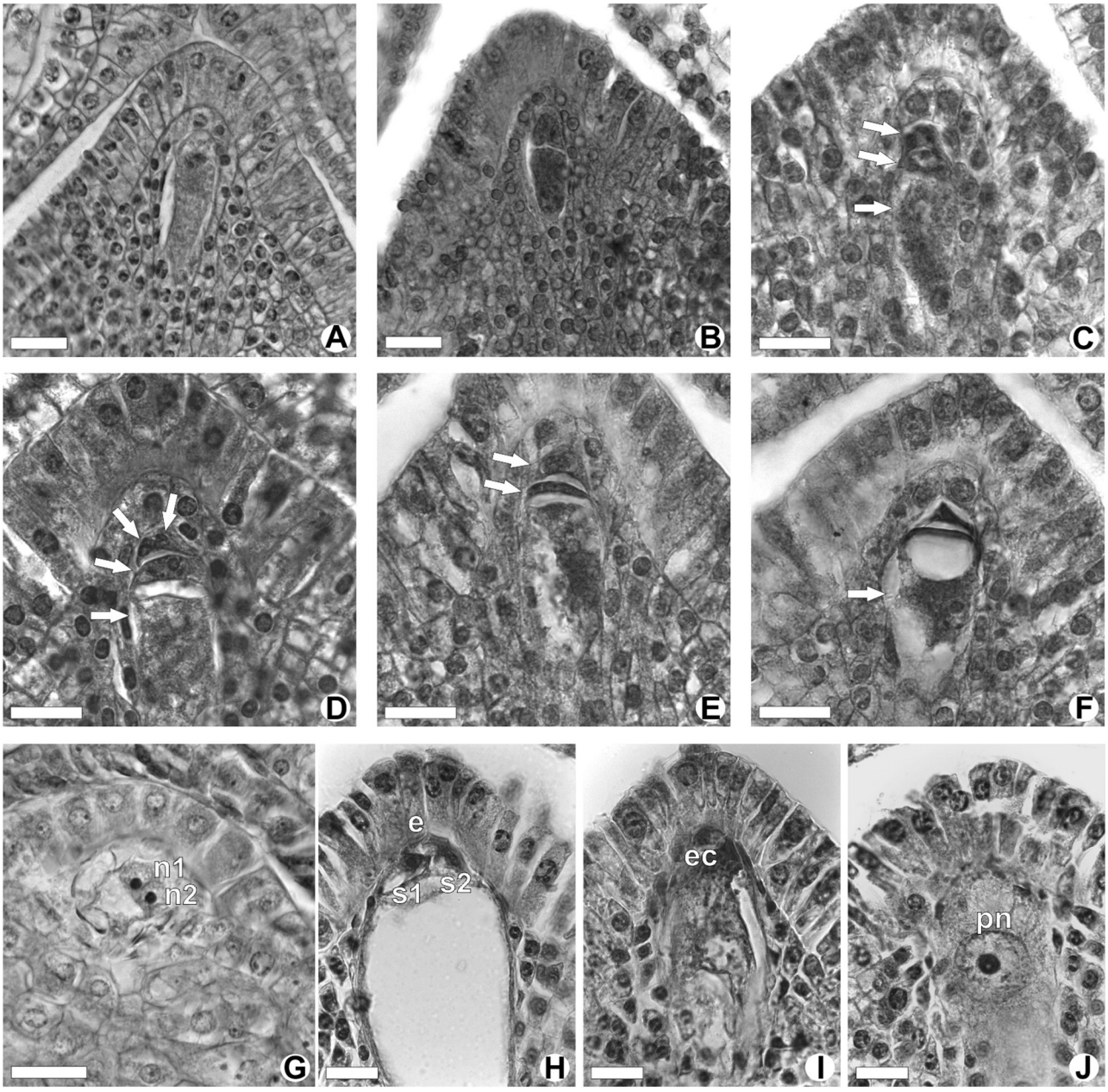

Fig. 6. LM photomicrographs of megasporogenesis and female gametophyte development in *Victoria cruziana*. (A) A mature megaspore mother cell below single hypodermal cell layer. (B) Dyad stage of the megaspore mother cell. (C) Triad of megaspores (arrows). (D) The same stage than that of (C), but showing the two nuclei of the micropylar dyad cell (two upper arrows), and the other two megaspores (two lower arrows). (E) The most micropylar dyad cell also with two nuclei (upper arrow), and the micropylar megaspore degenerating (lower arrow). (F) Functional chalazal megaspore (arrow). (G) Two-nucleate stage of the embryo sac. (H–J) Three serial longitudinal sections of the mature 4-celled female gametophyte. Scale bar = 20 μm (A–F, H–J); scale bar = 25 μm (G) (e, epistase; ec, egg cell; n1, nucleus 1; n2, nucleus 2; pn, polar nucleus; s1, synergid 1; s2, synergid 2).

