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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