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Cryptic haploid stages in the life cycle of *Leathesia marina* (Chordariaceae, Phaeophyceae) under in vitro culture[1]

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Abstract

We evaluated the life cycle of *Leathesia marina* through molecular analyses, culture studies, morphological observations and ploidy measurements. Macroscopic sporophytes were collected from two localities in Atlantic Patagonia and were cultured under long day (LD) and short day (SD) conditions. Molecular identification of the microscopic and macroscopic phases was performed through the *cox*3 and *rbc*L genes and the phylogeny was assessed on the basis of single gene and concatenated data sets. Nuclear ploidy of each phase was estimated from the DNA contents of individual nuclei through epifluorescence microscopy and flow cytometry. Molecular results confirmed the identity of the Argentinian specimens as *L. marina* and revealed their conspecificity with *L. marina* from New Zealand, Germany and Japan. The sporophytic macrothalli (2n) released mitospores from plurilocular sporangia, which developed into globular microthalli (2n), morphologically similar to the sporophytes but not in size, constituting a generation of small diploid thalli, with a mean fluorescent nuclei cross-sectional area of 3.21 ± 0.7 μm^2. The unilocular sporangia released meiospores which developed two morphologically different types of microthalli: erect branched microthalli (n) with a nuclear area of 1.48 ± 0.07 μm^2 that reproduces asexually, and prostrate branched microthalli (n) with a nuclear area of 1.24 ± 0.10 μm^2 that reproduces sexually. The prostrate microthalli released gametes in LD conditions, which merged and produced macroscopic thalli with a nuclear cross-sectional area of 3.45 ± 0.09 μm^2. Flow cytometry confirmed that the erect and prostrate microthalli were haploid and that the globular microthalli and macrothalli were diploid.

Keywords: Microthalli, Molecular identification, Ploidy, Flow cytometry, Fluorescent nuclei.

Abbreviations: RAxML-NG, Next Generation Randomized Maximum Likelihood; ML, Maximum Likelihood; BI, Bayesian Inference; GTR, General Time Reversible Model; I, proportion of invariable sites; Γ, distribution; LD, Long day; SD, Short day; DAPI, Fluorescent dye 4', 6-diamidino-2-phenylindole; PI, Propidium iodide.

INTRODUCTION

The brown algae (Phaeophyceae) constitute a taxonomically diverse and ecologically important group in marine environments (Mann 1982, Andersen 1992). These algae are of particular interest in evolution studies as they have evolved complex characteristics (complex multicellular and metabolic organization, cell walls containing various polysaccharides and high resistance to osmotic stress) independent of other lineages, such as Plantae, Fungi and Metazoa (Charrier et al. 2008).

Furthermore, brown algae exhibit a diverse range of life cycles with wide variety of sexual traits and reproductive strategies, where the transitions between different types of life cycle could reveal key adaptive events in the evolution of this group (Cock et al 2014).

Ancestral brown alga appears to have had a diplohaplontic life cycle with similar dominance of the diploid phase (sporophyte) and haploid phase (gametophyte), in size and complexity (Phillips et al. 2008, Heesch et al. 2019). However, over evolutionary time, in some clades the diploid phase became dominant; while in others, they evolved towards greater haploid dominance, exhibiting in both phases a wide variety of morphologies and complexity (Heesch et al. 2019).

This variability ranges from diploid life cycles, to the isomorphic haploid- diploid life cycles with morphologically similar generations, and heteromorphic haploid–diploid life cycles with more or less marked differences between the two generations, in terms of size and morphology, where either the sporophyte or the gametophyte generation being dominant in terms of size (Clayton 1988, Cock et al. 2014). Also, the correspondence between the sporophyte and gametophyte generations and the ploidy levels of each generation is not absolute and mutations or natural variations that uncouple ploidy and life cycle generation intervene in life cycle progression in brown algae (Cock et al. 2014).

These life cycle variations occur at the order level in brown algae. For example, in the order Ectocarpales, we find families with isomorphic generations (i.e., Acinetosporaceae, Ectocarpaceae) and families with heteromorphic generations, in which either the sporophyte (Scytosiphonaceae, except the genera *Myelophycus* and *Melanosiphon*) or the gametophyte (Chordariaceae, Adenocystaceae) is microscopic (Peters and Ramirez 2001). Also, evidence of mutations was detected in *Ectocarpus* that produce gametophytes, when the non-mutated algae would have produced sporophytes (Arun et al. 2018).

It has been proposed that life history variations are a result of complex adaptation as well as a successful solution to a particular ecological problem (Russell 1986). On the other hand, advances in molecular studies suggest that specific developmental programs are deployed at precise points in the life cycle to generate either a sporophyte or a gametophyte, where the intervention of the TALE (three amino acids loop extension)

homeodomain transcription factors (very ancient protein family) and chromatin modification seem to participate in the regulation of the life cycle (Cock et al. 2014, Arun et al. 2019, Bourdareau et al. 2020).

For this reason, brown algae represent a particularly interesting group to explore life cycle functions, since they exhibit a broad range of phenomena involving developmental processes, reproductive skills, dispersal modes, and adaptation to the local environment (Cock et al. 2014).

The family Chordariaceae with heteromorphic life cycles is the largest and most morphologically diverse lineage within the order Ectocarpales (Van den Hoek et al. 1995). In heteromorphic life cycles, the phases exhibit a high degree of independence and differentiation; usually, each phase has unique ecological and evolutionary constraints (Schiel and Foster 2006). In many species of marine macroalgae with heteromorphic life cycles, the microscopic generation is usually the most tolerant to unfavorable light, temperature, and nutrient availability (Carney and Edwards 2006).

Leathesia marina (Chordariaceae) is widely distributed in cold subtropical to temperate waters of both hemispheres, occurring as epiphytes in rocky intertidal communities (Oates 1989). However, it is not found in the tropics nor in Antarctica (Quartino and Boraso de Zaixso 1996). Life history studies on *Leathesia marina* have shown that the alga exhibits a typical heteromorphic life cycle, where a prostrate microscopic gametophyte alternates with a macroscopic sporophyte (Dangeard 1965, 1969, Peters 1987). Under culture conditions, *L. marina* microthalli have been reported, but neither sexual reproduction nor young macrothalli have been observed (Damman 1930, Kylin 1933). Therefore, the life cycle of *L. marina* is yet to be elucidated.

The most common approach to elucidate algal life cycles is by observing the development of their life stages under variable culture conditions. Additionally, several methods have been developed to determine the ploidy of the different stages of algae. Chromosome counting is a classical method to determine the nuclear phase; however, this technique is difficult to perform on algae due to the rapid cell division, and the results are often not precise due to the small size of the nuclei and chromosomes (Cole 1990). Another method is based on the correlation between the DNA content and nuclear size (Sparrow and Miksche 1961, Price 1976, Whittick 1986), wherein relative ploidy is determined in stained nuclei using a fluorometer and an image analysis system (Choi et al. 1994). In recent years, flow cytometry has been used as a convenient alternative to quantify the nuclear DNA content in marine algae (Le Gall et al. 1993), allowing the analysis of thousands of nuclei in a few minutes. Additionally, molecular analyses based on mitochondrial and plastidial markersproved to be excellent tools to assist in the identification of macroalgae (Le Gall and Saunders 2010) and are very useful for assisting with the identification of the microscopic generations.

