Megagametogenesis and Nuclear DNA Content Estimation in *Halophila* (Hydrocaritaceae)

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ABSTRACT

Megagametogenesis has been identified in the seagrass *Halophila johnsonii*, a species with no known seeds, using DAPI staining and manual sectioning methods. Developmental stages were documented and compared with megagametogenesis stages in *Halophila decipiens*, a related species widely reported to produce viable seeds. Present observations suggest that meiosis occurs in the megasporocyte and, therefore, sexual reproduction should be possible in *H. johnsonii*. Results of this study, which was part of a multi-level threatened-species recovery plan sponsored by NOAA, are encouraging and suggest that two additional management-related inquiries, 1) the search for male reproductive structures and 2) hybridization with related species are worthwhile. Quantification of the nuclear DNA of nine *Halophila* taxa was conducted to better understand interspecific genome size variation within the genus. Results suggest that three polyploidy events may have accompanied evolution in this genus. Data for 2C and 4C values are reported with *H. engelmanii* having the largest content of 2C = 27 pg and *H. stipulacea* having the lowest, 2C = 7.5 pg.

ACKNOWLEDGEMENTS

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CHAPTER I GAMETOGENESIS

INTRODUCTION

The seagrass genus *Halophila* (Thouars) is pantropical with 13 species presently recognized (den Hartog, 1970; Phillips and Menez, 1988; Larkum, 1995). *Halophila* species grow on a variety of substrates and are often the first to colonize newly-available sediments. They can withstand large variations in temperature (eurythermal) and are distributed from tropical to warm-temperate waters. This genus has a wide depth distribution as well, with plants growing from the intertidal to 85m (den Hartog, 1970; Phillips and Menez, 1988; Kuo et al., 2001). Ecological benefits (Josselyn et al., 1986; Dawes et al., 1989; Preen et al., 1995; Nakaoka and Aioi, 1999; Kenworthy, 2000), physiological characteristics (Hemminga et al., 1999; Ralph, 1999; Durako et al., 2003) and reproduction of *Halophila* species (Pettitt, 1981; Johnson and Williams, 1982; Herbert, 1986; McMillan and Jewett-Smith, 1988; Kuo and Kirkman, 1992; Zakaria et al., 1999) have been widely reported.

Three *Halophila* species are reported from the intertidal and coastal waters of south Florida: *Halophila decipiens* Ostenfeld, *Halophila engelmanii* Ascherson, and *Halophila johnsonii* Eiseman. *Halophila decipiens* appears to be the only pantropical species in the genus, inhabiting both oceanic waters and subtidal waters of coastal lagoons and estuaries. It is reported to be monoecious, with perfect flowers (McMillan and Soong, 1989). *Halophila engelmanii*, which is dioecious , occurs in shallow depths with larger seagrasses such as *Thalassia testudinum* Banks ex König and *Halodule wrightii* Ascherson. *Halophila johnsonii* is endemic within the lagoonal system of southeast Florida from Sebastian Inlet (approximate lat. 27°50'N) to Virginia Key (approximate lat. 25°45'N) (Eiseman and McMillan, 1980), giving it the smallest reported distribution of any seagrass species. *Halophila johnsonii* has been found in disjunct and

patchily distributed populations and occurs from the intertidal to 3m depths. Both *H. decipiens* and *H. johnsonii* also occur in subtidal mixed meadows (Kenworthy, 1997).

The mechanism of reproduction in *Halophila johnsonii* is of special interest. Sexual reproduction has been documented in all other *Halophila* species (Lakshmanan, 1961; McMillan, 1976; Johnson et al., 1982; Herbert, 1986; Lakshmanan and Poornima, 1991; Kuo and Kirkman, 1992; Larkum, 1995; Zakaria et al., 1999), but it has never been observed in *H. johnsonii.* *Halophila johnsonii* reproduction is thought to be dioecious, because observed plants produce only pistillate (imperfect female) flowers. Neither staminate (male) flowers nor seeds have been found (Kenworthy, 1997).

Limited geographic range and unknown mode of sexual reproduction are two reasons *H. johnsonii* was the first marine plant listed as threatened on the United States threatened and endangered species list (FR28392, 1999). As a result, a species-recovery team was convened to develop and implement an eight step recovery plan for the National Oceanic and Atmospheric Administration (Recovery Plan for *H. johnsonii,* 2002). The main objective of this study was to investigate the life history of *Halophila johnsonii* (priority 4). Since neither *H. johnsonii* staminate (male) flowers nor seeds have been found; only the presence of pistillate (female) flowers gives any indication of the potential for sexual reproduction in this species. and the ability of the pistillate flowers to produce haploid egg cells remains unknown.

In normal pistil development, ovules are defined as the sporophyte tissue, which produces and retains the female gametophyte (megagametophyte). An immature ovule contains a single megasporocyte, which goes through a reduction-division sequence (megasporogenesis) to produce a megagametophyte (Fig. 1). The first division is meiotic, and results in the megasporocyte developing into four haploid megaspores. Only one of these spores is functional,

while the other three are abortive. The next three divisional stages are mitotic, the nucleus divides but the cell does not go though cytokinesis. After the first mitotic division the cell is now a premature megagametophyte. The end product is a cell with eight haploid nuclei. In a mature megagametophyte the eight nuclei differentiate into seven cells: a central cell with two nuclei, three antipodal cells, two synergid cells, and an egg cell between them. Meiosis is required to generate a functional haploid gametophyte, but whether or not this occurs in *Halophila johnsonii* is unknown.

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, was used to observe gametophyte developmental stages of both *H. johnsonii* and *H. decipiens*. *Halophila decipiens* was used as a reference in this study because megasporogenesis has been described in this species (Lakshmanan and Poornima, 1991). Identification of a linear tetrad in *H. johnsonii* (Fig. 1B) would indicate that meiosis has occurred and that the ovules could be functional up to the megaspore formation stage of sexual development.

MATERIALS AND METHODS

Plant collection

Specimens of *H. johnsonii* and *H. decipiens* flowers were collected in the field from three locations within the range of *H. johnsonii* (Table 1). Samples were collected with two techniques: 1) direct collection by gently up-rooting portions of ramets with flowers, and 2) culture of plant plugs containing sediments collected from the field site using a 100 cm^2 sod-plugger. These plug samples were placed into 100 cm^2 peat pots and transferred to the UNCW

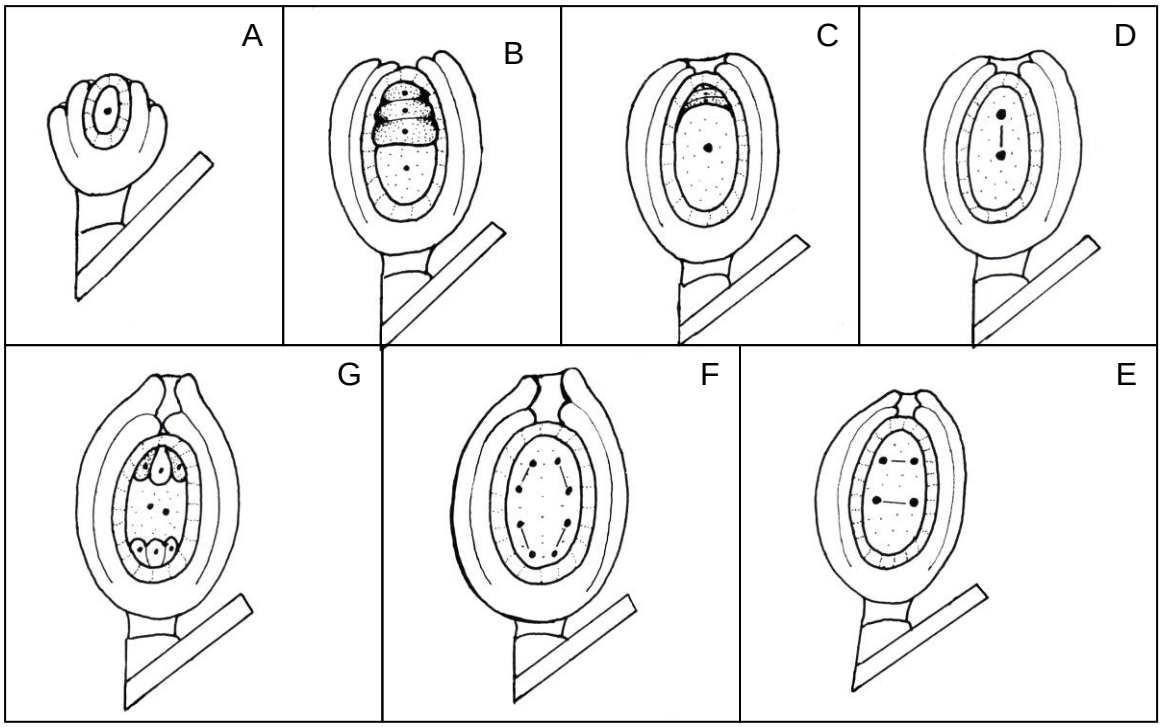

Figure 1. Megasporogenesis. A) A single megasporocyte cell surrounded by integument tissue. B) Megasporocyte that has gone through meiosis resulting in a linear tetrad of megaspores. C) Functional megaspore is enlarged while three abortive megaspores dissolve. D, E, and F) Three mitotic divisional stages. G) Eight nuclei in seven cells; a mature megagametophyte.

Table 1
Locations of collection sites for *H. decipiens* and *H. johnsonii* in Florida USA.

Location	Latitude	Longitude	*H. decipiens*	*H. johnsonii*
Sebastian Inlet	27°51.37'N	80°27.04'W	No collection	collected
Jupiter Inlet	26°57.68'N	80°04.76'W	collected	collected
Haulover park	25°55.22'N	80°07.54'W	collected	collected

Center for Marine Science greenhouse under the conditions of 16:8 L:D, 24°C, and 32ppt to induce flowering (McMillan, 1976).

Fixation and Embedding

The up-rooted samples were placed directly into 3:1 95% ETOH : Glacial Acetic Acid, for 24hrs., then placed into 70% ETOH for storage (see Appendix A for outline of sampling protocols). 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 hour, 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 hrs.) 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) for approximately 2hrs to allow resin to harden. Hardened resin blocks were stored in a drying oven at 37°C until they were sectioned.

Sectioning and DAPI staining

Resin blocks were trimmed and 10µm 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). The consecutive sections were placed, one at a time, on Fisherbrand frosted microscope slides. The sections were flattened with a dilute solution of ammonium hydroxide and dried at room temperature.

Analysis

Prepared slides were examined using an Olympus BH2-RFK fluorescence microscope equipped with a high-pressure mercury vapor lamp (HBO, 100W), and a 420-nm suppression

filter, which is specific for DAPI-bound emissions. Digital images of sectioned ovules that were determined to be in the correct orientation and intact were captured using Spot RTke or Spot RTcolor digital cameras and Spot Advanced imaging software. Scaling and structural dimensions were obtained using a digital image of 2 mm slide micrometer.

