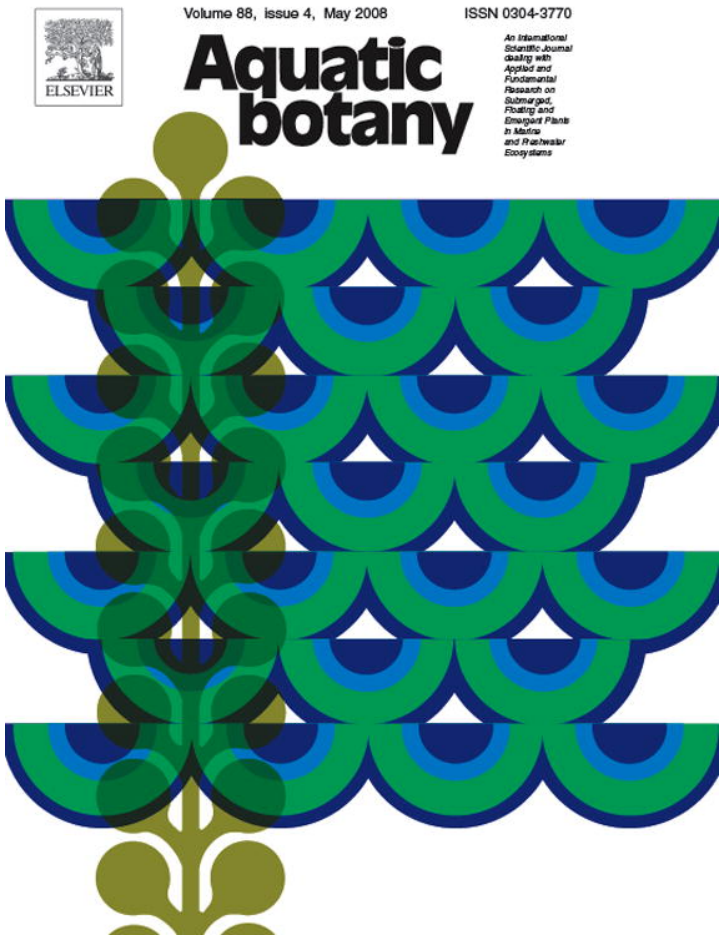

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Megagametogenesis in *Halophila johnsonii*, a threatened seagrass with no known seeds, and the seed-producing *Halophila decipiens* (Hydrocharitaceae)

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Abstract

Megagametogenesis, the development of a megaspore into an embryo sac, has been identified in the seagrass *Halophila johnsonii*, a threatened species with no known sexual reproduction or seeds. Megagametogenesis in *H. johnsonii* was compared with megagametophyte development in *Halophila decipiens*, a related species known to readily produce viable seeds. In both species, ovules were structurally similar, megaspore mother cells were seen in premeiotic ovules, and linear tetrads and megagametophytes with two to eight nuclei were present in postmeiotic ovules. However, *H. decipiens* postmeiotic ovules had a chalazal pouch that was absent in the postmeiotic ovules of *H. johnsonii*. Late-stage *H. decipiens* ovules also contained embryos, indicating that they had been fertilized, whereas all late-stage *H. johnsonii* ovules were degrading and showed no signs of fertilization. These observations suggest that meiosis does occur in *H. johnsonii* megasporocytes, leading to the formation of viable megagametophytes and egg cells that could be fertilized if pollination occurred. Thus, the lack of seed set is due to a lack of pollination rather than any loss of capacity to produce seeds in this species.

Keywords: Seagrass; *Halophila* sp.; Flowering; Megagametogenesis

1. Introduction

The seagrass genus *Halophila* (Thouars) is pantropical with 14 species presently recognized (den Hartog, 1970; Phillips and Meñez, 1988; Larkum, 1995; Uchimura et al., 2006). *Halophila* includes a number of ruderal species capable of growing on a wide range of substrates and in marginal environments incapable of supporting the growth of larger seagrass species (Dawes et al., 1989; Kenworthy, 1997; Durako et al., 2003; Den Hartog and Kuo, 2006). This genus has a wide depth distribution as well, with plants growing from the intertidal down to 85 m, where they are capable of thriving in fluctuating, low light environments (den Hartog, 1970; Phillips and Meñez, 1988; Kenworthy et al., 1989; Gallegos and Kenworthy, 1996; Kuo et al., 2001; Hammerstrom et al., 2006).

