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Morphological and molecular evidence for the recognition of *Hypoglossum sabahense* sp. nov. (Delesseriaceae, Rhodophyta) from Sabah, Malaysia

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Culture isolates of the genus *Hypoglossum* (Delesseriaceae, Rhodophyta) were obtained and their development and morphological structure over many years were followed in the laboratory. Molecular data (*rbc*L, large subunit ribosomal DNA, and cytochrome *c* oxidase subunit I) were obtained from these strains and evidence presented to recognize the new species: *Hypoglossum sabahense* from Sabah, Malaysia. Because various aspects of morphology in culture specimens differ significantly from types based on field specimens we have to rely mainly on the molecular criteria in ascribing a new taxonomic name here. This also is complicated by the major lack of molecular phylogenetic evidence for *Hypoglossum* and other Delesseriaceae. The 'Germling Emergence Method' and 'serendipity' are proving valuable in discovering significant new taxa from laboratory cultures which otherwise might never be known.

Key Words: COI; Delesseriaceae; *Hypoglossum*; LSU; Malaysia; new species; *rbc*L

INTRODUCTION

The world's coasts are underexplored for their macroalgal diversity. In remote locations, phycologists are often constrained by limited time in the field and / or rudimentary laboratory facilities. Also, in tropical coral reef locations, much of the actual macroalgal diversity may not be conspicuous during diving surveys due to the naturally intense grazing activity in such ecosystems (e.g., Freshwater et al. 2017). In this context, the development of the Germling Emergence Method has enabled numerous new records and discoveries of macroalgal taxa around the world (Peters et al. 2015). The approach consists of collecting substratum samples in sterile tubes

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during fieldwork or diving surveys, followed by incubation in the laboratory, and isolation of unialgal cultures, which are subsequently characterized by microscopic and molecular means (Peters et al. 2015). The methodology has enabled the recent isolation and study of *Phrix spatulata* (E. Y. Dawson) M. J. Wynne, M. Kamiya & J. A. West from Easter Island (Muñoz et al. 2018), a novel *Desmarestia* species and a number of Ectocarpales species from the Canadian Arctic (Küpper et al. 2016) and many other cryptic macroalgae.

The genus *Hypoglossum* Kützing (Delesseriaceae, Rhodophyta) with 31 'accepted' species (Huisman and Lin 2018, Guiry and Guiry 2020) is widely distributed. Up to now only three species of *Hypoglossum* have been recorded from Malaysia: *H. caloglossoides* M. J. Wynne & D. L. Ballantine, *H. rhizophorum* D. L. Ballantine & M. J. Wynne, and *H. simulans* M. J. Wynne, I. R. Price & D. L. Ballantine (Masuda et al. 2001). Morphological observations have traditionally served as the primary method for systematic investigations in the genus. Up to now molecular phylogeny in this genus has been poorly investigated. With the removal of *Hypoglossum geminatum* Okamura to the segregate genus *Wynneophycus* by Jeong et al. (2016), data for only three species of *Hypoglossum* are currently available in GenBank (https://www.ncbi.nlm.nih.gov). We have examined the relationships of *Hypoglossum* culture strains, which were isolated from Sipadan Island, Malaysia, with other Delesserioideae algae using plastid ribulose-1,5-bisphosphate carboxylase / oxygenase large subunit (*rbc*L), nuclear large subunit ribosomal DNA (LSU rDNA), and mitochondrial cytochrome *c* oxidase subunit I (COI). Our present investigation (laboratory culture, morphology / reproduction and molecular analyses) of *Hypoglossum* specimens obtained in Malaysia expands the taxonomic knowledge of the genus (*rbc*L tree).

MATERIALS AND METHODS

Sipadan Island (Sabah, Malaysia 4.1150° N, 118.6287° E) was visited by FCK in December 2007. The collected samples were incubated at the Culture Collection of Algae and Protozoa (CCAP, Oban, Scotland) at 20°C, in Provasoli-enriched seawater (Starr and Zeikus 1987) with GeO_2 to suppress diatom growth. Algal thalli started to appear after around 1 month. Two of the samples from Turtle Patch, collected on 9 and 10 December, respectively, one from 25 m depth (# 091207-4) and one from 16 m depth (# 101207-1), yielded foliose red algal thalli, which were further investigated by JW at the University of Melbourne starting in June 2008. The first was designated as 4731 (CCAP 1344/1, https://www.ccap.ac.uk/) and the second as 4732 (CCAP 134/2) in JW culture records and determined to belong to the genus *Hypoglossum* based on vegetative morphology. Primary isolation and culture research methods used by JW are explained in West (2005).