RESULTS

Fifty flowers of each species were embedded in resin, however only twenty flowers of each species were intact and oriented correctly. Soft resin, sectioning problems, and improper orientation caused thirty samples to be non-useful. A total of 3600 sections of each species were visually scanned. 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 considered to be: premeiotic (*Pr*) (Fig. 1A) gametogenic/postmeiotic (*Pm*) (Fig. 1B-G) and senescent or post fertilization (*Pf*). In addition to exhibiting common stages of development, ovules of both species exhibited parietal placentation and were composed of two bistromatic integuments surrounding a layer of nucellus tissue that covers the embryo sac, in both species.

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. 2). The *Pr* ovaries of *H. decipiens* measured 0.25 mm wide. They contained 20 to 30 ovules, measuring 75 µm in diameter. The ovaries were considered *Pr* primarily because the ovules contained a single megaspore mother cell (Fig.3). The *Pm* ovaries measured 1 mm wide, with the ovules 100 µm in diameter. The ovules contained immature megagametophytes, which consisted of a developing embryo sac containing a linear tetrad with four nuclei within (Fig. 4). Later-stage *Pm* ovaries

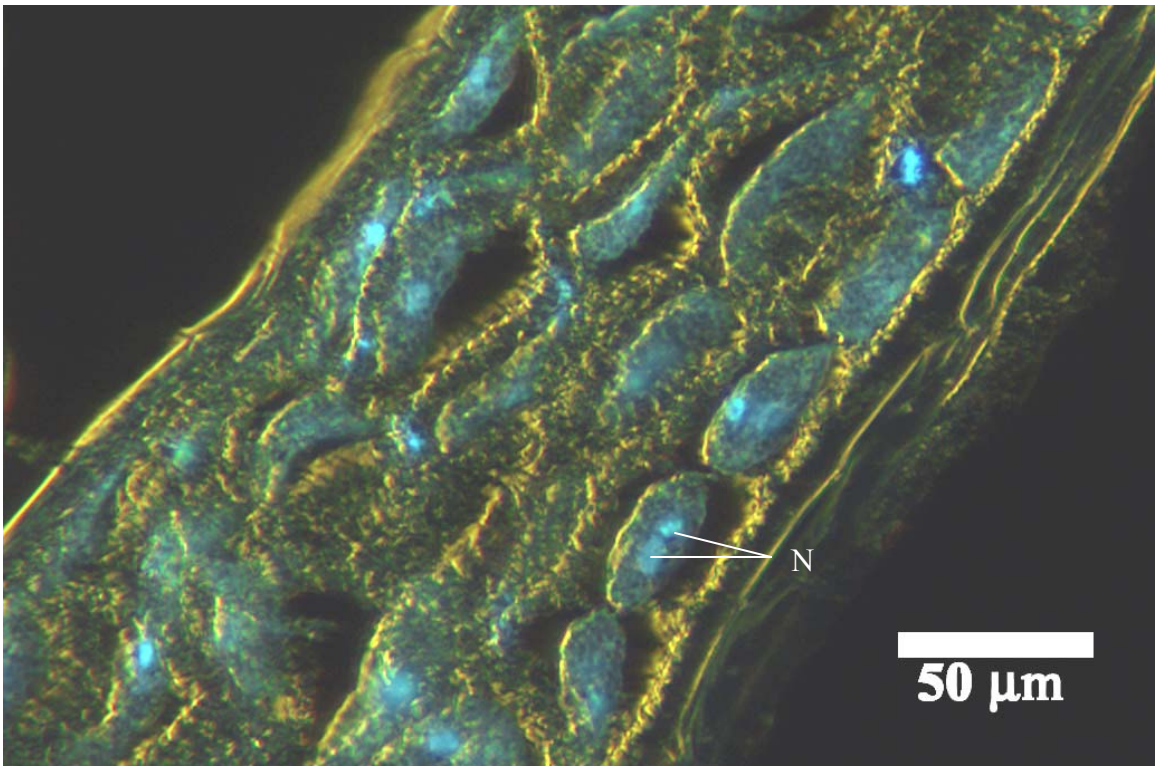

Figure 2. Binucleate pollen cells within a mucilage tube of *Halophila decipiens* stamen; N: nuclei.

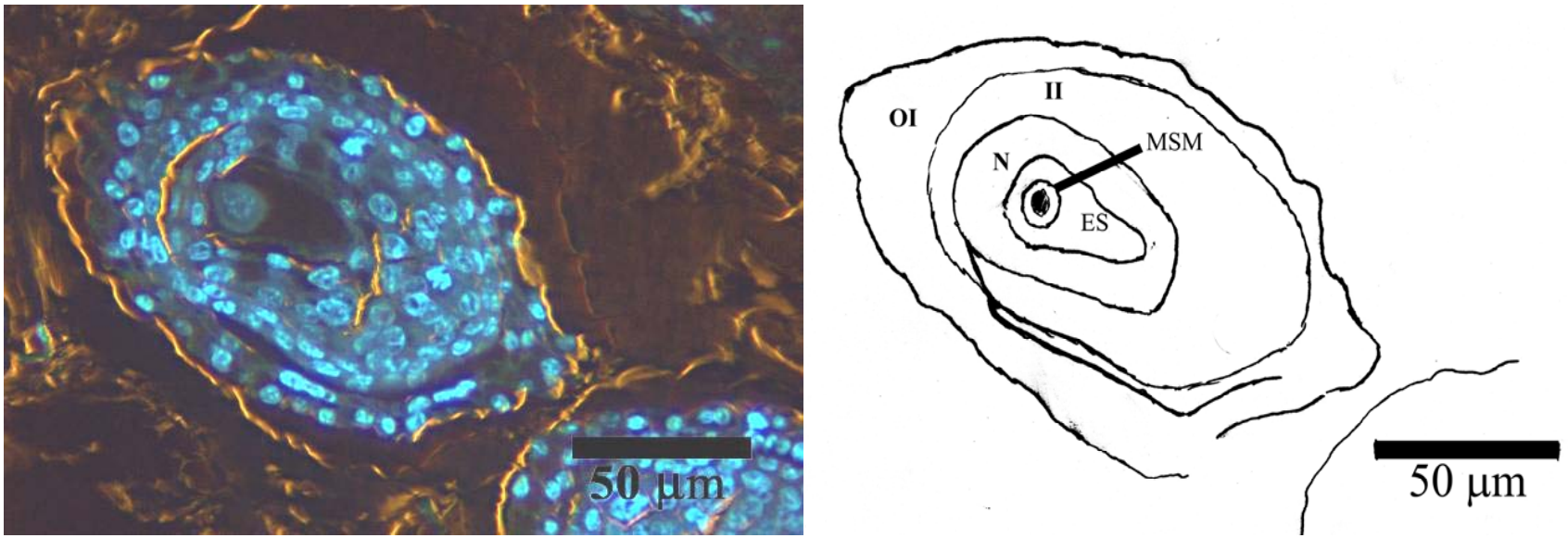

Figure 3. Young ovule of *Halophila decipiens* containing a meiospore. MSM: megaspore mother cell; ES: embryo sac; N: nucellus; II: inner integument; OI: outer integument

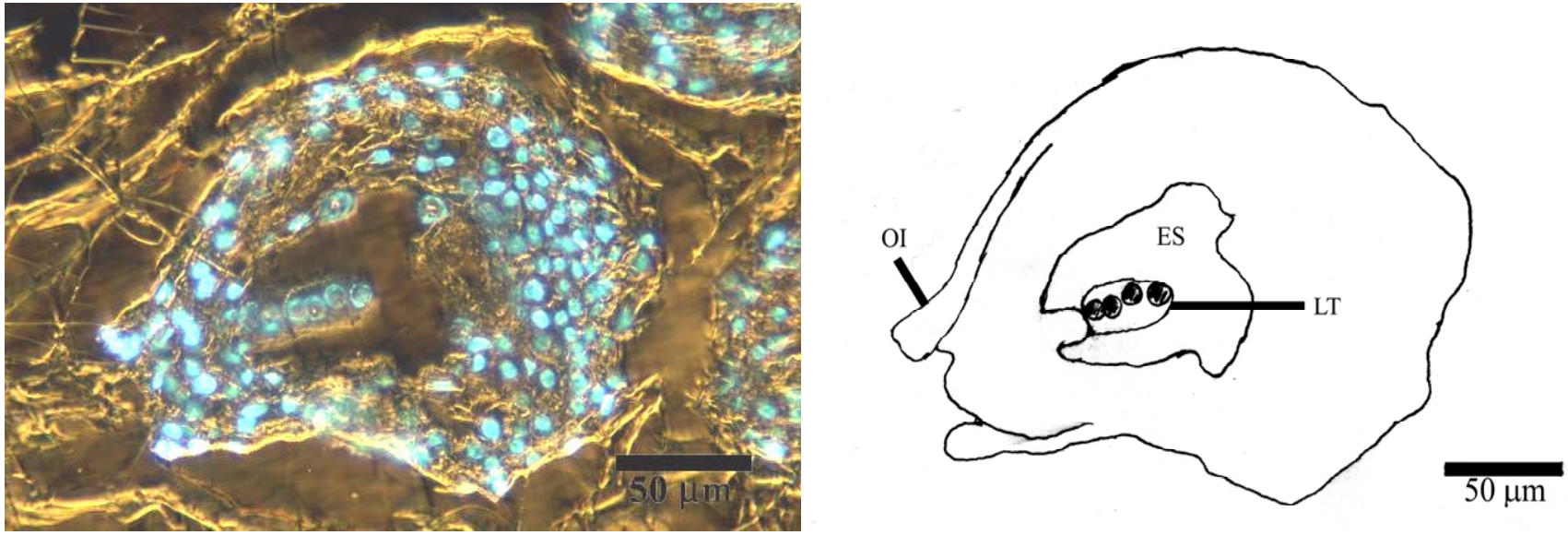

Figure 4. A linear tetrad of *Halophila decipiens*. LT: linear tetrad; ES: embryo sac; OI: outer integument

contained immature-to-mature ovules with megagametophytes containing two-to-eight nuclei (Fig. 5). The *Pf* ovaries had ovules that, for the majority, had been fertilized. The ovaries measured 2-3 mm wide. The fertilized ovules were 150 µm in diameter. The ovules contained embryos at various stages of development (Fig. 6). Previous embryology work on *H. decipiens*, showed similar results (Lakshmanan and Poornima 1991).

Halophila johnsonii

The *Pr* ovaries of *H. johnsonii* were 0.5 mm wide. The ovaries contained ten-to-fifteen ovules, which measured 150 µm in diameter, and had single megaspore mother cells inside (Fig. 7). The *Pm* ovaries measured 1.5 mm wide, with ovules of 150 µm in diameter. Ovules of the first stage of *Pm* are characterized by the presence of a linear tetrad (Fig. 8). The later-stage *Pm* ovules in *H. johnsonii* contain megagametophytes with two to eight nuclei, similar to the later *Pm* stages found in *H. decipiens*. The two species differ in that *H. decipiens* exhibits a large, spacious embryo sac, which is absent in *H. johnsonii* (Figs. 5 and 9). The *Pf* ovaries had ovules, which had completed or were in the process of senescence. The ovaries measured 2 mm wide. The ovules were 250 µm in diameter but showed sign of evacuation or death of inner gametophytic tissue, (Fig. 10).