Three *Halophila* species are reported from the coastal waters of southeast Florida: *Halophila decipiens* Ostenfeld, *Halophila engelmannii* Ascherson, and *Halophila johnsonii* Eiseman. Among these species *H. decipiens* is monoecious, with perfect flowers that release three-celled ellipsoid pollen grains (McMillan and Soong, 1989; Lakshmanan and Poornima, 1991). It is the only pantropical species in the genus, inhabiting both oceanic waters and the subtidal waters of coastal lagoons and estuaries. *H. engelmannii* is dioecious and occurs in both shallow and deep water, sometimes growing with larger seagrasses such as *Thalassia testudinum* Banks ex König and *Halodule wrightii* Ascherson. *H. johnsonii* is endemic within the lagoon systems of southeast Florida, USA from Sebastian Inlet (approximate lat. 27°51′N) to Virginia Key (approximate lat. 25°45′N), the smallest reported distribution of any seagrass species (Eiseman and McMillan, 1980). *H. johnsonii* has been found in disjunct and patchily distributed populations and occurs from the intertidal to 4 m depths. Both *H. decipiens* and *H. johnsonii* also occur together in mixed species subtidal

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meadows (Gallegos and Kenworthy, 1996; Kenworthy, 1997; Virnstein et al., 1997, 2007).

H. johnsonii is believed to be a dioecious species because only pistillate (imperfect female) flowers have been observed in both plants collected from nature and those grown in culture (Eiseman and McMillan, 1980; Jewett-Smith et al., 1997; Kenworthy, 1997). Phylogenetic analyses of *Halophila* species also resolve *H. johnsonii* within a species complex including *H. australis* Doty & Stone, *H. hawaiiana* Doty & Stone, *H. minor* (Zollinger) den Hartog, and *H. ovalis* (R. Brown) Hooker f., species that are all dioecious (Waycott et al., 2002; Freshwater et al., unpublished). Although sexual reproduction has been documented in other *Halophila* species (Lakshmanan, 1963; McMillan, 1976; Johnson and Williams, 1982; Herbert, 1986; Lakshmanan and Poornima, 1991; Kuo and Kirkman, 1992; Larkum, 1995; Zakaria et al., 1999), it has never been reported in *H. johnsonii*.

Its unknown mode or lack of sexual reproduction is a significant contributing reason why *H. johnsonii* is the only marine plant listed as threatened on the United States threatened and endangered species list (Federal Register, 1998). The presence of pistillate flowers is the only indication of the potential for sexual reproduction this species exhibits. However, it is unknown if the pistillate flowers retain the ability to produce viable haploid egg cells that could potentially be fertilized.

In typical pistil development, ovules are defined as sporophyte tissue that produce and retain the female gametophyte (megagametophyte). An immature ovule contains a single megasporocyte (or megaspore mother cell), which goes through a reduction-division sequence (megagametogenesis) to produce a megagametophyte. The first division is meiotic, and results in the megasporocyte developing into four haploid megaspores (linear tetrad). Three of the megaspores degenerate and the remaining one undergoes three mitotic divisions to produce an eight-nucleate megagametophyte.

Meiosis is required to generate a functional haploid gametophyte, but whether or not this occurs in *H. johnsonii* is unknown. Eiseman and McMillan (1980) suggested that *H. johnsonii* might reproduce by apomixis. In apomixis, a somatic or germinal cell in the ovule, with unreduced (somatic) chromosome number, develops into an embryo (Asker and Jerling, 1992). Thus, fertilization is not required for seed production and seeds are 'clones' of the mother plant. Meiosis does not typically occur during the generation of apomictic seeds (Nogler, 1984). In the few species of apomictic plants that exhibit meiotic diplospory or premeiotic chromosome doubling through endoreplication or endomitosis, linear tetrads are not formed (Asker and Jerling, 1992). Establishing the presence (i.e., linear tetrad formation) or absence of meiosis and a functional megagametophyte is key to understanding if *H. johnsonii* has the ability to reproduce sexually or through apomixis.