In this study, we aim to evaluate the life history and the characteristics of the different phases of *L. marina* under culture conditions. For this purpose, we integrate ploidy quantification by epifluorescence microscopy and flow cytometry, and molecular studies to complement the morphological observations.

MATERIALS AND METHODS

Macroscopic sporophytes of *Leathesia marina* (henceforth referred to as globose macrothalli) were collected from the lower intertidal region of two coastal localities on Atlantic Patagonia. Las Grutas (40°50' S, 65°6' W), the northern location, is about 260 km away from the southern location at Puerto Madryn (42°46' S, 62°59'W). Vouchers of all collected specimens were deposited in the Herbarium of Universidad Nacional del Sur (BBB), Bahía Blanca, Buenos Aires, Argentina.

Mitochondrial *cox*3 and plastidial *rbc*L markers were used to infer the phylogeny of the Argentinian specimens of *L. marina* collected in the two localities and to confirm the identity of the microscopic generations obtained in culture. Sections of macrothalli and microscopic thalli were dried in silica gel, and the total genomic DNA was extracted using QuickExtract™ FFPE DNA Extraction Kit according to the manufacturer's instructions. Amplification and sequencing of the *cox*3 and *rbc*L genes were carried out as described in Santiañez et al. (2018). All newly generated sequences were submitted to GenBank (Table S1 in the Supporting Information).

Global alignment for the analyses of single gene (*cox*3: 650 bp) and concatenated data set (*rbc*L: 1399 bp + *cox*3: 509 bp = 1908 bp) were constructed together with available sequence data of the Chordariaceae in GenBank. Phylogenetic analyses based on single gene and concatenated dataset (partitioned by gene and codon position) were performed using maximum likelihood (ML) and Bayesian Inference (BI) under the GTR + I + Γ model. ML analyses were conducted in RAxML v.8 (Stamatakis 2014) with 1000 bootstrap pseudoreplicates through the Cipres Phylogenetic Portal (Miller et al. 2010). BI was performed in MrBayes v.3.2.1 (Huelsenbeck and Ronquist 2001) wherein 25% burn-in was set prior to calculating the trees. In addition, pairwise sequence differences (p-distances) within the *cox*3 data of *L. marina* was also calculated using MEGA v.6 (Tamura et al. 2013).

All in vitro culture used autoclaved seawater enriched with 10 mL $\cdot$ L^{-1} Provasoli medium (Provasoli 1968). Light irradiance of 25 µmol photons $\cdot$ $m^{-2} \cdot s^{-1}$ was provided to the cultures by cool white fluorescent tubes and monitored using a quantum flux meter (Apogee MQ-200, Logan, UT, USA). Fertile sections of 0.5 mm^2 obtained from 10 globose macrothalli from each geographical location were used to initiate cultures. Accordingly, 10 cultures from meiospores were obtained from unilocular sporangia and 10 cultures from mitospores were obtained from plurilocular sporangia. Each clean fragment containing mature unilocular sporangia (82 ±18, mean ± SD) or plurilocular sporangia (138 ± 26, mean ± SD) was incubated in a sterile tube

with a coverslip on the bottom for spore settlement. Ten replicates of each culture type were subjected to two light and temperature regimens: long-day (LD) with 16:8 h (light: dark) at 20°C and short day (SD) with 8:16 h (light: dark) at 8°C to simulate summer and winter conditions, respectively.

Morphological and anatomical observations of the globose macrothalli were done and the size of the thalli was estimated through its height (mm), diameter (mm), and drained wet weight (g). The development of the different stages of the life cycle after the germination of meiospores, mitospores, and zygotes, as well as the morphologies of the thalli obtained were monitored on coverslips under a Nikon Eclipse TE 300 microscope (Tokyo, Japan) equipped with a Nikon FDX 35 camera. Twenty visualizations were performed for each replica by photographing and quantifying the development of individuals in culture. All microscopic measurements were estimated from the images using the software ImageJ v. 1.46 (National Institutes of Health, Bethesda, MD, USA).

The nuclear ploidy of each phase of the life cycle was estimated by epifluorescence microscopy and flow cytometry. The DNA of individual nuclei was stained with the fluorescent dye 4', 6-diamidino-2-phenylindole (DAPI). Samples from different stages of the life cycle were fixed in Carnoy's solution (ethanol: 100% acetic acid = 3:1) for at least 24 h at 5°C and were then transferred to Eppendorf tubes containing 100% ethanol and kept at 4°C until the analysis. The nuclear ploidy level was estimated based on the correlation between DNA content and nuclear size (Kapraun and Nguyen 1994). The nuclear size was estimated by measuring the relative area of DAPI-stained DNA in nuclei using ImageJ software. To assess the differences in DNA contents of individual nuclei stained with DAPI, analyses of variance (ANOVA) were conducted to establish the nuclear size differences. Each data set was examined for homoscedasticity using Bartlett's test and normality using the Shapiro-Wilk test with a 0.05 significance level using the statistical program R Studio (R CoreTeam 2016). Statistical analyses were performed using a "WRS2" (Mair and Wilcox 2019) and "multcomp" (Hothorn et al. 2008) packages in R.

To perform flow cytometry, the nuclei were isolated from mature thalli of different morphology corresponding to the different life-cycle phases. Small portions of thallus were chopped with a razor blade in ice-cold buffer (30 mM MgCl, 120 mM trisodium citrate, 120 mM sorbitol, 55 mM 4-[2-hydroxyethyl] piperazine-1-ethanesulfonic acid [HEPES], 5 mM EDTA supplemented with 0.1% [v/v] Triton X-100 and 5 mM sodium bisulfite; pH 8.0), following the methods of Peters et al. (2004). The chopped thalli were filtered through a 30 µm and 10 µm mesh nylon filter. An aliquot of 100 µL of PI-RNase solution (propidium iodide [PI], 50 $\mu g \cdot mL^{-1}$, RNase 50 $\mu g \cdot mL^{-1}$ in distilled water) was added to the suspension of nuclei, and after 20 min of incubation their DNA content was measured in a flow cytometer FACS Calibur equipped with an Argon LASER (emission at 488nm) of INBIOSUR-CONICET. The nuclear population measurements were identified

and separated from noise measurements in biplots of side-scattered light (SSC) vs. green fluorescence (FL2). Data were processed using FlowJo V.10.0.7r2 software (Tree Star, Inc.).