DISCUSSION

Halophila decipiens was used as a reference in this study for three reasons: 1) it is known to have functional flowers that produce viable seeds; 2) it is a species within the same genus as *Halophila johnsonii*; 3) it co-occurs with *H. johnsonii* in the field, and therefore was an obtainable source. Additionally, a previous embryological study of *H. decipiens* (Lakshmanan and Poornima, 1991) provided a source to verify the observations made in this study. One observation here that differs from those of Lakshmanan and Poornima (1991) is the presence of

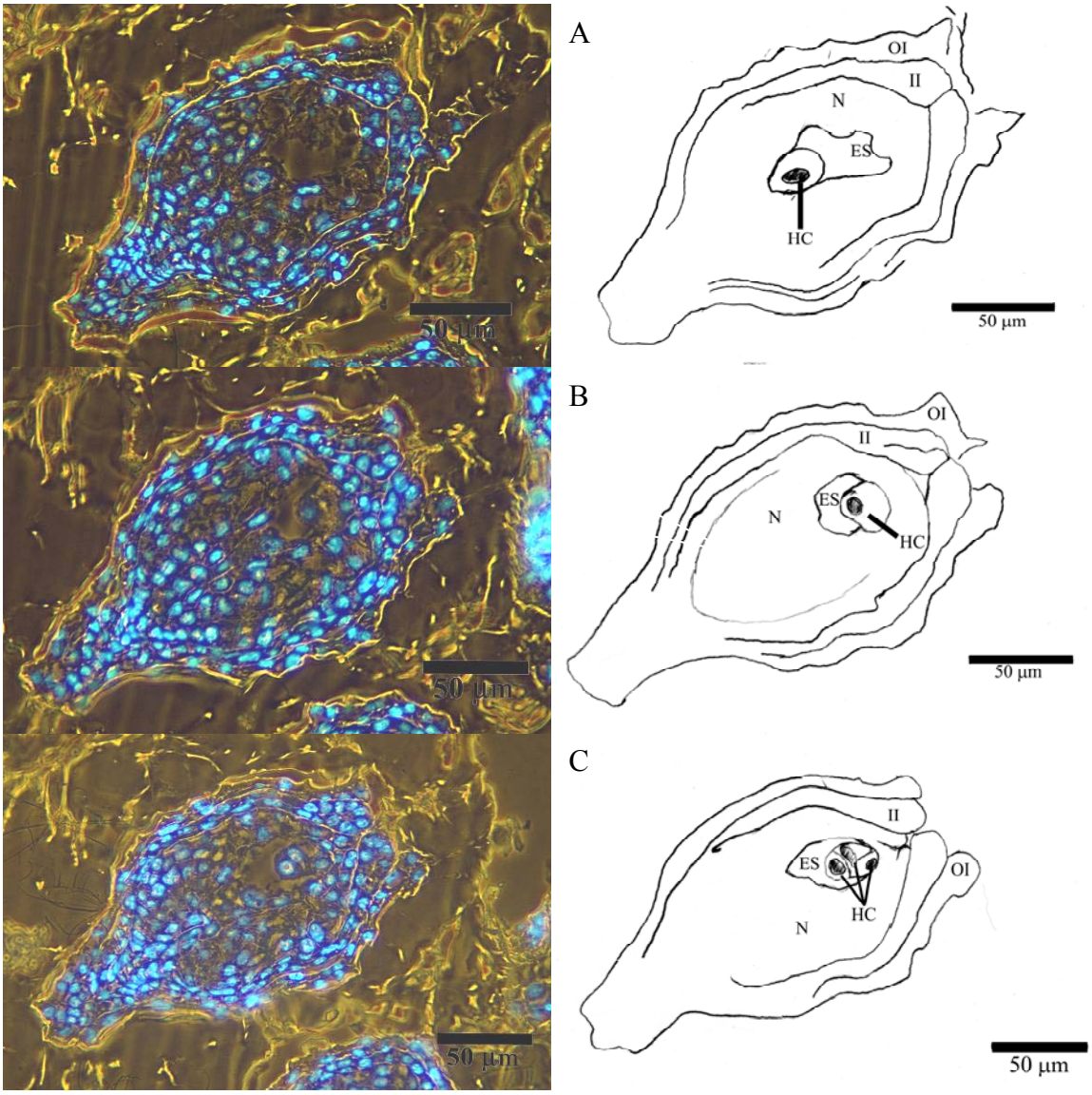

Figure 5. Three serial sections of a *Halophila decipiens* ovule with a developed gametophyte. A) one of the antipodal cells is near the chalazel pole; B) a cell near the micropyle and another antipodal cell near the chalazel pole; C) three cells, the antipodal from B, and two near the micropyle. HC: haploid cell; ES: embryo sac; N: nucellus; II: inner integument; OI: outer integument

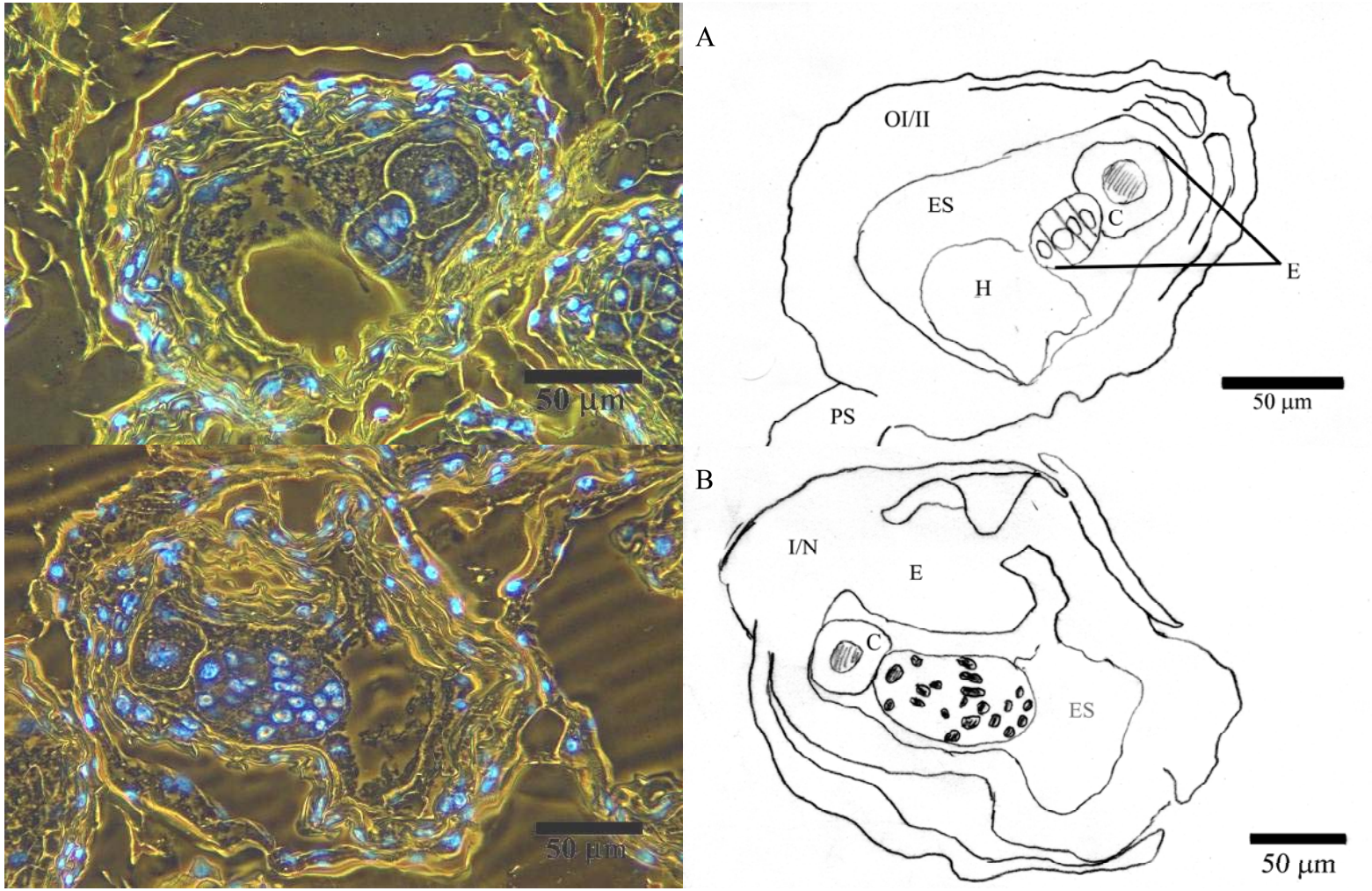

Figure 6. Post fertilized *Halophila decipiens*. A) A five-celled proembryo with the suspensor cell at the micropyle; B) A globular embryo with suspensor cell. E: embryo; C: basal cell; H: hole in section; ES: embryo sac; N: nucellus; II: inner integument; OI: outer integument; PS: placental stalk

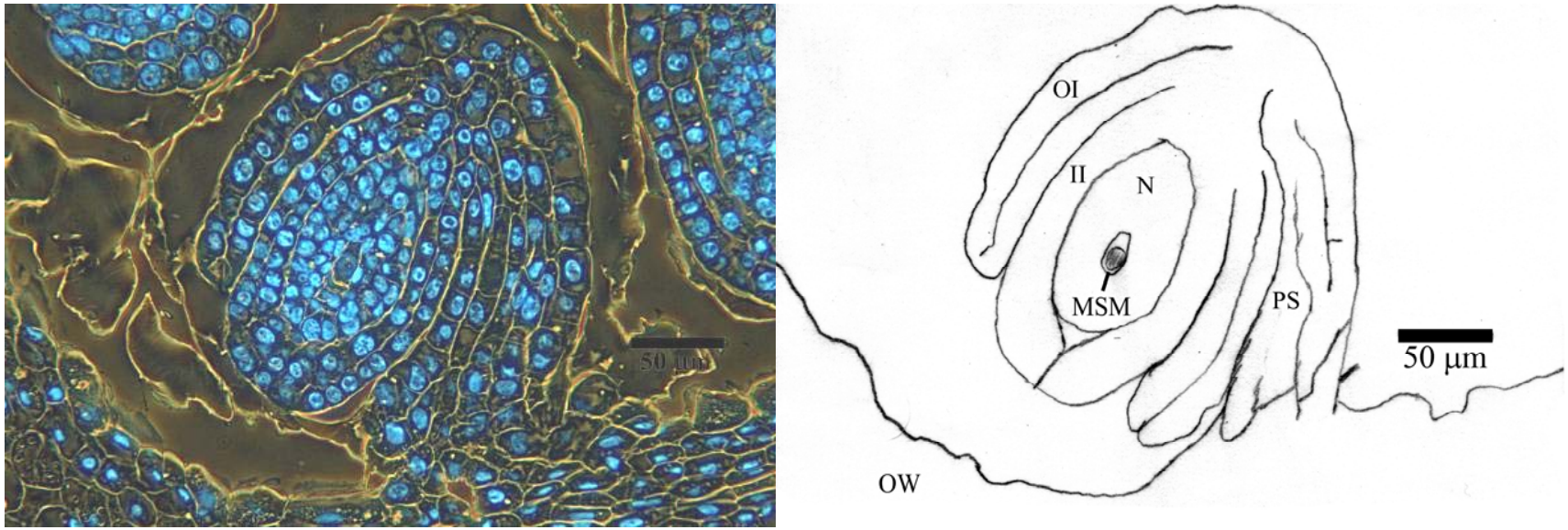

Figure 7. An ovule containing a *Halophila johnsonii* meiospore. MSM: megaspore mother cell; N: nucellus; II: inner integument; OI: outer integument; PS: placetal stalk; OW: ovary wall.