Microscopy

All photography by JW for Figs 1-3 was taken with a Zeiss GFL bright field microscope (Zeiss Australia, Sydney, Australia) and Canon G3 camera (https://www.canon.com.au/). Most images by JW were of living cultured specimens. Fig. 3A was of a specimens fixed in 5% formalin, washed in distilled water, microwaved for 10 s at 800 mW to soften filaments, then placed on slides with 1-2 drops of 0.02% aniline blue in 50% corn syrup with 0.1% phenol to prevent fungal growth, excess AB imbibed around coverslip and twice sealed with nail polish.

For Fig. 2, the pressed herbarium specimen of cultured *H. sabahense* sp. nov. 4732 (sent from University of Melbourne to University of Michigan), MW soaked off blades, floated them on 2 glass slides, put on coverslips, added diluted Karo syrup around the edges, and gently warmed in an oven to dry the slides. Images were taken by MW and Pavel Klimov with a Leica DMC4500 camera attached to a Leica DM 2500 LED compound microscope (Jena, Germany) using brightfield illumination. All images were edited by SLG with Adobe Photoshop CS6 (http://www.adobe.com/au/) for publication.

Molecular phylogenetic analyses

The DNA extraction from live material was as described by Hayakawa et al. (2012). The primer sequences to amplify and sequence almost the entire *rbc*L, partial LSU, rDNA, and 5′ partial COI are the same as those in Muñoz et al. (2018). The polymerase chain reactions (PCR) were conducted in a total volume of 25 µL containing about 40 ng of template DNA, 0.4 µM of each primer, 0.25 mM of each dNTP, 1 PCR buffer and 0.5 units of KOD FX neo DNA polymerase (Toyobo, Osaka, Japan). The thermal profile for PCR was as follows: initial denaturation at 94°C for 2 min, 30 cycles at 98°C for 30 s, 50°C for 30 s and 68°C for 90 s, and final extension at 68°C for 7 min. Excess primers and dNTPs were removed from the PCR products using Exosap-it (Affymetrix, Santa Clara, CA, USA). DNA sequencing was performed by Eurofins Genomics (Tokyo,