RESULTS

Phylogeny of Leathesia marina *from Patagonia*

A total of nine new DNA sequences were generated in this study from eight *Leathesia* specimens, eight *cox*3 (two from the microscopic gametophytes obtained in culture and six from the macroscopic sporophytes collected in the field) and one *rbc*L. Based on our *cox*3 and concatenated trees (Figs. 1 and 2), the Argentinian *L. marina* formed a well-supported clade with its conspecifics from New Zealand, Germany, and Japan. The specimens from both collection sites were genetically similar and were closely related to two specimens from New Zealand, having only 0.3% sequence divergence in *cox*3. Intraspecific sequence divergence in *cox*3 was up to 7.7%. The identity of the gametophyte was confirmed as *L. marina* by the phylogeny of *cox*3. The phylogeny inferred from the concatenated *rbc*L and *cox*3 revealed that the clade of *L. marina* is a sister clade of *Elachista tenuis* Yamada and *Cladosiphon okamuranus* Tokida.

Morphology of diploid macrothalli

The macrothalli of *Leathesia marina* were found predominantly in the low intertidal zone, only between late winter and early autumn (i.e., from August to April in Las Grutas and from September to April in Puerto Madryn). In both populations, *L. marina* was often found as an epiphyte in beds of *Corallina officinalis* Linnaeus (Fig. 3a).

Macrothalli of *Leathesia marina* had a diameter of 1.7–4.5 cm, a height of 0.8–1.2 cm, and a drained wet weight of 0.3–3.7 g. In cross-section, the medulla was composed of three layers of colorless, thin-walled cells that were smaller and dichotomously branched towards the surface (Fig. 3b). The lowest medullary layer cells were relatively larger, 60.8–72.2 × 37.8–45 μm in size, irregularly shaped, and anastomosing (Fig. 3c). Directly above the latter, the cells were oblong to broadly oblong, 38.2–49 × 27.6–32.7 μm and are basal to two smaller oblong cells (27–32 × 18–19.5 μm), forming a first order dichotomy (Fig. 3b). Two smaller subsurface cells were also borne on each of the latter cells, 13.3–17.3 μm long and 9.6–11 μm wide, forming the second order dichotomy. Each subsurface cell bore cortical filaments, and the fertile specimens also have either uni- or plurilocular sporangia. Cortical filaments were solitary or in groups of up to three. Each filament possessed an inflated ovoid terminal cell containing numerous phaeoplasts (Fig. 3b). The hyaline hairs were scattered on the macrothalli surface, developing from the outermost medullary cells, and were 32.5–45.3μm long and 8–9 μm wide (Fig 3d). Plurilocular sporangia (24.5–35 × 5.2–6 μm) were linear and uniseriate, usually of 3–6 locules,

and were borne solitary or in groups of three (Fig 3d). Unilocular sporangia were ovoid and sessile, 19.4–35.2 μm long and 12.4–16.7 μm wide (Fig 4a).

Life cycle of **Leathesia marina** *under culture conditions*

Diploid generation produced from plurilocular sporangia

In both LD and SD regimes, the plurilocular sporangia on the globose macrothallus released mitospores of 1.8–2.3 μm long after 12 h of incubation. The majority of these mitospores settled after 12 h of swimming. Once settled, they exhibited bipolar germination, forming two germ tubes. Some divided profusely in different directions forming a disc from the central cell, whereas others formed loose discs (Fig. 3, e-g). Young germlings developed into minute thalli with the same morphology as the macrothalli. They never exceeded 1.5 mm in diameter constituting a generation of small diploid thalli (Fig. 3, h-j). This diploid generation was called globular microthalli. These thalli had a medullar structure formed by three layers of colorless cells, decreasing in size towards the surface (Fig. 3k). The lower cells were large, oblong, measuring 24–32 × 13–17 μm; the cells of the second layer were also oblong and 16–21 × 13–17 μm in size; and the superficial cells were oblong to broadly oblong and smaller 9–12 × 9–10 μm. Phaeophycean hairs were associated with the cortical layer. The cortical layer was typically composed of short assimilatory filaments formed from two to three cells, 5–7 μm in length. After 20 days of incubation, the globular microthalli developed plurilocular sporangia (15–30 μm long × 3–5 μm wide) located among cortical filaments, which were linear to linear-lanceolate and had one to three rows of loculi (Fig. 3k). Unilocular sporangia were never observed in these thalli under LD and SD conditions. Settled spores from plurilocular sporangia of globular microthalli showed similar developmental patterns as the original mitospores. These grew into new globular microthalli (Fig. 3l) and repeated the same asexual cycle indefinitely.

Haploid generation produced from unilocular sporangia

Meiospores were released from unilocular sporangia (Fig. 4a) on the globose macrothallus after 12 h of incubation. They were 1–1.3 μm long and they settled after 12 h. They had unipolar germination (Fig. 4b) and developed a germ tube with transverse divisions forming uniseriate branched filaments (Fig. 4c). These meiospores formed two types of microthalli with different growth patterns. Some formed a basal structure before developing into erect branched microthalli (Fig 4, d-g) whereas others formed prostrate branched microthalli (Fig 4, h-j).

The erect branched microthalli grew up in LD and SD regimes, representing 100% and 37.8% of the individuals in the LD and SD regimes, respectively. These were characterized by upright filaments with lateral

branches of 9–11 × 2–4 μm, that emerged from rounded basal cells of 6–4 × 2–4 μm (Fig. 4e). After four to five weeks, the basal or intercalary cells of lateral branches differentiated into plurilocular reproductive structures (10–20 × 4–5 μm) with one to three rows of loculi (Fig. 4g). This morphology generated itself during successive generations.

Prostrate branched microthalli only grew under the SD regime, representing 62.2% of the individuals from germling in SD regimes and had lateral branches extending horizontally with cells of 9–13 × 4–6 μm, forming a dense matrix of interwoven prostrate filaments (Fig. 4h). These prostrate microthalli did not form any reproductive structures, remaining latent for 18 months. However, when these microthalli were transferred to LD conditions, they formed plurilocular gametangia. The gametangia were linear, 20–30 × 6–8 μm and possessed one to three rows of loculi (Fig. 4j). Gametogenesis occurred two weeks after the thalli were transferred from SD to LD conditions. Gametes (1–2 μm diameter) were released after 24 h of incubation and later the prostrate microthalli were detached from the substrate. Relatively larger female gametes settled first, becoming spherical, and were subsequently fertilized by persistently motile male gametes. The resulting zygotes were larger (4–5 μm diameter; Fig. 4k). Zygotes developed into diploid macrothalli under LD conditions, which in young stages exhibited bipolar germination. Successive divisions in different directions resulted in symmetrical disc; later, this developed into the typical macroscopic globose structure (Fig. 4, l-o). Both mitospore-producing plurilocular sporangia and meiospore-producing unilocular sporangia developed on each mature macrothallus upon reaching a size of 0.8-1.2 cm. Approximately 6.3% of unfertilized female gametes developed parthenogenetically to produce new prostrate branched microthalli.