Meiosis can be confirmed by observing the structural development of the megagametophyte (Lakshmanan and Poornima, 1991; Sherwood, 1995). In this study, the DNA-localizing fluorochrome DAPI, along with bright-field fluorescence microscopy, were used to observe gametophyte developmental stages of both *H. johnsonii* and *H. decipiens*. *H. decipiens* was used as a comparative reference in this study because megasporogenesis has been described in this species (Lakshmanan and Poornima, 1991). Identification of a linear tetrad in *H. johnsonii* would indicate that meiosis has occurred and that the ovules could be functional up to the megaspore formation stage of sexual development.

2. Materials and methods

2.1. Plant collection

Specimens of *H. johnsonii* and *H. decipiens* were collected in the field from three locations within the range of *H. johnsonii* on the east coast of Florida: Sebastian Inlet (27°51.37′N, 80°27.04″W); Jupiter Inlet (26°57.68′N, 80°04.76″W); and Haulover Park (25°55.22′N, 80°07.54″W). Samples were collected using two techniques: (1) direct collection in the field by gently excavating portions of ramets with flowers, and (2) culture of plant plugs containing sediments collected from the field sites using a 100 cm^2 sod-plugger. Plug samples were placed into 100 cm^2 peat pots and transferred to the UNCW Center for Marine Science greenhouse under the conditions of 16:8 L:D, 24 °C, and a salinity of 32 to induce flowering (McMillan, 1976).

2.2. Fixation and embedding

The excavated ramets were placed directly into 3:1 95% ETOH: glacial acetic acid, for 24 h, then placed into 70% ETOH for storage. Developing flowers were excised from cultured samples with forceps and fixed as above. Pistillate flowers of *H. johnsonii* and the perfect flowers of *H. decipiens* were embedded with a JB-4™ kit as follows: flowers were dehydrated in 100% ethanol for at least 1 h, then placed into an infiltration solution of 25 mL of JB-4™ Solution A (2-hydroxyethyl methacrylate) and 0.25 g of catalyst powder (benzoyl peroxide) until flowers sank to the bottom (24–120 h) of a 35 mL screw-cap specimen bottle. Each flower was then placed in a JB-4™ resin of 2 mL infiltration solution and 0.13 mL of JB-4 Solution B (polyethylene glycol), and the resin allowed to harden for approximately 2 h. Hardened resin blocks were stored in a drying oven at 37 °C until they were sectioned.

2.3. Sectioning and DAPI staining

Resin blocks were trimmed and 10 μm consecutive sections of each flower were made using a Sorval ''Porter-Blum'' MT-1 ultra-microtome. Approximately 180 sections were obtained from each flower (50 flowers per species). Consecutive sections were placed, one at a time, on frosted microscope slides. The sections were flattened with a dilute solution of ammonium hydroxide, air-dried at room temperature, stained in the dark for 20 min with DAPI (0.5 μg mL^{-1} 4′6-diamidino-2-phenylindole; Sigma Chemical Co., St. Louis, MO), and covered with a coverslip.

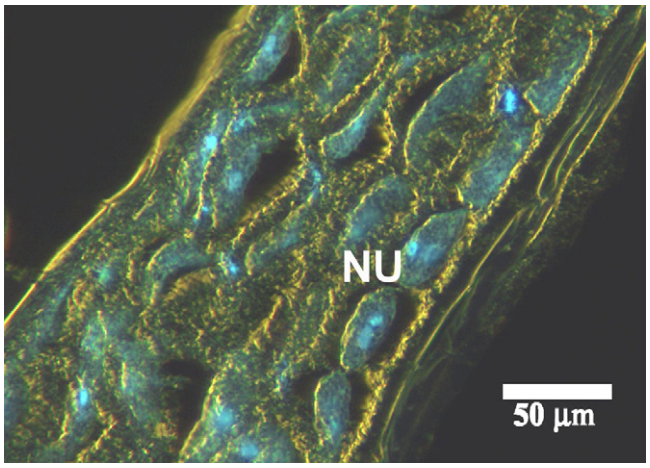

Fig. 1. *Halophila decipiens* binucleate pollen cells within a mucilage tube of the stamen; NU: nucleus.