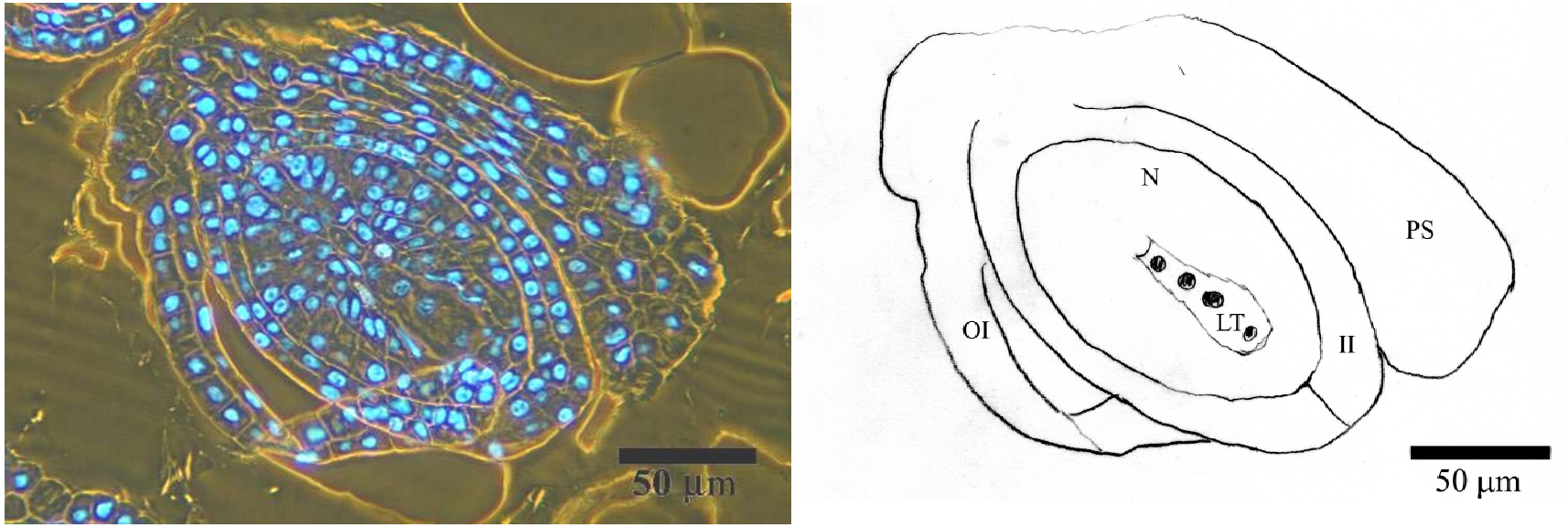

Figure 8. Linear tetrad of *Halophila johnsonii*. LT: linear tetrad; N: nucellus; II: inner integument; OI: outer integument; PS: placental stalk.

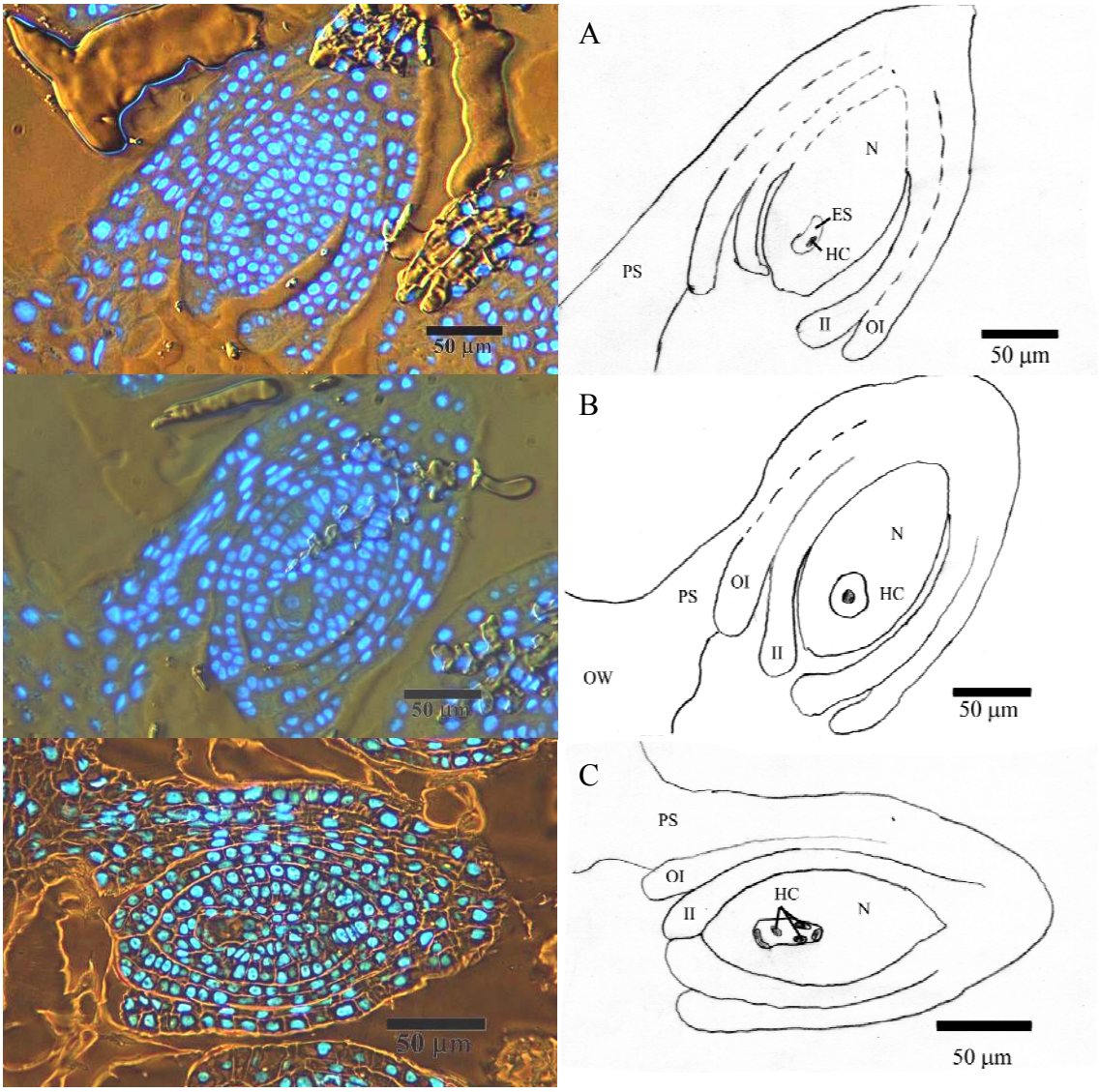

Figure 9. Serial sections of a developed *Halophila johnsonii* megagametophyte. A) embryo sac just opening up by the section, barely visible nucleus in the middle of opening; B) snyergid cell visible near the micropyle; C) two polar nuclei shown in the middle of embryo sac, a synergid cell near the micropyle, and one or two antipodal cells in the V of the chalazal. ES: embryo sac; HC: haploid cell; N: nucellus; II: inner integument; OI: outer integument; PS: placental stalk; OW: ovary wall.

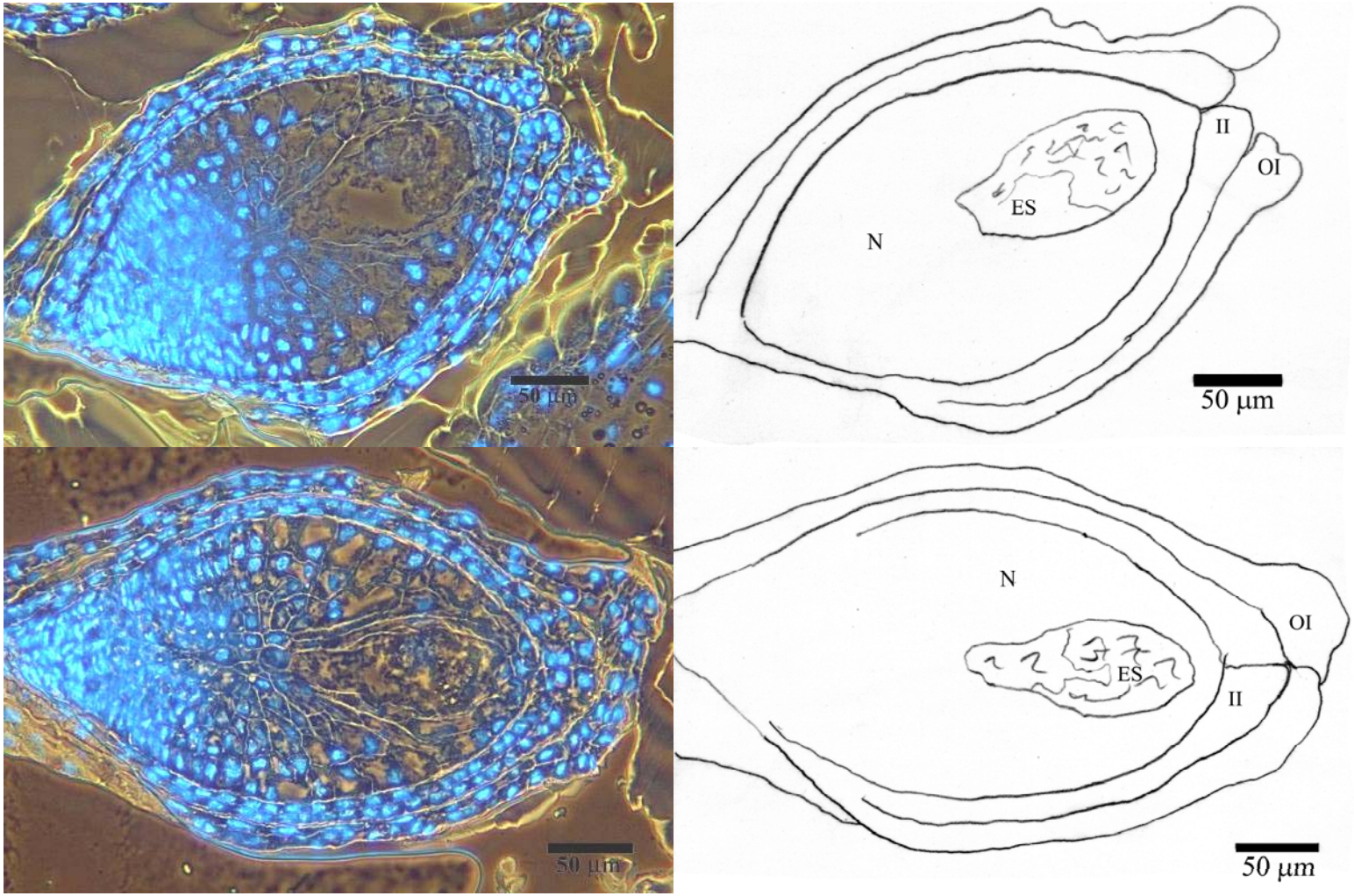

Figure 10. *Halophila johnsonii* micrographs showing two senescent ovules, with clear embryo sac intact, but the megagametophyte have died as shown by the degradation of the DNA (wispy). ES: embryo sac; N: nucellus; II: inner integument; OI: outer integument.

binucleate pollen. Their study observed trinucleate pollen in *H. decipiens*. An explanation for this discrepancy is that the pollen examined here had gone through meiosis and one mitotic division but had not fully matured to contain one tube nucleus and two sperm nuclei.