All *Leathesia marina* specimens from both collection localities exhibited similar development and life cycles patterns in in vitro culture. They completed the cycle in six months developing different morphologies. During the haploid generation, two types of microthalli that developed from meiospores were observed: one erect and branched and other prostrate. The prostrate branched microthalli behaved like true gametophytes, since they produced gametes that developed into macrothalli after fertilization. Meanwhile, the erect morphology (i.e., erect branched microthalli) only regenerated itself through mitospores. The diploid generation was also represented by two other morphologies; a globular microthallus derived from mitospores, which remained in a dwarf state and repeated the same asexual cycle indefinitely; and, a globose macrothallus, which is a product of fertilization and commonly found in nature (Fig. 5).

Nuclear ploidy of the different phases of* Leathesia marina *life cycle

When determining the ploidy by flow cytometry the presence of one peak represents the cells in the G1 phase of the cell cycle, followed by a plateau or S phase, whereas the occurrence of a second peak (with twice the channel value) corresponds to post-DNA replication cells in the G2/M phase.

The nuclei isolated from the macrothalli presented three populations of cells, producing one 2C peak in G1 cellular phase, a smaller 4C peak in G2/M and a small 1C peak, possibly caused by haploid spores produced by meiosis (Fig. 5a).

Young germlings produced from mitospores had a nuclear cross-sectional area of 3.85 ± 1.2 μm^2 (mean ± SD; Fig. 6c). The nuclei isolated from the globular microthalli were 2C, followed by S phase and a 4C peak (Fig. 5b) and had a cross-sectional area of 3.21 ± 0.7 μm^2 (Fig. 6, j and l). Young germlings from the meiospores had a nuclear cross-sectional area of 1.64 ± 0.16 μm^2 (mean ± SD; Fig. 6a). The nuclei isolated from the erect microthalli had a 1C peak and a smaller 2C peak, suggesting active cell growth (Fig. 5c). Their nuclear cross-sectional area was 1.48 ± 0.07 μm^2 (Fig. 6, e–h). On the other hand, the nuclei from prostrate microthalli under latency conditions (SD), were only in phase G1, without cell division (Fig. 5c) and their nuclear area was 1.24 ± 0.10 μm^2 (Fig. 6, m–p). The nuclei of the erect microthalli were significantly larger ($F_{1,197}$= 33.64; P =0.001) than those of the prostrate microthalli, possibly due to active cell growth.

The gametes that developed on prostrate microthalli under LD conditions had a nuclear cross-sectional area of 0.9 ± 0.02 μm^2 (Fig. 6, q–r). The zygotic nuclear cross-sectional area (3.6 ± 0.12 μm^2; Fig. 6, s and t) was significantly larger than that of the unfertilized gametes ($F_{1,76}$= 792.4; P < 0.001; Fig.6, d–f). The young filaments that developed from the zygotes had a nuclear cross-sectional area of 3.45 ± 0.09 μm^2 (2n; Fig. 6, u-x).

DISCUSSION

Leathesia marina populations from the two sites in the South Atlantic Ocean exhibited the same life cycle, development, and morphologies. This is corroborated by our molecular data confirming that populations at both macroscopic (obtained from nature) and microscopic (obtained in culture) stages are conspecifics. The inferred phylogeny revealed that *L. marina* from Atlantic Patagonia is closely related to *L. marina* from New Zealand.

Many organisms with heteromorphic life cycles exhibit marked morphological, physiological, and ecological divergence between the different haploid-diploid phases (Couceiro et al. 2015). The present study revealed that four different morphologies are involved in the life history of *L. marina* under in vitro culture. That is, diploid phases occur as globular macrothalli or globular microthall, whereas haploid morphologies occur as branched erect microthalli and branched prostrate microthalli.

Other authors have reported the occurrence of these morphologies in separate studies of *L. marina* originated from meiospores and mitospores under in vitro culture. However, our current study is the first to report gamete fusion and the development of resulting zygote into the globose macrothalli that we observe in the field.

Prostrate microthalli in *Leathesia marina* (as *L. difformis*) cultures have been found by Dangeard (1965) and Cole et al. (1968) generated from unilocular sporangia at low temperatures. Dammann (1930) observed that spores released by unilocular sporangia develop into asexual microscopic thalli that bear plurilocular sporangia. This reproductive behavior coincides with the erect branched microthalli found in this study. Kylin (1933) and Sauvageau (1925) reported globular microthalli that regenerated for several generations through plurilocular sporangia. Kylin (1933) considered that the globular microthalli as an early macroscopic state, although he could not prove it; while Sauvageau (1925) suggested that it was a different morphology, since never re-generated new macrothalli from plurilocular sporangia.

The globular microthalli that we obtained in culture from plurilocular sporangia of the globose macrothallus remained in a dwarf state for several generations, never reaching the size and shape of the globose macrothalli in culture (originated by fecundation). In natural populations of *Leathesia marina* from Puerto Madryn, the globular macrothalli appear in spring, constituting a single cohort developing through the growing season without recruiting any new thalli (Poza et al. 2017). This suggests the absence of successive generations of macrothalli (originated from plurilocular sporangia) in this Patagonian population. However, it is likely that Poza et al. (2017) were not able to detect globular microthalli as new recruitment in their study populations due to the small size. Thus, we consider that globular microthalli in nature are as small as those that grew in culture. Careful search is probably required to find globular microthalli in nature. Studies on natural *L. marina* populations in Nahant, Massachusetts reported the presence of dwarf morphotypes that were growing as epiphytes on *Zostera marina* Linnaeus (Webber 1981). These dwarf morphotypes that grow to 2 mm in diameter were recognized as *Leathesia nana*- type. Webber (1981) alluded to the possibility that *L. nana*-type may be a growth form of *L. marina* (as *L. difformis*) based on the morphological, anatomical, and seasonal distribution data. In another species of the family Chordariaceae, *Litosiphon laminariae* (as *Litosiphon pusillus*) were also observed to have dwarf sporophytes that originated from plurilocular sporangia under winter culture conditions (Nygren 1975).

Leathesia marina displayed a haplodiplontic life cycle with marked differences between the two generations in terms of size, morphology, and reproductive behavior, which appears to be determined by temperature and photoperiod, as observed in our current culture studies. Some authors hypothesize that the different generations are adapted to different niches, allowing these species to survive in a fluctuating

environment differing in temperature, daylength, competitors, or herbivore pressures (Lubchenco and Cubit 1980, Zupan and West 1990, Cunningham et al. 1993).