2.4. Analysis

Prepared slides were examined using an Olympus BH2-RFK fluorescence microscope equipped with a high-pressure mercury vapor lamp (HBO, 100 W), and a 420-nm suppression filter, which is specific for DAPI-bound emissions. Digital images of correctly oriented and intact sectioned ovules were captured using Spot RTke or Spot RTcolor digital cameras and Spot Advanced imaging software (Diagnostic Instruments, Inc., Sterling Heights, MI).

3. Results

Fifty flowers of each species were embedded in resin. However, only 20 flowers of each species were intact and oriented correctly following the embedding and sectioning procedures; soft resin, sectioning problems, and improper orientation caused 30 samples to be discarded. A total of 3600 sections of each species were visually scanned. *H. decipiens* pollen was used as a control to verify that staining of haploid nuclei within a cellular structure could be observed (Fig. 1). Pollen cells were observed in the stamens of *H. decipiens*, while pistils for both species were observed in three main stages of development based on the size of the ovary and the stage of development at which the ovules were observed. The stages were: premeiotic, postmeiotic, and senescent or post-fertilization. In addition to exhibiting common stages of development, ovules of both species exhibited parietal placentation and were

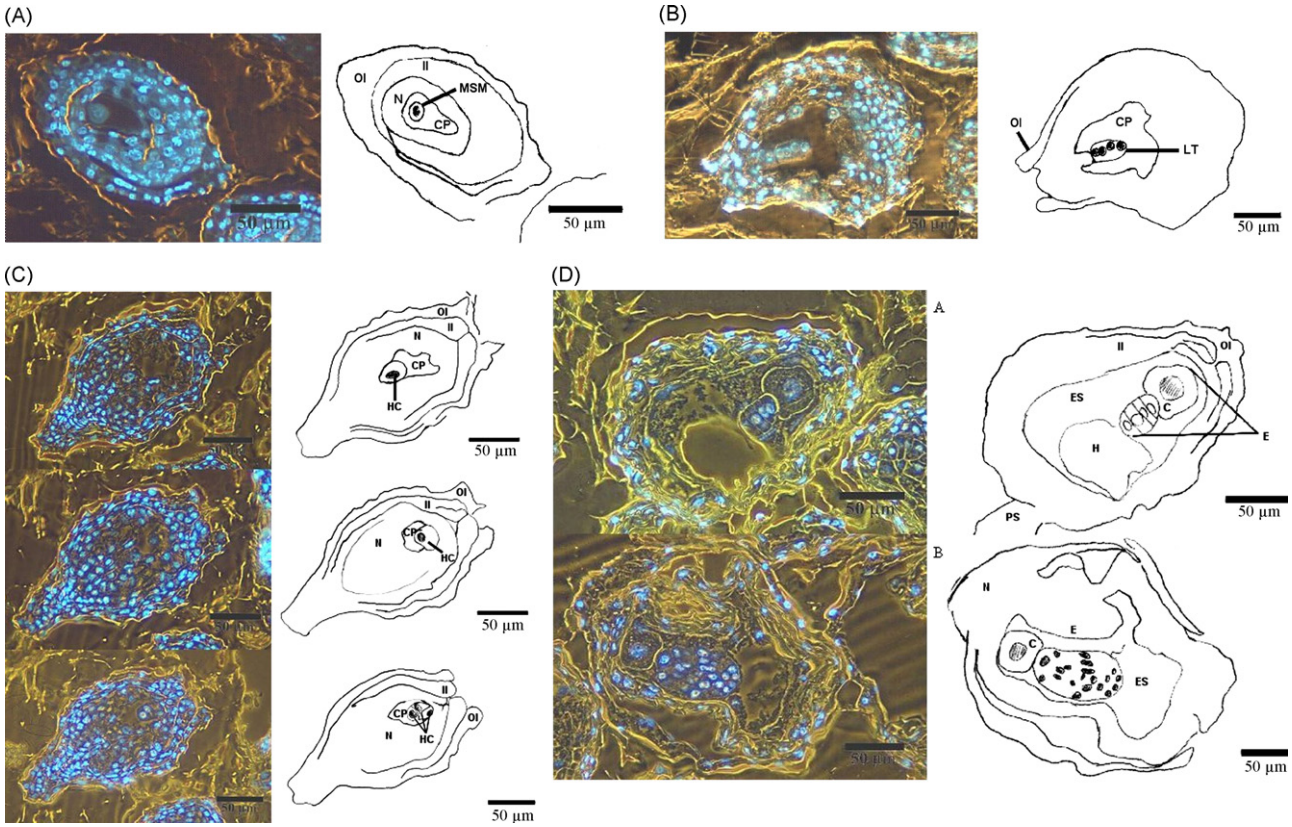

Fig. 2. *Halophila decipiens*. (A) Premeiotic ovule containing a megaspore mother cell. MSM: megaspore mother cell; CP: chalazal pouch; N: nucellus; II: inner integument; OI: outer integument. (B) Early postmeiotic ovule with a linear tetrad; LT: linear tetrad; CP: chalazal pouch; OI: outer integument. (C) Three serial sections of a *Halophila decipiens* ovule showing a developed gametophyte. (i) One of the antipodal