Halophila decipiens pollen was used as a control to verify that staining of haploid nuclei within a cellular structure could be observed (Fig. 2). The staining and embedding technique allowed for all stages of pistil development from megagametogenesis to embryo development to be observed in *H. decipiens*. Micrographs of *H. johnsonii* ovules showed developmental structures similar to those of *H. decipiens*, up to later stages of the *Pm* ovules (Fig. 7-9). 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*. These observations strongly suggest that normal megagametophyte development and the production of a haploid egg cell occurs within the ovules of *H. johnsonii.*

The presence of meiosis and megagametophyte development in *H. johnsonii* precludes apomixis as the means by which this species reproduces. Apomicts do not go through meiosis. Therefore, no haploid cells are formed and fertilization of the egg cell cannot occur. Apomixis may still result in structures resembling a female gametophyte (Fig. 9). However, post-meiotic linear tetrad development will not be observed (Fig. 8). In apomicts, the central cell may or may not be fertilized by a sperm cell (Koltunow, 1993; Bicknell and Koltunow, 2004). In this study, older ovules (Fig. 10) were observed in the process of dying as would be expected in unfertilized gametophytes (Erdelská and Ovečka, 2004). If *H. johnsonii* is an apomict, the ovules should resemble the older *H. decipiens* ovules (Fig. 6). The observation of a structural linear tetrad (Fig. 8) does not support a previous report of apomixis in *H. johnsonii* (Eiseman and McMillan ,1980).

Finally, absence of seedlings and seed banks provide further corroborative evidence that *H. johnsonii* does not reproduce by apomixis. Therefore, the most plausible mode of reproduction available to *H. johnsonii* is vegetative fragmentation (Kenworthy (1997). Consequently, we conclude that *H. johnsonii*'s female gametophytes are structurally viable and that sexual reproduction could occur in the presence of pollen.

CHAPTER II Nuclear DNA Quantification in *Halophila*

INTRODUCTION

Halophila johnsonii female flowers undergo megagametogenesis and, based on structural evidence, should produce viable haploid egg cells (Chapter 1). However, actual ploidy level and DNA content of the egg cell is unknown. In the present study, nuclear DNA contents of sporophytic tissue (which should be diploid = 2N = 2C/4C) were compared to the nuclear DNA contents of gametophytic tissue (which should be haploid = 1N = 1C/2C). Identification of haploid egg cells would raise the possibility of attempting pollination using pollen donors from closely-related species within the genus. Consequently, nuclear DNA contents were determined to screen for potential genome compatibly based on genome size equivalency

Nuclear DNA content studies of angiosperms are best appreciated in the larger context of our emerging understanding of the role of the nucleotype on phenotypic expression (Baetcke et al., 1967; Bennett et al., 1998; Van't Hof, 1999; Bennett et al., 2000). Specifically, an up to 200,000-fold variation in nuclear DNA content (C-value) has been reported in eukaryotes (Gregory, 2001). Although little correlation generally exists between nuclear genome size and an organism's complexity (the C-value paradox) (Thomas, 1971)), there is substantial evidence that the nucleotype affects the phenotype in a non-genic manner in response to environmental demands (Cavalier-Smith, 1985; Ohri and Khoshoo, 1986). In both plants and animals (Price, 1988) genome size and cell size extend their influence to ecological selection types. Larger genome size is associated with k-selection that favors slower development, delayed reproduction and larger body size. Smaller genome size is associated with r-selection that favors rapid development, high population growth rate, early reproduction and small body size (Begon *et al*., 1990).

C-values are increasingly useful in a phylogenetic context. Much research has looked for evolutionary trends in DNA amount at the species, genus and family level, but an understanding of these trends has been hampered by the lack of rigorous phylogenetic frameworks (Bennett et al., 2000). The development of a phylogeny for *Halophila* species and the determination of their DNA C-values would permit superimposing the former on the latter as has been reported for other angiosperms (Leitch et al., 1998).

Genetic data have been used previously to better understand population dynamics (Procaccini et al., 1999) and to aid in the identification of species (Jewett-Smith, 1997; McDermid et al. 2002) in *Halophila*. Phylogenetic trees yield more useful information on evolutionary relationships, which is needed to detect any trends related to DNA content. Recently, a phylogenetic tree based on analyses of DNA sequences of the nuclear-encoded internal transcribed rRNA spacers (ITS) was published for *Halophila* species (Waycott et al., 2002). The relationships of early diverging *Halophila* lineages were clearly shown in the ITS tree, but relationships among more recently evolved species, including *H. johnsonii*, *H. hawaiiana*, and *H. ovalis,* were not resolved. Additional sequence data from the chloroplast-encoded intergenic spacers and intron of the *trnL* locus (*trnL*) have been generated to further resolve the evolutionary relationships among *Halophila* species (Freshwater, York, and Melton, unpublished).

The objectives of the present investigation were to 1) identify haploid and diploid nuclei in reproductive and vegetative tissue, respectively, 2) determine the nuclear DNA contents and nuclear areas of eight species (nine taxa) within the genus *Halophila*, and 3) superimpose these values on the *trnL* based phylogenetic tree to better understand how nuclear DNA contents may have evolved within the genus. These objectives relate to the goals of understanding the life

history and genetic diversity within the genus *Halophila*, priorities 4 and 6 of the list of recommended actions to promote the recovery of *H. johnsonii*.

MATERIALS AND METHODS

Plant collection

Halophila johnsonii and *H. decipiens* flowers were collected in the field from three locations within the *H. johnsonii* range (Table 1). Samples were collected by gently up-rooting portions of ramets with flowers by hand. In addition, plant plugs containing sediments were collected from the field site using a 100 cm^2 sod-plugger and placed into 100 cm^2 peat pots and cultured in the Center for Marine Science greenhouse under the conditions of 16:8 L:D, 24^oC, and 32ppt to induce flowering (McMillan, 1976). Other species were collected from various locations (Table 2) and quick dried with silica gel desiccant to stop any DNA degradation.

Fixation and staining preparation

The up rooted samples were placed directly into 3:1 95% ETOH : Glacial Acetic Acid, for 24 h, then placed into 70% ETOH for storage (see Appendix A for outline of sampling protocols). Developing flowers from cultured samples were snipped off and fixed as above. Vegetative tissue (meristems and young leaves) from dried samples were rehydrated in dH_2O for 20 min and then fixed as above. Samples were then placed into dH_2O for 20 min and further dissected into vegetative tissue (all taxa), male flowers (*H. decipiens* anthers), and female flowers (*H. decipiens*, *H. johnsonii,* and *H. stipulacea*). Female flowers were further dissected to remove ovules from the ovaries. Tissue was then placed into 5% EDTA for 24 h to soften the tissue and allow the stain to infiltrate the cells.

DAPI staining

A subbing solution was put on the cover slips and allowed to dry. *Halophila* tissue to be stained were placed on the cover slips and allowed to air dry. DAPI stain (0.5ug/mL 4'–6'

Table 2
Locations of *Halophila* species collections

Species	Abbreviations	Collection site	Collector
H. engelmannii	He	Florida USA	D. W. Freshwater
H. spinulosa	Hsp	Malaysia	S. M. Phang
H. capricorni	Hc	New Caledonia	D. W. Freshwater
H. stipulacea	Hst	Jordan	O. De Clerck
H. ovalis	Ho BB	Botany Bay AUS	D. W. Freshwater
H. hawaiiana	Hh	O'ahu USA	D. W. Freshwater
H. ovalis	Ho R	Rottnest Is. AUS	D. W. Freshwater
H. johnsonii	Hj	Florida USA	York / Freshwater
H. decipiens	Hd	Florida USA	York / Freshwater

diamidodino-2-phenylindole)(Sigma Chemical Co., St. Louis, MO 63178) was applied to the tissues for 30-40 minutes in the dark at room temperature as previously described (Goff and Coleman, 1990). After staining, excess DAPI was removed, the cover slips mounted on slides, sealed with nail polish and then stored at 4^{o}C for 24hrs before examination. Detailed procedures for microspectrophotometry with DAPI and requirements for reproducible staining have been specified previously (Kapraun and Nguyen, 1994; Kapraun, 2005). Microspectrophotometric data for *Gallus* (chicken erythrocytes or RBC) with a DNA content of 2.4 pg (Clowes *et al*., 1983) were used to quantify mean fluorescence intensity (I_f) values for plant specimens. DAPI binds by a non-intercalative mechanism to adenine and thymine rich regions of DNA which contain at least four A-T base pairs (Portugal and Waring, 1988). Consequently, RBC are best used as a standard for estimating amounts of DNA when the A-T contents of both standard and experimental DNA are equivalent (Coleman *et al*., 1981). *Gallus* has a nuclear DNA base composition of 42-43 mol % G + C (Marmur and Doty, 1962). *Halophila* specimens investigated in this study are assumed to have a similar range of base pair compositions (Sueoka, 1961), and linearity is accepted between DAPI-DNA binding in both RBC and plant samples.

Fluorescence Analysis

Cytophotometric measurements were made with an Olympus BH2-RFK fluorescence microscope equipped with a high-pressure mercury vapor lamp (HBO, 100W), and a 420-nm suppression filter, which are specific for DAPI-bound emissions. The DNA data obtained for the RBCs were used to quantify the mean fluorescence intensity (I_f) values for the plant tissues, as: mean I_f RBC / I_f plant tissue = 2.4pg / pg of plant DNA. The DNA content of cells from different tissues, (vegetative, pollen, and ovules), were compared within *H. decipiens*, *H. johnsonii*, and

H. stipulacea, to determine ploidy level of each tissue. Vegetative tissues of the additional 6 taxa were prepared as above to determine their DNA content.

Area Analyses

DAPI stained vegetative tissue from 9 species of *Halophila*, including *H. johnsonii* and *H. decipiens* were analyzed by fluorescence and through digital imaging analysis. Digital photographs were taken with an RTke spot digital camera. Approximately eight optically dissected images from each field of view were processed with Image-Pro Plus software to extend the depth of view. The nuclei in each image were masked in Photoshop 7.0 and counts along with area measurements were taken with Image-Pro Plus software. Area measurements were compared to the DNA content analyses to identify any association between nuclear size versus content. DNA contents and nuclear area were then mapped on a *trnL* sequence-based phylogenetic tree in order to detect evolutionary trends in *Halophila* nuclear DNA contents.