The sporophytic generation was dominant in size and is more conspicuous. This prevalence of the diploid phase should not be interpreted as an evolutionary advantage because in many taxa the haploid-diploid life cycles are an evolutionarily stable strategy, with the genetic benefits of both phases (Klinger 1993). The genetic advantage of the diploid phase is that nearly all deleterious mutations within the genome are masked, as the vast majority of mutations that adversely affect fitness are partially recessive (Crow and Kimura 1965). However, mutations are more efficiently eliminated in haploids. As haploid populations tend to carry fewer mutations, they tend to have a higher average fitness at equilibrium than diploid populations (Otto 1996, Mable and Otto 1998).

The presence of different haploid and diploid microthalli with different reproductive behavior suggests high plasticity in the development of *L. marina*. The cultured globular microthalli and erect branched microthalli of *L. marina* produced fertile structures after 20–30 d and were regenerated during several generations, behaving as diploid and haploid sporophytes (i.e., asexual phases), respectively, since no zooid fusions were observed under the culture conditions evaluated. However, this reproductive behavior could be fostered by laboratory conditions; therefore, it is necessary to document these morphologies in natural populations of Patagonia in order to reveal their true reproductive strategies. A high level of morphogenic and reproductive heterogeneity has also been noted in *Ectocarpus siliculosus* under controlled conditions in laboratory culture (Müller 1967, 1980, Le Bail et al. 2008). Architectural plasticity with variation in growth rate and branching pattern are common features in algae, especially in response to environmental changes (Le Bail et al. 2008). Hence, further studies focused on exploring the occurrence and function of these microthalli in nature and its possible links in the life cycle of *L. marina* is needed.

Meanwhile, the prostrate branched microthalli can be maintained in latency for more than one year and can exhibit gametogenesis, but only under higher temperatures and longer photoperiods (LD; summer conditions). The same behavior has also been reported in other widely distributed brown algae such as *Dictyota kunthii* (=*Glossophora kunthii)* and *Dictyota dichotoma*, where low temperatures inhibited formation of reproductive structures (Hoffmann 1988, Hwang et al. 2005). It is known that algae respond to many cues for the timing of gametogenesis, where light, photoperiod, and temperature play a major role in the induction of reproduction (Brawley and Johnson 1992).

The prostrate branched microthalli, also called “banks of microscopic forms” (Chapman 1986), would be crucial to the persistence of populations during periods when the macroscopic stages are absent (Carney and Edwards 2006). The delayed development in juvenile or alternate life stages may be an adaptation to surviving

in temporally variable environments and in establishing spatial patterns in other stages (Swanson and Druehl 2000). Macroalgal microscopic stages generally tend to be more tolerant to unfavorable light, temperature, and nutrient conditions, being able to persist for extended periods (such as during winter) and promote rapid recruitment once the conditions become favorable (Chapman and Burrows 1971, Nakahara 1984, Wiencke and Dieck 1989, 1990, Hoffmann and Santelices 1991, Edwards 2000). In turn, the macroscopic diploid phase produced by gamete fusion, appears only under summer conditions, provide the genetic advantages of having two sets of homologous chromosomes and a high rate of beneficial mutations (Crow and Kimura 1965).

Macroscopic thalli of *Leathesia marina* from Patagonia have high reproductive output due to the great increase in the number of sporangia and high release of spores at the end of the macrothalli season, coinciding with the months when the prostrate branched microthalli appeared (Poza et al. 2017). The high reproductive output of the macroscopic phase could ensure massive recruitment of the haploid phases, establishing banks of microscopic stages with unrelated individuals that accrue over time. This could allow the exchange of genetic material between unrelated individuals.

The life history characteristics of *Leathesia marina* that we described here appear to be an adaptive strategy of the species to the extreme climate of Patagonia, a region in the southern hemisphere with a well-marked seasonality. This arid region is characterized by a predominance of strong west winds and year-round low humidity (Paruelo et al. 1998). These strong dry winds, combined with low rainfall, give the Patagonian intertidal zone the highest desiccation stress recorded for rocky shore communities (Bertness et al. 2006). In this way, globular macrothalli with irregular morphology, such as having numerous interstices and large assimilatory filaments, could favor water retention and thus avoid desiccation during the summer. Whereas, prostrate microthalli are favored during winter as such small morphologies are more tolerant to cold temperatures and have lower energy requirements (Hurd et al. 2014).

Leathesia marina is a good example of the heteromorphic life cycle, with great morphological plasticity that involves resting states (prostrate branched microthalli). These characteristics may allow the species to survive long periods of unfavorable environmental conditions and to recover following severe disturbances. The understanding of life cycles and microscopic macroalgal development is critical for the protection of the natural populations.

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Figure 1. Phylogeny of *Leathesia marin*a based on *cox3* sequences. Values shown at each node are Bayesian posterior probabilities (PP) and maximum likelihood bootstrap percentages (BP), respectively. Thickened lines indicate highly supported nodes (PP: ≥ 0.98 and BP: ≥ 95%). Values <80% BP and <0.80 PP have been removed. Bold names = newly generated sequences. * = used in life history studies.

Figure 2. Phylogeny of *Leathesia marin*a based on concatenated (*cox*3 + *rbc*L) sequences. Values shown at each node are Bayesian posterior probabilities (PP) and maximum likelihood bootstrap percentages (BP), respectively. Thickened lines indicate highly supported nodes (PP: ≥ 0.98 and BP: ≥ 95%). Values <80% BP and <0.80 PP have been removed. Bold names = newly generated sequences.

Figure 3. Macrothalli and the generation from plurilocular sporangia of *Leathesia marina* in culture. **a.** Diploid macrothalli (black arrow) living in epiphytic association with *Corallina officinalis* (arrowhead). Scale bar: 1 cm. **b.** Thallus section showing medullary cells that are progressively smaller and dichotomously branched to two orders towards the surface, marked with numbers 1-3. Subsurface medullary cells bear cortical filaments with inflated and pigmented terminal cell (arrowhead). Scale bar: 20 µm. **c.** Detail of irregularly shaped and anastomosing basal medullary cell in transverse section. Scale bar: 20 µm. **d.** Hairs born from medullary cells (black arrow) and plurilocular sporangia with numerous loculi (arrowhead). Scale bar: 20 µm. **e.** Bipolar germination of mitospore released from plurilocular sporangia. Scale bar: 5 µm. **f.** Bilobal (black arrow) and bipolar germination (arrowhead) from mitospore. Scale bar: 10 µm. **g.** More advanced stage presenting divisions in different directions forming small discs. Scale bar: 10 µm. **h.** Advanced stage of diploid globular microthalli, beginning to develop the medullar structure (arrowhead). Scale bar: 20 µm. **i**. Mature globular microthalli. Scale bar: 200 µm. **j.** Detail in transverse section of medullary cells of globular microthalli. Scale bar: 20 µm. **k.** Plurilocular sporangia (black arrow) and assimilatory filaments of globular microthalli in transverse section. Scale bar: 10 µm. **l.** Regeneration of globular microthalli. Scale bar: 50 µm.