cells in the chalazal pouch; (ii) a haploid cell near the micropyle; (iii) three cells, an antipodal near the chalazal pouch and two haplid cells near the micropyle. HC: haploid cell; CP: chalazal pouch; N: nucellus; II: inner integument; OI: outer integument. (D) Later-stage post-fertilization ovules with developing embryos. (i) A five-celled proembryo with the suspensor basal cell at the micropyle end; (ii) a globular embryo with suspensor. E: embryo; C: suspensor basal cell; H: hole in section; ES: embryo sac; N: nucellus; II: inner integument; OI: outer integument; PS: placental stalk.

composed of two bistromatic integuments surrounding a layer of nucellus tissue that covers the megagametophyte.

3.1. Halophila decipiens

Mature pollen grains were binucleate 10 μm wide and 50 μm long. They were arranged linearly and contained within mucilage tubes, enclosed by the anther wall (Fig. 1). The premeiotic ovaries of *H. decipiens* measured 0.25 mm wide. They contained 20–30 ovules, measuring 75 μm in diameter. The ovaries were considered premeiotic primarily because the ovules contained a single megaspore mother cell within a chalazal pouch (Fig. 2A). The postmeiotic ovaries measured 1 mm wide, with the ovules 100 μm in diameter. The early postmeiotic ovules contained linear tetrads with four nuclei (Fig. 2B). Further-developed postmeiotic ovaries contained immature to mature ovules with megagametophytes containing two-to-eight nuclei (Fig. 2C). The majority of later-stage ovaries contained developing, fertilized ovules, rather than unfertilized senescent ones, and were 2–3 mm wide. The fertilized ovules were 150 μm in diameter, and contained embryos at various stages of development (Fig. 2D). These characteristics confirm the results of Lakshmanan and Poornima's (1991) study of *H. decipiens* embryology.