RESULTS

Fluorescence

Fluorescence (I_f) data for reproductive cells were obtained to determine the utility of the DAPI staining and fluorescence technique. Specifically, could DAPI be used 1)to distinguish between haploid and diploid cells, and 2) to identify meiotic cells in *H. johnsonii* pistillate structures. I_f values for both reproductive and vegetative tissue of *H. decipiens*, and *H. stipulacea*, and vegetative tissue of *H. johnsonii* were compared (Fig. 11). Differences were observed in calculated DNA contents (pg) between the sexual tissues, *H. decipiens* mature pollen cells and *H. stipulacea* ovule tissue, and the vegetative tissue of these two species. The *H. decipiens* pollen yielded a 2C DNA content value of approximately 5.5 pg while its vegetative tissue had an estimated 4C-value of 10 pg DNA. In *Halophila stipulacea* ovule tissue, nuclear

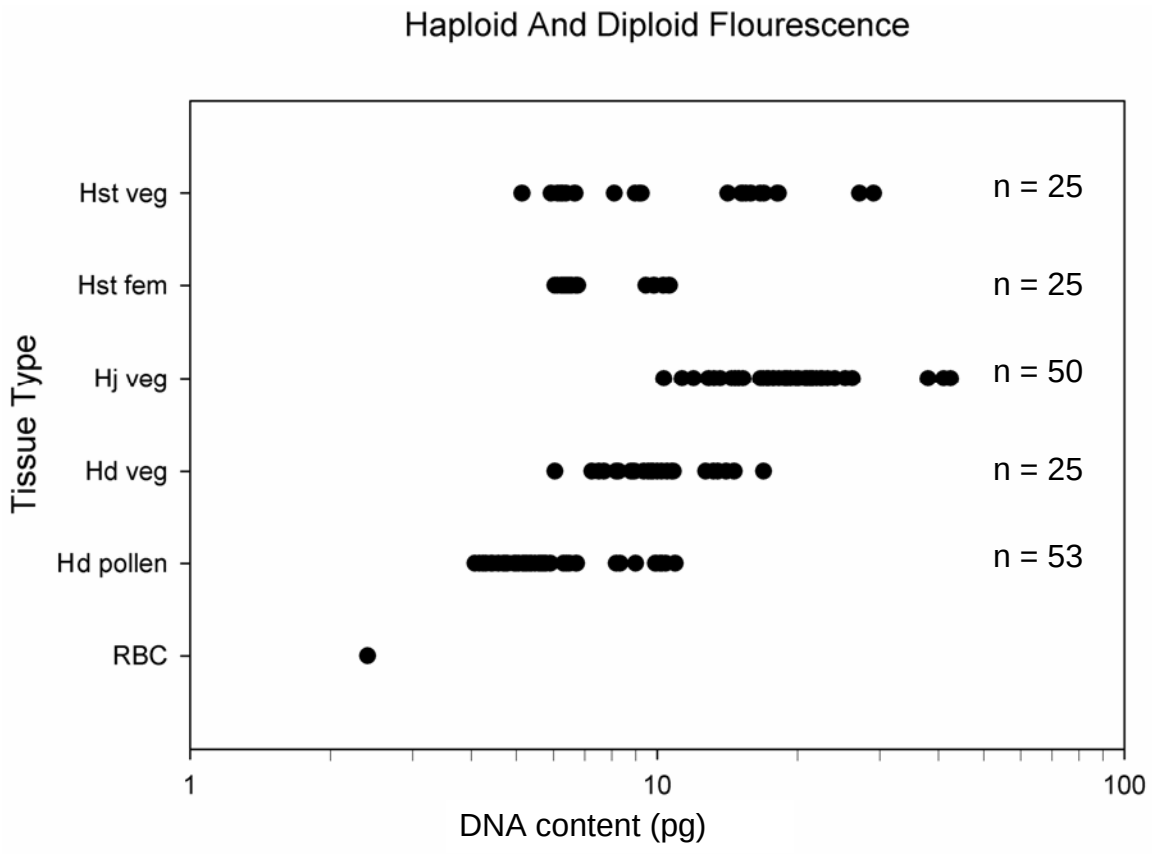

Figure 11. Nuclear DNA contents in picograms of haploid and diploid tissue *Halophila stipulacea* (Hst), *H. johnsonii* (Hj), and *H. decipiens* (Hd). n = number of nuclei measured

DNA content estimates yielded an average 2C of 7 pg DNA compared to a 4C of 16 pg DNA for the vegetative tissue. For *H. johnsonii* only nuclear DNA content estimates for vegetative tissue were plotted because no haploid female cells were identified using this technique.

Nuclear DNA content estimates based on I_f data for nine *Halophila* taxa are summarized in Table 3. These data have been submitted to the Royal Botanical Gardens, Kew Angiosperm DNA C-values database (http://www.rbgkew.org.uk/cval/database1.html) for electronic publication. Comparison of fluorescence (I_f) data from the vegetative tissue of 9 taxa indicated a large range of DNA contents for each species (Fig. 12). This can be explained in two ways. First, the amount of DNA varies from G1 to S to G2, of interphase. Second, variation involved with the fluorescence technique can be expected from potential degradation of DNA during quick drying. Nuclear orientation, and background fluorescence can result in variation of fluorescence readings as well. Consequently, I_f data should be considered accurate only to $\pm$ 0.1 pg (Kapraun, 2005). Ranges of nuclear DNA contents (pg) were evaluated using frequency histograms to allow for separation and estimation of 2C and 4C-values (Fig. 13). Table 3 summarizes the estimated 2C and 4C-values based on the frequency histograms. *Halophila engelmannii* had the highest 2C-value estimated at 27pg DNA with *H. decipiens* estimated as the lowest with 5.5pg DNA. Change in DNA-content values with the evolution of Halophila species may be seen by mapping the species average 2C and 4C-values on the *trnL* phylogeny (Fig. 14). This analysis suggests that polyploidy has occurred three separate times during the evolution of Halophila species.

Nuclear Area

Nuclear areas were determined with Image-Pro Plus software measuring the masked nuclei from photoshop 7.0. Resulting average areas were superimposed on the phylogenic tree to

Table 3
Average C-values derived from frequency histograms of fluorescence and nuclear areas.

Species	2C average (pg)	4C average (pg)	Nuclear Area (μm^2)
H. engelmanii	27	38	106.86
H. spinulosa	7.5	14	41.42
H. capricorni	13	30	56.97
H. decipiens	5.5	10	34.52
H. stipulacea	7.5	17.5	77.27
H. ovalis (BB)	22	40	126.35
H. hawaiiana	15	35	58.04
H. ovalis (R)	13	21	188.69
H. johnsonii	12	22	54.93

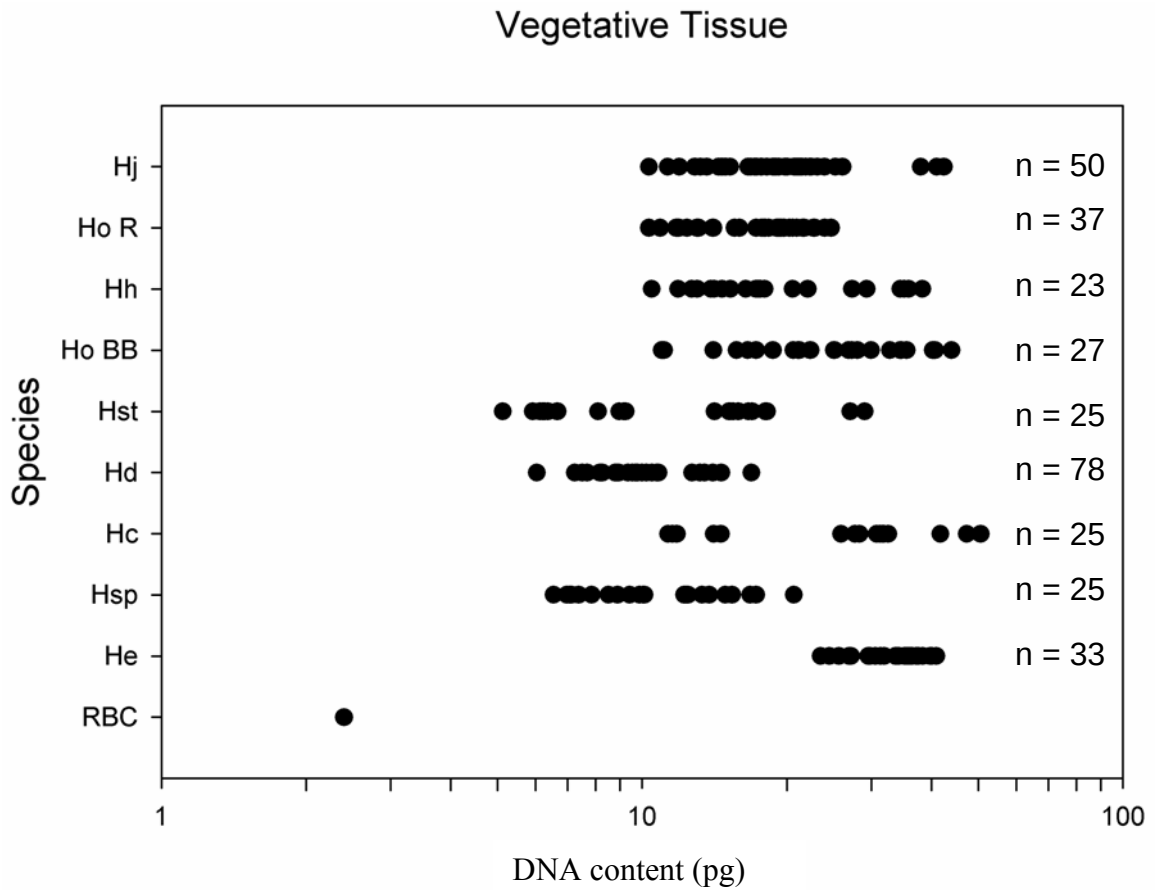

Figure 12. Nuclear DNA contents in picograms of vegetative tissue of 9 species of *Halophila*