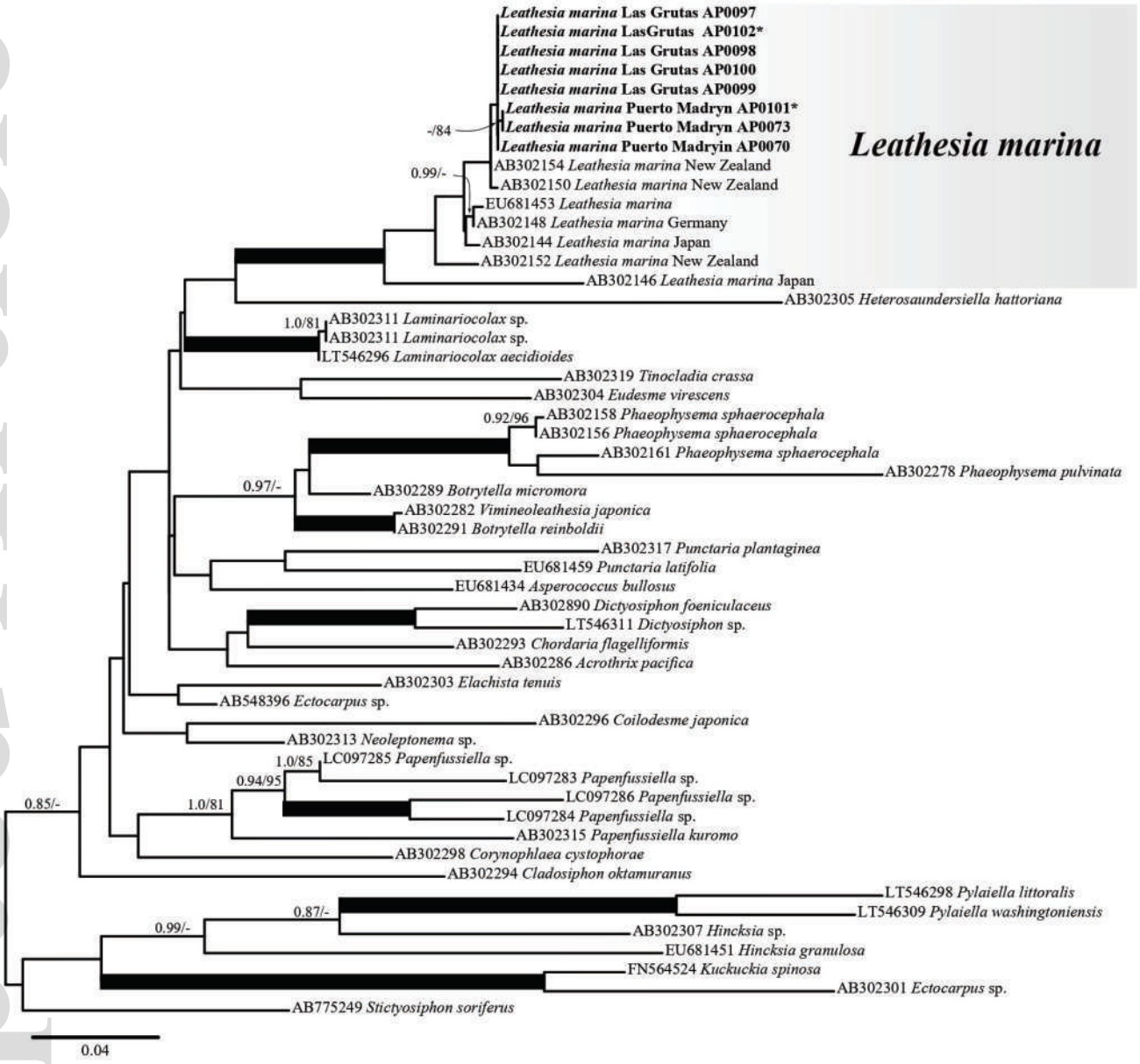
Figure 4. Generation from unilocular sporangia and fertilization of *Leathesia marina* in culture. **a-g.** Erect branched microthalli formation. **h-j**. Prostrate branched microthalli formation (gametophyte)**. k-o**. Macrothalli formation **a.** Unilocular sporangium on diploid macrothalli. Scale bar: 10 µm. **b.** Unipolar germination from meiospore. Scale bar: 5 µm. **c.** Germling with cellular divisions. Scale bar: 10 µm. **d.** Advanced stage of erect branched microthalli. Scale bar: 250 µm. **e.** Mature erect branched microthalli. Scale bar: 2.5 mm. **f.** Detail of erect branched microthalli in transverse section. Scale bar: 20 µm. **g.** Reproductive structure plurilocular of erect microthalli. Scale bar: 20 µm. **h.** Prostate microthalli forming a dense matrix of

interwoven filaments. Scale bar: 4 mm. **i.** Detail of the prostate microthalli. Scale bar: 40 µm. **j.** Plurilocular gametangia on prostrate microthalli. Scale bar: 10 µm. **k.** Zygote (arrowhead) and paired gametes (black arrow). Scale bar: 5 µm. **l**. Macrothalli germling after syngamy. Scale bar: 30 µm. **m**. Young macrothalli. Scale bar: 3 mm. **o.** Cross section of a young macrothalli. Scale bar: 20 µm.

Figure 5. Nuclear DNA content estimated by flow cytometry in adult thalli and schematic *Leathesia marina* life cycle. R!, reduction of the chromosome number by meiosis; LD, long days; SD, short days; PG, plurilocular gametangia. **a.** DNA content of the nuclear population of sporophyte (macrothalli), containing C, 2C and 4C nuclei. **b.** DNA content of globular microthalli, containing primarily 2C followed by S phase nuclei and 4C nuclei. **c.** DNA content of the erect branched microthalli, containing primarily 2C and a small number of nuclei 4C. **d.** DNA content of the gametophyte populations in latency (prostrate branched microthalli), presenting only C nuclei.

Figure 6. Light and epifluorescence of nuclear cross-sectional areas measured with DAPI, showing the ploidy changes of different life cycle phases of *Leathesia marina* in young and adult stages. Histograms showing DNA content, measured as fluorescent nuclear cross-sectional areas. The vertical dotted line separates the diploid peaks (2n) to the right, and the haploid peaks (n) to the left. The horizontal dotted line separates the direct or asexual cycle in the upper part from the sexual phase in the lower part. **a, b.** Differential interference contrast images of early germling produced from meiospores. Fluorescent dye visible in the nucleus. Scale bar: 5 µm. **c, d**. Differential interference contrast images of germling produced from mitospores. Fluorescent dye visible in the nuclei. Scale bar: 5 µm. **e- h.** Erect branched microthalli (n): view of a seven day old germling produced from meiospores, cross section of the adult stage and fluorescent dye visible in nuclei. The circular arrow indicates successive regenerations of this phase. Scale bar: 10 µm. **i-l.** Globular microthalli (2n): view of a seven day old germling produced from mitospores, cross section of the adult stage and fluorescent dye visible in nuclei. The circular arrow indicates successive regenerations of this phase. Scale bar: 10 µm. **m-x.** The sexual phase follows the direction of the arrow. **m-p.** Prostrate branched microthalli (n): germling of a seven day old, adult stage showing prostrate ramifications and view of small fluorescent nuclei. Scale bar: 10 µm. **q, r.** Free gametes from prostrate microthallus before fecundation. Scale bar: 5 µm. **s, t.** Zygotes which have undergone nuclear fusion. Scale bar: 5 µm. **u-x.** Young macrothalli of three day old, cross section of the adult stage and fluorescent dye visible in nuclei. Scale bar: 10 µm.