Barclaya showing orthotropous ovules (Schneider, 1978; Igersheim and Endress, 1998), ontogeny and thickness of outer integument, micropyle formation, and nucellus thickness. The ontogenetic and morphological ovule similarities in *V. cruziana* and *E. ferox* encompass a further set of phenotypic characters that support the phylogenetic association between both genera (Conard, 1905; Weidlich, 1980; Les, 1988; Moseley et al., 1993; Les et al., 1999). On the other hand, all *Nymphaea* species studied so far share the presence of an outer integument semiannular with subsequent annular development, and a linear triad of megaspores.

Although previous works distinguished several differences and evolutionary trends among genera of Nymphaeales, the new evidences about different ovule morphologies from selected species of *Nymphaea* suggest that diversification had occurred also among the subgenera. Many characters such as nucellus and outer integument thickness, and pattern of development of the outer integument were inferred to have changed rapidly in a first step as suggest estimates of divergence time (Löhne et al., 2008b) on the branch leading to *Victoria-Euryale* and *Nymphaea*. Indeed, the characters studied show no definite evidences to support the hypothesis of a monophyletic genus *Nymphaea*. Therefore, only further morphological studies in species from all subgenera may contribute to the resolution of the phylogenetic relationships of the genus.

Table 1
Records of some ovules characters in representatives of the order Nymphaeales.

Taxon	Number of species examined/total number species	Nucellus	Outer integument	Thickness of outer integument	Micropyle	Megaspores number and arrangement	References
Trithuria	5/12	Pseudo-crassinucellar	Semiannular (hood-shaped)	2	Distomic	Linear tetrads	Rudall et al. (2008)
Brasenia	1/1	Weakly crassinucellar	Semiannular	2	Endostomic	Linear tetrads	Khanna (1965) and Yamada et al. (2001a)
Cabomba	2/5	Weakly crassinucellar	Semiannular	2	Endostomic	Linear tetrads or triads	Galati (1985) and Endress (2011)
Nuphar	4/15	Crassinucellar	Semiannular at early stages, and annular at maturity	4–5	Endostomic	Linear triads or tetrads	Zhou and Fu (2008), Yamada et al. (2001a) and Friedman and Williams (2003)
Barclaya	1/4	Unknown	Annular (cup-shaped)	2	Endostomic	Unknown	Schneider (1978) and Igersheim and Endress (1998)
N. alba (subg. *Nymphaea*)	3/8	Crassinucellar	Semiannular at early stages, and annular at maturity	4–5	Distomic	Linear triads	Yamada et al. (2001a) and Winter and Shamrov (1991)
N. odorata (subg. *Nymphaea*)		Crassinucellar	Unknown	Unknown	Unknown	Linear triads?	Cook (1902)
N. tetragona (subg. *Nymphaea*)		Unknown	Annular at maturity	3	Endostomic	Unknown	Igersheim and Endress (1998)
N. amazonum (subg. *Hydrocallis*)	2/14	Weakly crassinucellar	Semiannular at early stages, and annular at maturity	2	Distomic	Linear triads	Present paper
N. gardneriana (subg. *Hydrocallis*)		Weakly crassinucellar	Semiannular at early stages, and annular at maturity	2	Distomic	Linear triads	Present paper
N. lotus (subg. *Lotos*)	1/3	Weakly crassinucellar	Annular at maturity	2	Distomic	Linear triads	Dai and Zhou (2010)
N. nouchali (subg. *Brachyceras*)	1/12	Weakly crassinucellar	Annular at maturity	Unknown	Distomic	Linear triads	Khanna (1967) and Orban and Bouharmont (1998)
N. gigantea (subg. *Anecphya*)	3/11	Crassinucellar	Annular at maturity	Unknown	Distomic	Linear triads?	Winter and Shamrov (1991)
N. ondinea (subg. *Anecphya*)		Unknown	Annular at maturity	2	Distomic	Unknown	Williamson and Moseley (1989)
N. heudelotii (subg. *Anecphya*)		Crassinucellar	Unknown	Unknown	Distomic	Linear triads	Van Miegroet and Dujardin (1992)
Euryale	1/1	Weakly crassinucellar	Annular	c. 20	Distomic	Linear or T-shaped tetrads	Igersheim and Endress (1998) and Yamada et al. (2001a)
Victoria	1/2	Weakly crassinucellar	Annular	c. 20	Distomic	Linear triads or tetrads	Igersheim and Endress (1998) and Yamada et al. (2001a); present paper

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