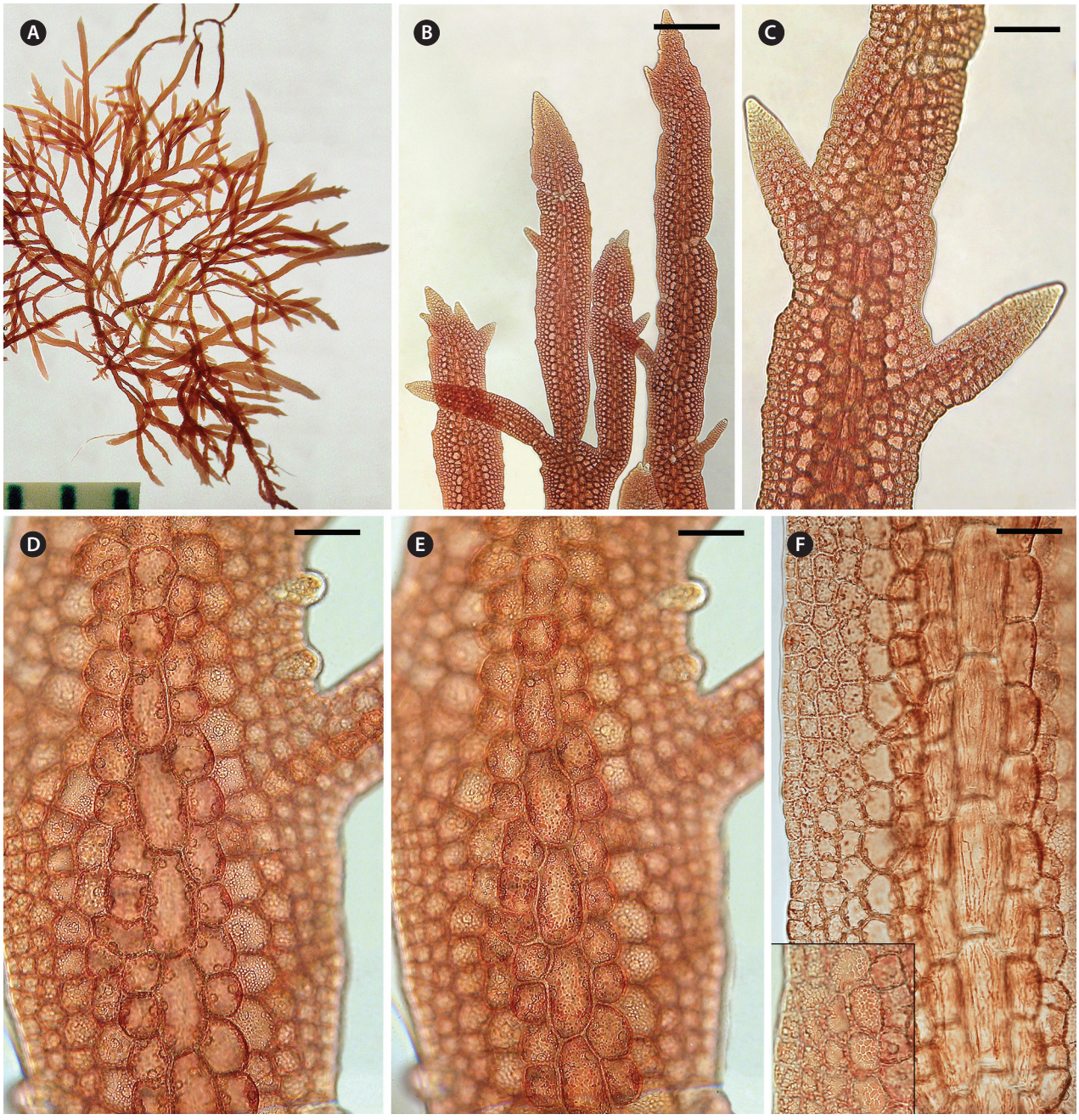

Fig. 1. *Hypoglossum sabahense* (4731). (A) Photo with stereoscope showing wide blades (190-260 µm) in brighter light and shaker growth. (B) Blades of variable widths have clear midrib (central and periaxial cells) and flank of second and third cell rows extending to margins. Margins irregular. (C) Main blade, two lateral blades (not opposite as seen in low light) appear to arise from periaxial cell or axial cell. (D) Periaxial cells, each with peripheral 4-6 nuclei, flank cells with 1-2 nuclei. Each 4-5 µm diameter nucleus outlined by tiny refractile particles (putative starch grains). Tiny plastids variable in shape (discoid to short irregular ribbons) and size (2-3 µm width). (E) Similar to Fig. 1D, periaxial and adjacent flank cells have numerous very small plastids visible. (F) Focus on spectacular long ribbon plastids in axial and periaxial cells of thallus grown in lower light. Numerous small round plastids of wing cells at margin also shown in enlarged inset. Smaller cells at blade margins mostly uninucleate. Inset: compactly arranged peripheral plastids in blade margin cells. Scale bars represent: A, 1 mm; B, 250 µm; C, 80 µm; D-F, 25 µm.