Table S1. *Leathesia marina* specimens included in the molecular analyses, with GenBank accession numbers for their *rbc*L and *cox*3 sequences.



Shown: PP/BS; > 0.80/80, respectively
Thickened: PP > 0.98, BS > 95
Taxa with asterisk indicate those used in culture

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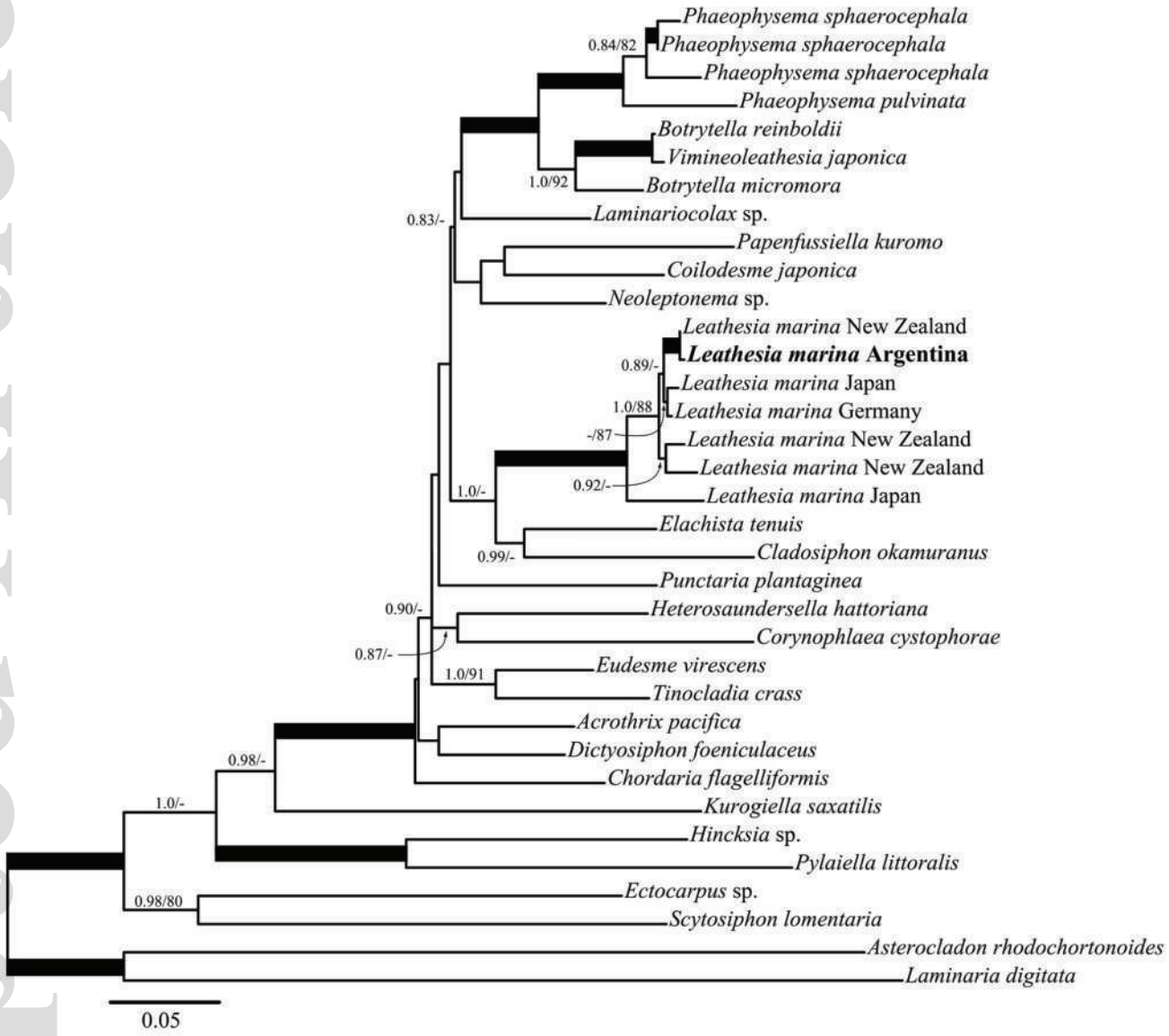

Shown: PP/BS; > 0.80/80, respectively
Thickened: PP > 0.98, BS > 95

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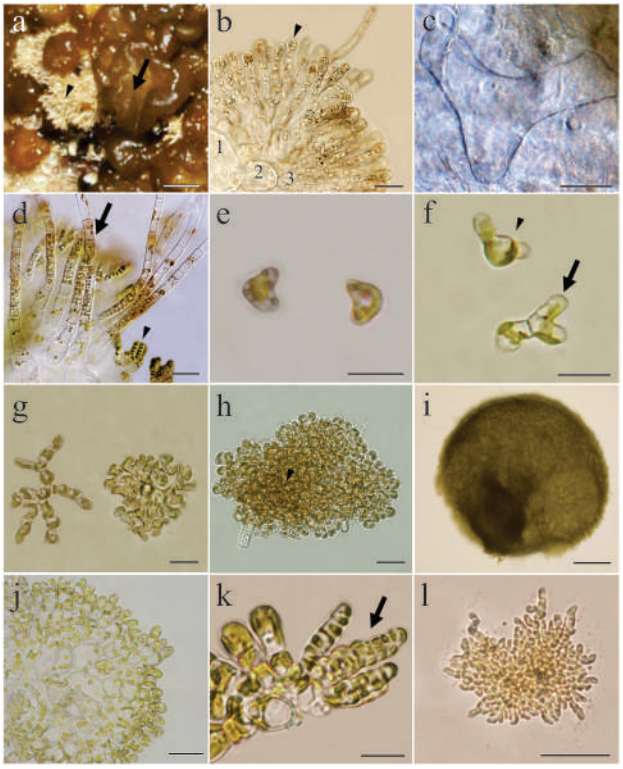