3.2. Halophila johnsonii

The premeiotic ovaries of *H. johnsonii* were 0.5 mm wide. The ovaries contained 10–15 ovules, which measured 150 μm in diameter, and had single megaspore mother cells inside (Fig. 3A). The postmeiotic ovaries measured 1.5 mm wide, with ovules 150 μm in diameter. Early postmeiotic ovules were characterized by the presence of a linear tetrad (Fig. 3B). Further-developed postmeiotic ovules in *H. johnsonii* contained megagametophytes with two to eight nuclei (Fig. 3C), similar to the later postmeiotic stages found in *H. decipiens*. The two species differed in that the megagametophyte for *H. decipiens* exhibited a large, spacious chalazal pouch, which is absent in *H. johnsonii* (compare Figs. 2C and 3C). The later-stage ovaries had ovules that had completed or were in the process of senescence, and they showed no signs of fertilization. The senescent ovaries measured 2 mm wide and contained ovules that were 250 μm in diameter but showed signs of evacuation or death of inner gametophytic tissue (Fig. 3D).

4. Discussion

H. decipiens was used as a reference in this study because: (1) it is known to have functional flowers that produce viable

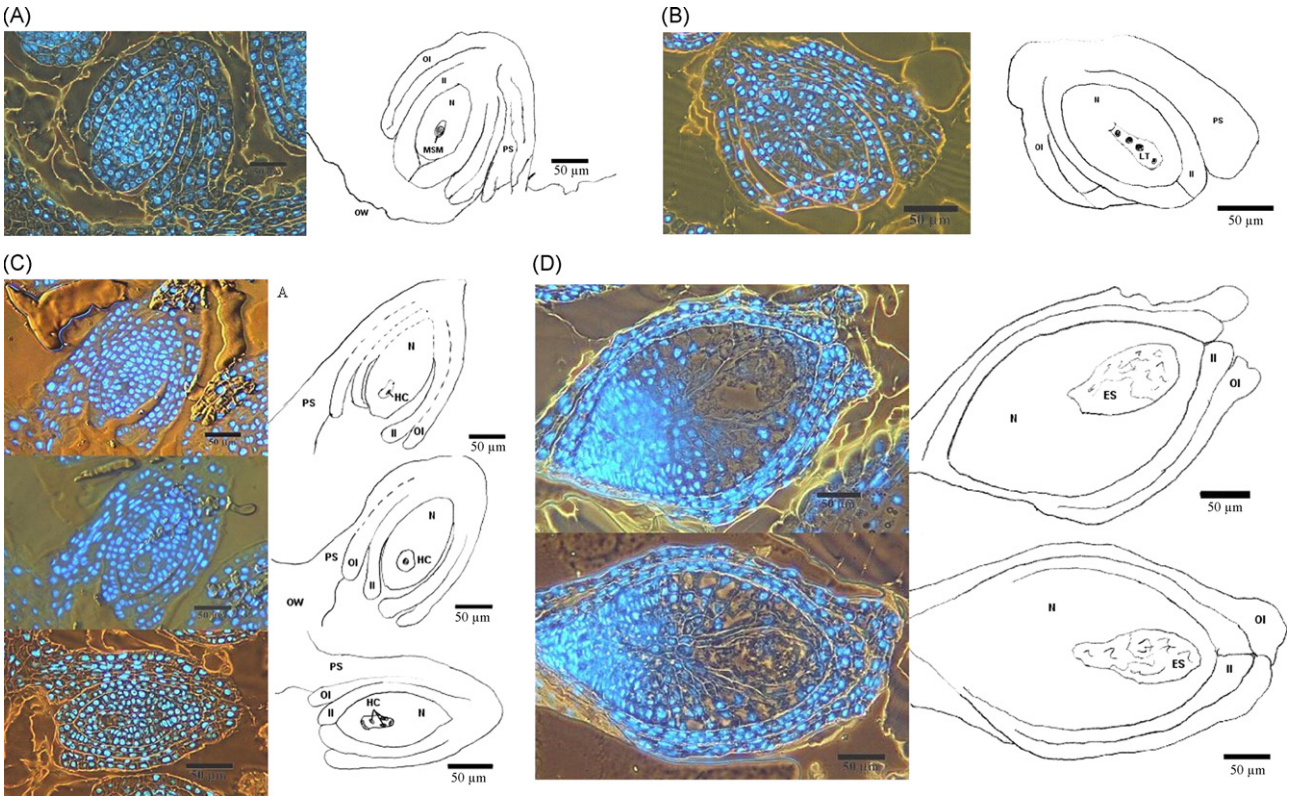

Fig. 3. *Halophila johnsonii*. (A) Premeiotic ovule containing a megaspore mother cell. MSM: megaspore mother cell; N: nucellus; II: inner integument; OI: outer integument; PS: placental stalk; OW: ovary wall. (B) Early postmeiotic ovule with a linear tetrad. LT: linear tetrad; N: nucellus; II: inner integument; OI: outer integument; PS: placental stalk. (C) Serial sections of a postmeiotic *Halophila johnsonii* ovule with a developed megagametophyte. (i) A section including mostly nucellus but with a single haploid nucleus of the megagametophyte visible; (ii) haploid synergid cell visible near the micropyle end; (iii) two polar nuclei shown in the middle of the megagametophyte, a synergid cell near the micropyle end, and one or two antipodal cells in the V of the chalazal pole. HC: haploid cell; N: nucellus; II: inner integument; OI: outer integument; PS: placental stalk; OW: ovary wall. (D) Later-stage senescent ovules with intact megagametophytes that have died as shown by the degradation of the DNA (wispy). N: nucellus; II: inner integument; OI: outer integument.

seeds; (2) it is a species within the same genus as *H. johnsonii*, and (3) it was the subject of a previous embryological study (Lakshmanan and Poornima, 1991) that provided a reference to verify the observations made in this study. One observation, different from those of Lakshmanan and Poornima (1991), was the presence of binucleate pollen in contrast to their report of trinucleate pollen in *H. decipiens*. This discrepancy was probably because the pollen examined in our study had gone through meiosis and one mitotic division but may have not fully matured to contain one tube nucleus and two sperm nuclei.