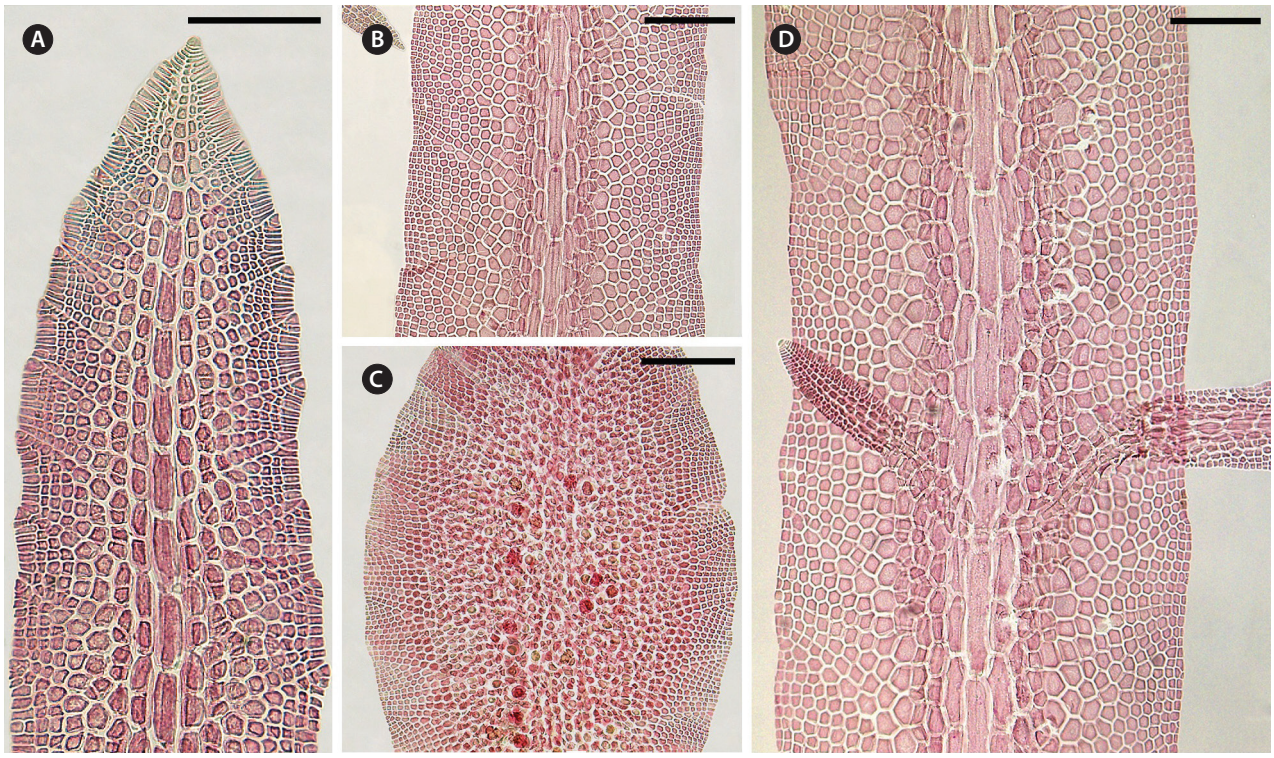

Fig. 2. *Hypoglossum sabahense* (4732). These images taken from HOLOTYPE in MICH. (A) Apical region of blade showing Type 1 pattern of growth. (B) Mid-region of blade with thickened midrib region. (C) Blade with tetrasporangial sorus extending over mid-region and sporangia lying in several layers. (D) Pair of branches arising from the parent blade away from the midline. Scale bars represent: A, 100 µm; B & C, 200 µm; D, 150 µm.

Japan) and the sequences were deposited in DDBJ / ENA / GenBank (accession numbers LC541575-LC541577). The acquired LSU rDNA sequences were aligned using the MAFFT ver. 7 online application (http://mafft.cbrc.jp/alignment/software/) based on the L-INS-i algorithm with default parameters, which reflected RNA secondary structure. Adjustments of the resulting alignments were performed manually (final length was 1,926 bp). No indels were present in *rbc*L (final length was 1,333 bp) and COI sequences (final length was 664 bp). The models for each codon of *rbc*L selected by the program Kakusan4 (Tanabe 2011) are shown in Supplementary Table S1.

Maximum likelihood trees were constructed using the TREEFINDER ver. March 2011 software (http://www.treefinder.de) and were evaluated by bootstrap analysis with 1,000 replicates. For Bayesian inference analysis, Markov chain Monte Carlo (MCMC) iterations were conducted for 5,000,000 generations, with sampling every 100 generations using MrBayes 3.2.6 (http://mrbayes.sourceforge.net). Two independent simultaneous MCMC runs were performed to assess appropriate chain mixing and to ensure topological convergence (split frequency " 0.01). After inspection of convergence and stabilization with Tracer 1.5 (http://tree.bio.ed.ac.uk/software/tracer), the first 10% of generations were discarded as burn-in and clade posterior probabilities were computed from the remaining trees.

RESULTS

Hypoglossum sabahense M. J. Wynne, M. Kamiya & J. A. West sp. nov. (Figs 1-3)

Holotype. designated herein West 4732. Deposited in MICH (Herbarium of the University of Michigan, U.S.A.) [MICH 660141].

Type locality. Sipidan Island, Sabah, Malaysia (4.1150° N, 118.6287° E). 10 Dec, 2007. Original material collected by Frithjof Kepper at 16 m depth.

Etymology. "*sabahense*" refers to the origin of the sample, from the state of Sabah, Island of Borneo, Malaysia. Original culture CCAP No. 1344/2; further isolated by John West and grown in culture (West No. 4732) (Figs