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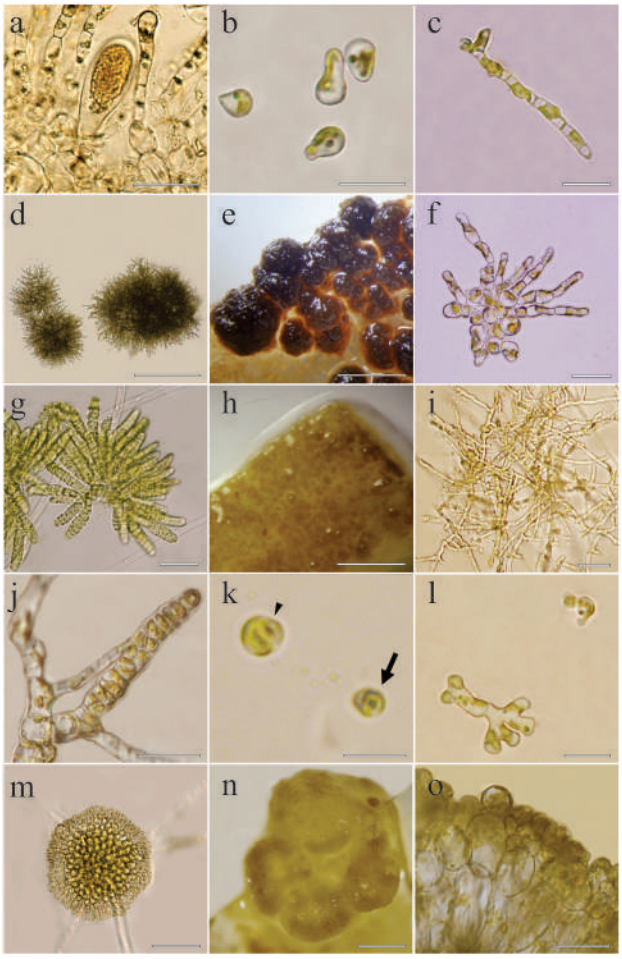

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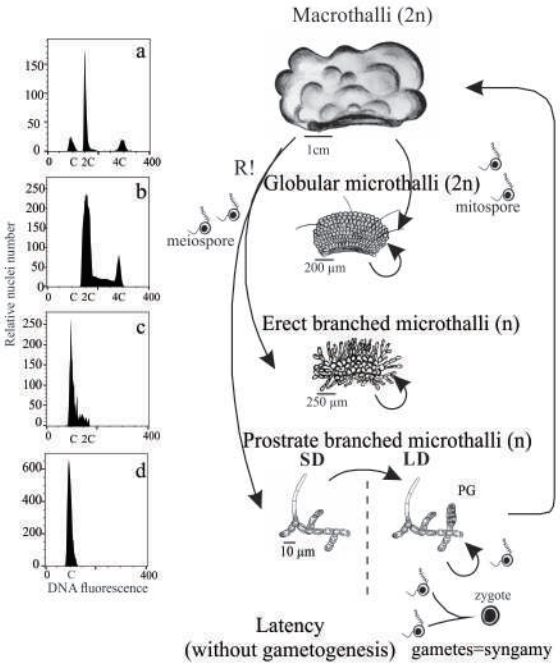

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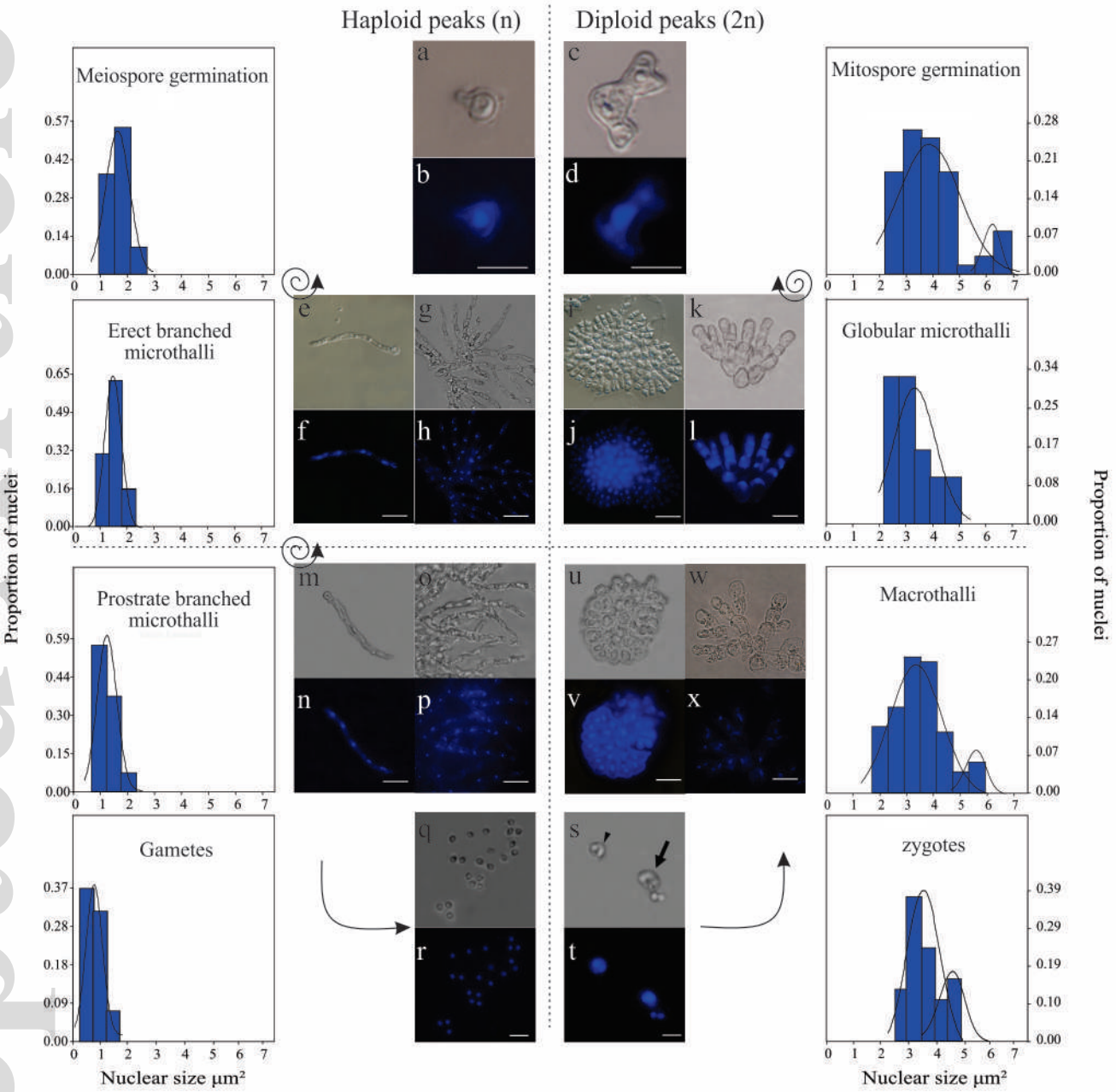

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