All stages of *H. decipiens* pistil development, from megagametogenesis to embryo development, were observed using the described staining and embedding techniques. Micrographs of *H. johnsonii* ovules showed developmental structures similar to those of *H. decipiens*, up to later stage postmeiotic ovules (Fig. 3A–C). Stages of megagametophyte development in *Halophila ovata* (Lakshmanan, 1963), a species more closely related to *H. johnsonii* than *H. decipiens*, also resemble those seen in *H. johnsonii*. The observations of parietal placentation and the presence of two bistromatic integuments surrounding a layer of nucellus tissue that covers the megagametophyte strongly suggest that normal megagametophyte development and the production of a haploid egg cell occurs within the ovules of *H. johnsonii*. Consequently, we conclude that *H. johnsonii*'s female gametophytes are structurally viable and we suggest that sexual reproduction should occur in the presence of pollen.

The presence of a linear tetrad and megagametophyte development in *H. johnsonii* precludes Eiseman and McMillan's (1980) suggestion of apomixis as the means by which this species reproduces. Apomicts usually do not undergo meiosis, and in those species that do, diploid dyads are formed due to asynapsis, endoreduplication or endomitosis (Asker and Jerling, 1992). Therefore, no haploid cells are formed in apomicts, and fertilization of the egg cell cannot occur. Apomixis may still result in structures resembling a female gametophyte (Fig. 3C). However, postmeiotic linear tetrad development will not be observed (Fig. 3B). The central cell may or may not be fertilized by a sperm cell in apomicts (Koltunow, 1993; Bicknell and Koltunow, 2004). In this study, older ovules (Fig. 3D) were observed in the process of dying as would be expected in unfertilized gametophytes (Sun et al., 2004). If *H. johnsonii* was an apomict, the ovules should resemble the older *H. decipiens* ovules (Fig. 2D). The absence of seedlings and seed banks provide further corroborative evidence that *H. johnsonii* does not reproduce by apomixis. Therefore, in view of the apparent absence of staminate flowers, the most plausible mode of reproduction available to *H. johnsonii* is vegetative fragmentation (Kenworthy, 1997; Hall et al., 2006). We conclude that *H. johnsoni*'s female gametophytes are structurally viable and that sexual reproduction could occur in the presence of pollen.

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