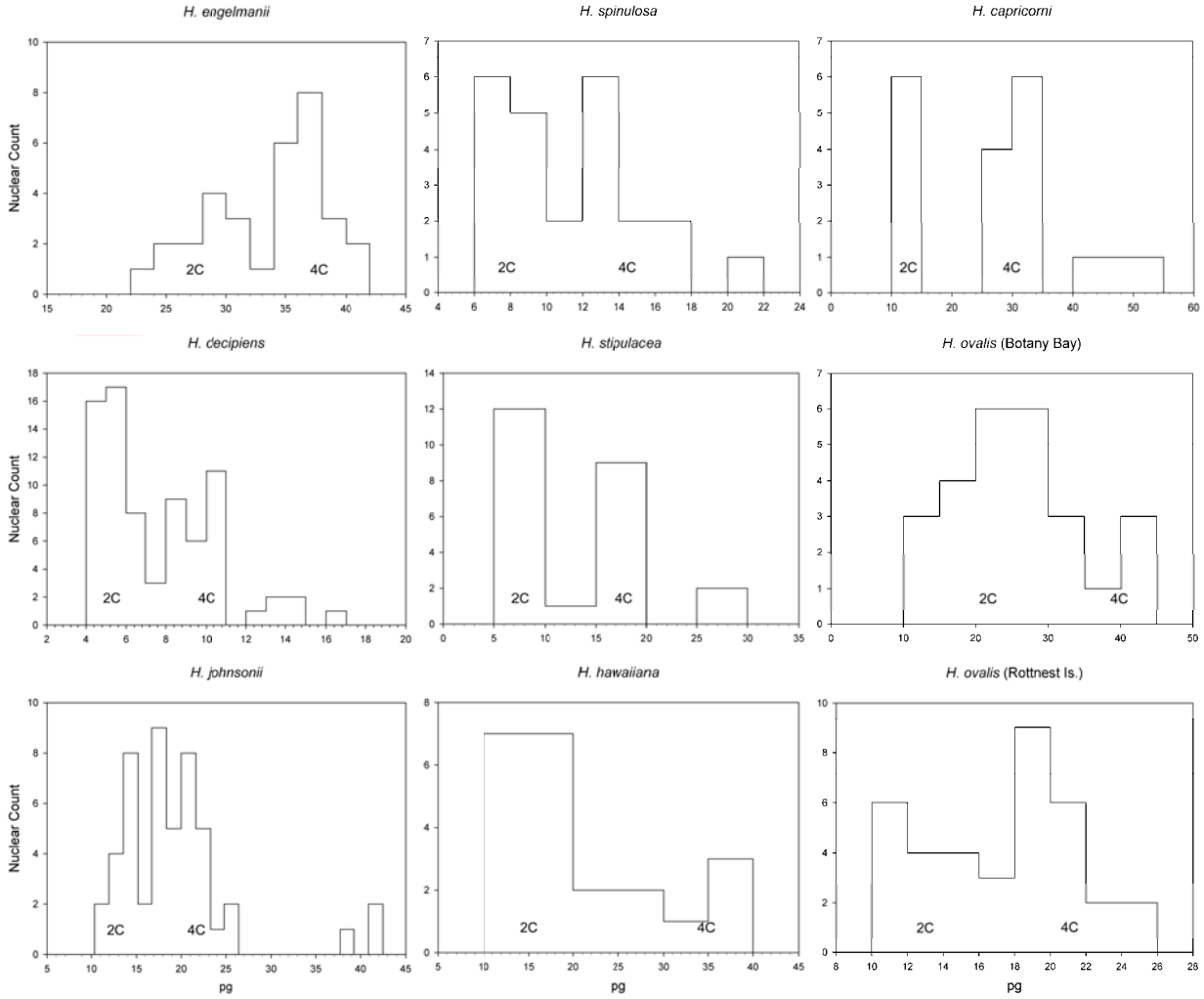

Figure 13: Frequency histograms of nuclear DNA contents for 9 species of *Halophila*; y axis: count of nuclei; x axis: picograms of DNA.

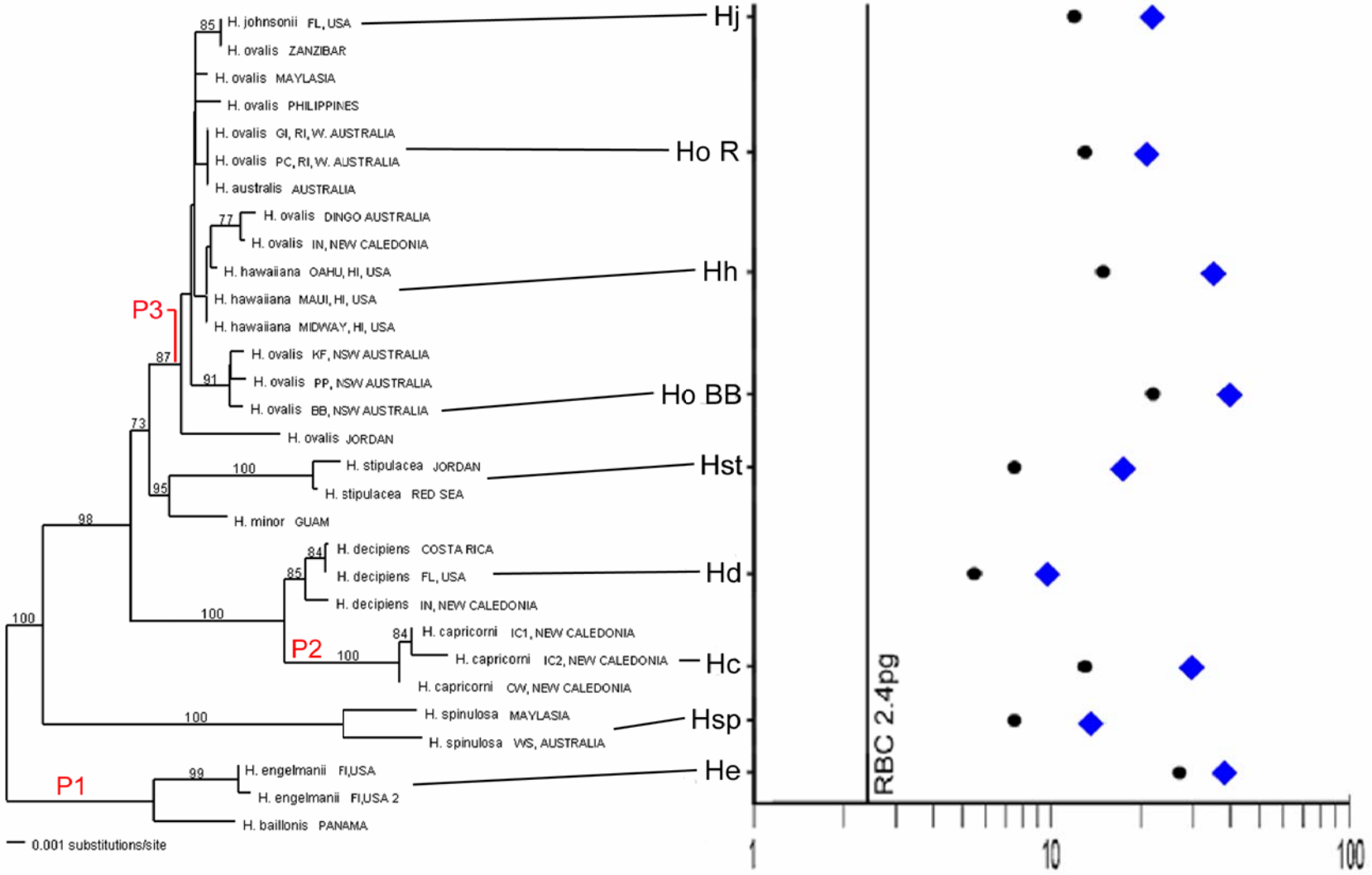

Figure 14. The average 2C-values (●) and 4C-values (◆) of nine species of *Halophila* mapped on the sequenced based phylogeny; the units for the logarithmic scale is picograms of DNA. Polyploidal events (P1, P2, P3).

show the changes in nuclear size as species evolve within the genus (Fig 15). The trend seen in the area comparison is almost the same as Figure 14, except that the *H. ovalis* sample from Rottnest Island had a larger nuclear area than *H. johnsonii*, *H. hawaiiana* and the Botany Bay *H. ovalis*, but its 2C and 4C DNA content values were less or equal to those for these same taxa (Fig. 15). The smallest average area occurred in *H. decipiens* at 34.52 μm^2 and the largest nuclear area was 188.69 μm^2 for *H. ovalis* (Rottnest) (Table 3).

DISCUSSION

This study had two main goals: 1) to identify haploid cells in the ovules of *H. johnsonii* and 2) to obtain a better understanding of nuclear genome size diversity within *Halophila*. *Halophila decipiens* was used as a reference taxon or standard because of its known sexual reproduction. The use of *H. decipiens* was beneficial in that pollen cells did not lose their identifiable traits in the squash slides. This permitted nuclear DNA content estimates for both 2C (haploid) and 4C (diploid) stages, thus verifying that the DAPI fluorescence technique can distinguish between gametes (haploid) and vegetative (diploid) cells. Problems with the technique arose when processing the ovules of both species. Specifically, in squash preparations, it was not possible to identify the location of potential haploid cells that might have been present on the slide. In preparations that were only lightly squashed, tissue would stay in an identifiable morphological conformation but would not stain properly or would not separate enough to permit accurate I_f readings.

Fluorescence data from vegetative tissue of 9 *Halophila* taxa suggest that large changes in DNA content occurred during the evolution of the genus. The changes in nuclear DNA content were corroborated by measurements of nuclear area in the same 9 taxa. Nuclear DNA content and nuclear size (volume) are highly correlated in most plants (Koce et al., 2003). Studies have used nuclear volume estimates to support nuclear DNA content estimates (Baetcke

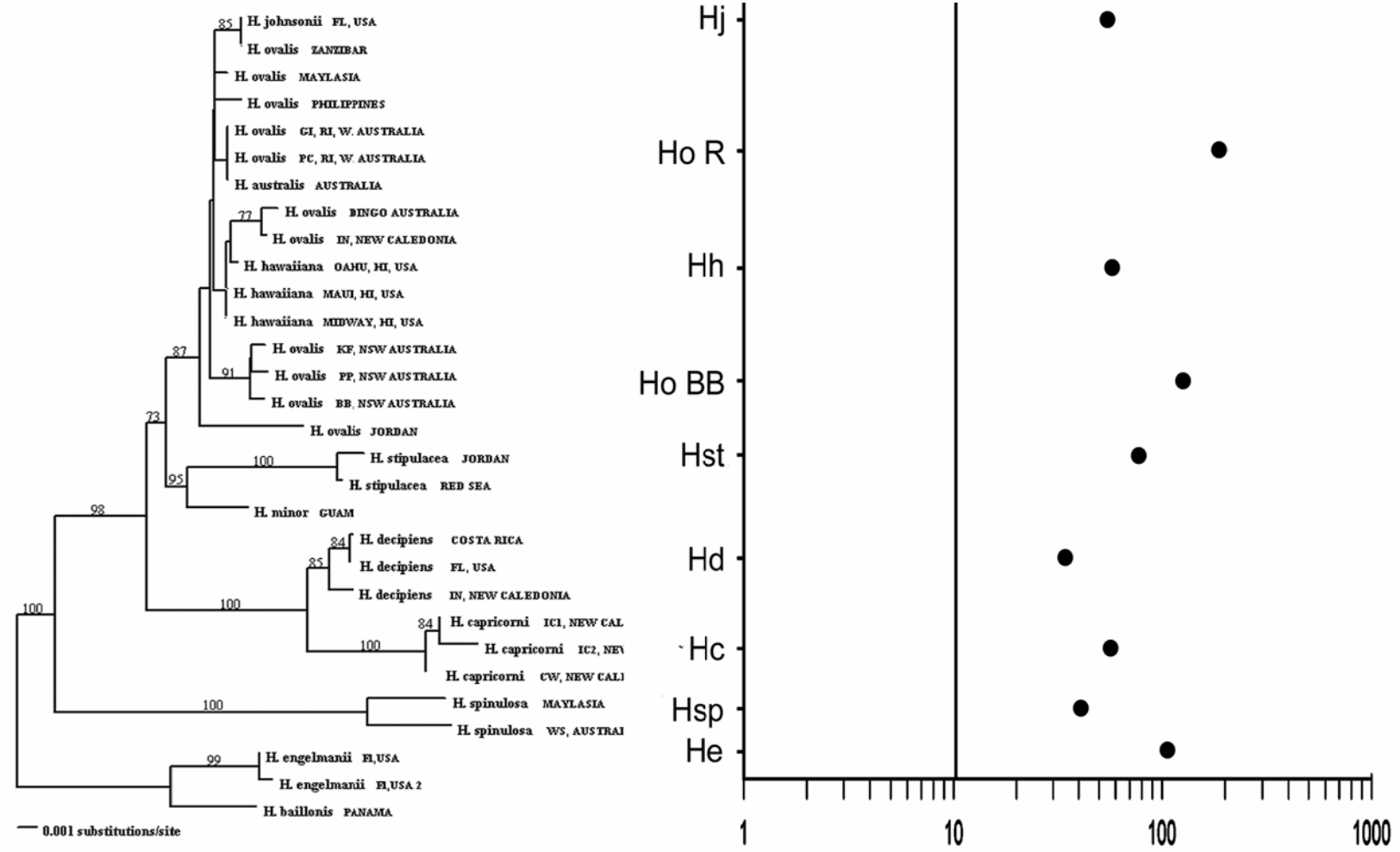

Figure 15. Average nuclear areas (●) of vegetative tissue of 9 species of *Halophila* superimposed on the phylogeny