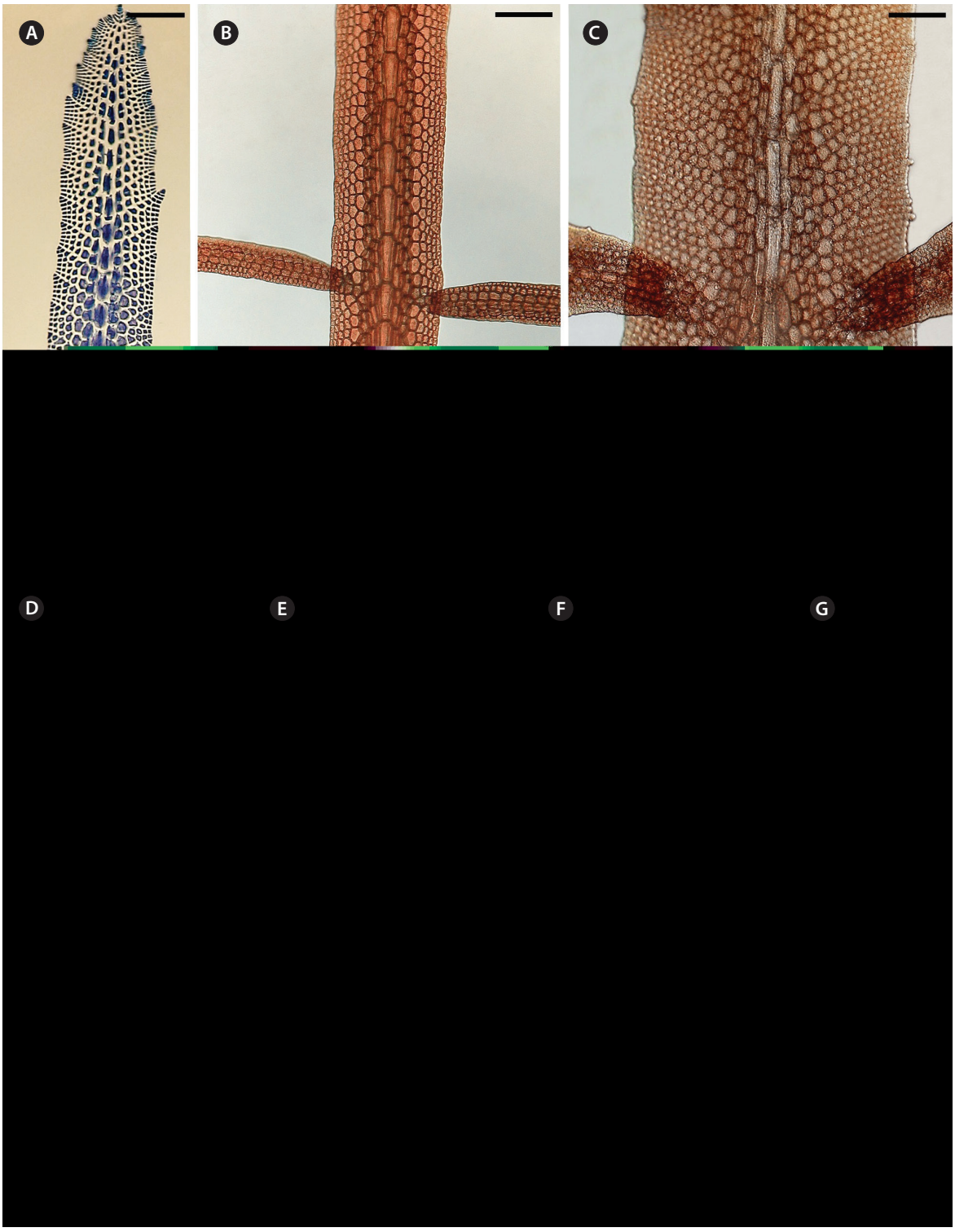

Fig. 3. *Hypoglossum sabahense.* (A-C & G, 4731; D-F, 4732). (A) Blade fixed, stained with aniline blue. 2nd order cell rows extend from periaxial cells forming short lateral blades and dentate margin. 3rd order cell rows extending to blade margin. Blade 240 µm wide. Grown in high light (8-9 µmol photons) and shaker. (B) Pair of opposite blades. Grown in low light (1-2 µmol photons) and stationary culture. (C) Pair of opposite blades extending from axial cell row (arrowheads). (D) Rhizoid initial with 6 nuclei, developing from wing cell at blade margin. Grown in high light (8-9 µmol photons) and shaker culture. (E) Blade grown in high light and shaker culture. (F) Blade with many rhizoids and some secondary blades (arrowheads) forming on blade margin; in low light and stationary culture. (G) Rhizoid arising from marginal wing cell, rhizoid cells 15-20 µm wide, many small discoid plastids in low light and stationary culture. Scale bars represent: A & B, 120 µm; C, 50 µm; D & G, 40 µm; E, 60 µm; F, 150 µm.