et al., 1967). In the present study, nuclear area, rather than estimated volume, was used as a measure of nuclear size because of technical difficulties in deriving the 3rd dimension of the nuclei. Comparison of 2C DNA content estimates with nuclear area for *Halophila* taxa shows that they are correlated as found in previous studies (Fig. 16).

How has the nuclear DNA content evolved within *Halophila*? Ancestral monocots in general can be characterized as having small chromosome complements, small chromosomal size and low DNA contents (Leitch et al., 1998, 2005;). Genera within the Hydrocharitaceae (Alismatidae), which includes *Halophila*, are likewise reported to have similar small chromosome numbers e.g. *H. stipulacea* with 2n = 18 (Den Hartog ,1987), and low DNA contents (Bennett et al., 1998;Bennett et al., 2001; Koce et al., 2003). These data suggest that the ancestral lineage leading to the genus *Halophila* had a low DNA content and small chromosomal number. Nuclear DNA content data superimposed on the *trnL* phylogeny supports three polyploidy events (Fig. 14). The first occurs after the initial divergence within the genus in the lineage leading to *H. engelmannii* (Fig 14, P1). The second occurs along the lineage which leads to *H. capricorni,* after its divergence from the *H. decipiens* (Fig. 14, P2). The third ploypoloidial event occurs along the lineage leading to the "*ovalis* complex" after its split from the *H. stipulacea* and *H. minor* clade (Fig. 14, P3).

These data help to clarify recent issues with the upper part of the phylogenetic tree that have been left unresolved using molecular sequencing data. The data show that within the "*ovalis* complex", the clade containing *H. ovalis* and four other taxa, there are similar DNA contents among these species. Another issue these data address relates to the life history of *H. johnsonii*. If no males exist within this species, can pollen from male flowers of another closely related species successfully pollinate the females? The results here suggest that the best

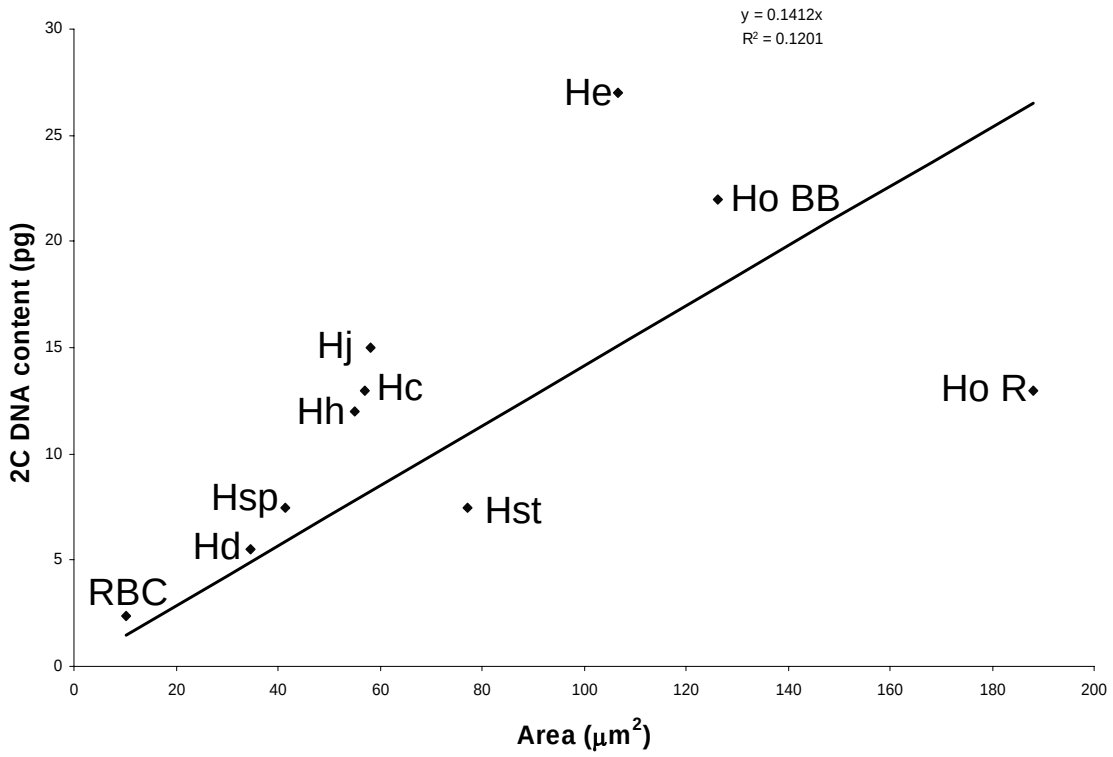

Figure 16. 2C DNA content (pg) verses nuclear area (μm^2), abbreviations of taxa (Table 2), and trend line set through 0, as $0pg = 0\mu m^2$.

candidates to test would be the closely related *H. ovalis* from the Western Pacific or Western Indian Ocean or *H. hawaiiana*.

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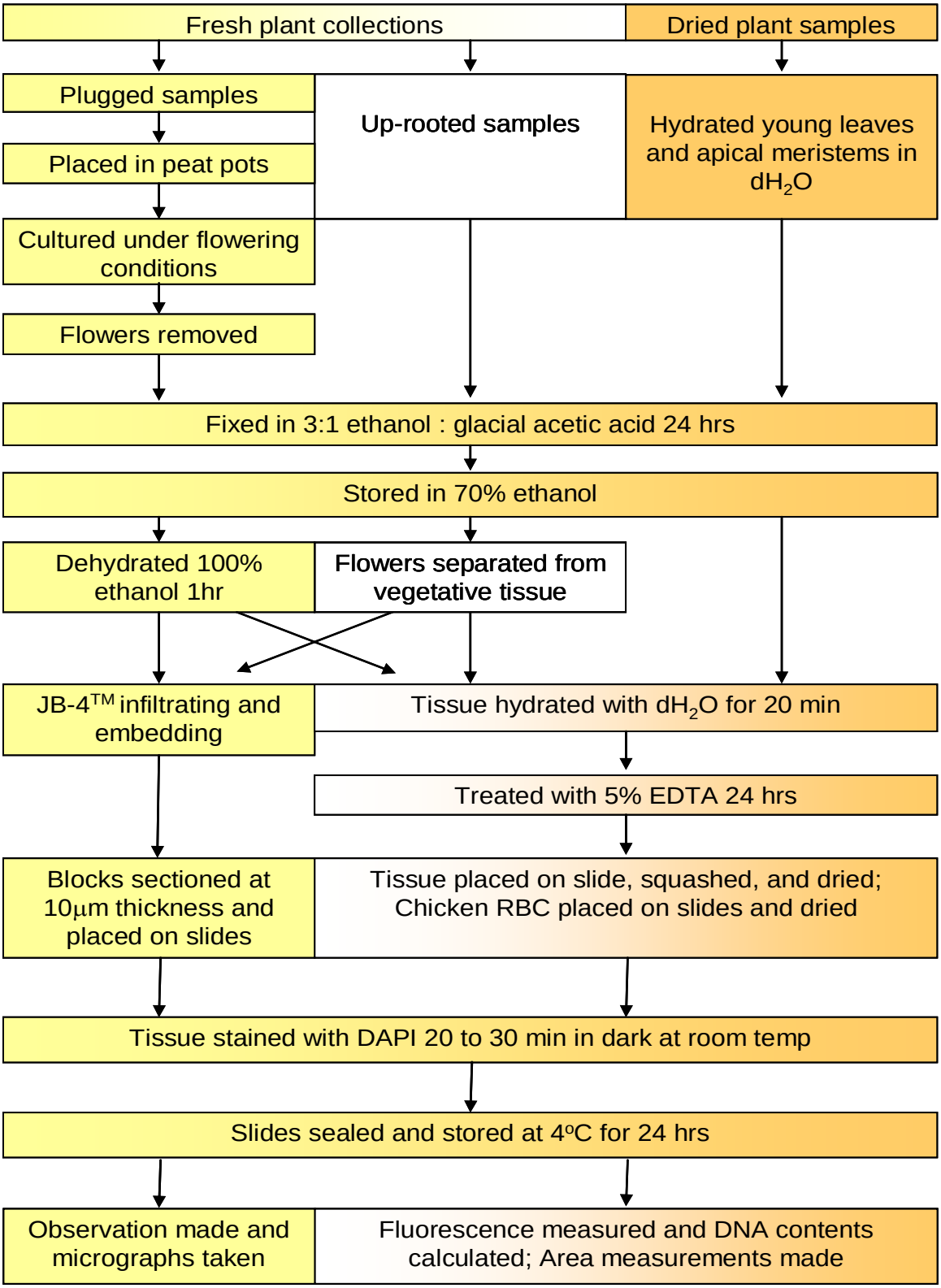
APPENDIX A


Fresh plant collections
Dried plant samples
Plugged samples
Up-rooted samples
Hydrated young leaves and apical meristems in dH_2O
Placed in peat pots
Cultured under flowering conditions
Flowers removed
Fixed in 3:1 ethanol : glacial acetic acid 24 hrs
Stored in 70% ethanol
Dehydrated 100% ethanol 1hr
Flowers separated from vegetative tissue
JB-4™ infiltrating and embedding
Tissue hydrated with dH_2O for 20 min
Treated with 5% EDTA 24 hrs
Blocks sectioned at 10μm thickness and placed on slides
Tissue placed on slide, squashed, and dried; Chicken RBC placed on slides and dried
Tissue stained with DAPI 20 to 30 min in dark at room temp
Slides sealed and stored at 4°C for 24 hrs
Observation made and micrographs taken
Fluorescence measured and DNA contents calculated; Area measurements made