Erythrolobus australicus sp. nov. (Porphyridiophyceae, Rhodophyta): a description based on several approaches

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The unicellular marine red alga *Erythrolobus australicus* sp. nov. (Porphyridiophyceae) was isolated into laboratory culture from mangroves in Queensland and New South Wales, Australia. The single multi-lobed red to rose-red plastid has more than one pyrenoid and lacks a peripheral thylakoid. Arrays of small electron dense globules occur along the thylakoids. The nucleus is peripheral with a central to eccentric nucleolus. Each Golgi body is associated with a mitochondrion. The spherical cells are positively phototactic with slow gliding movement. The *psaA* + *psbA* phylogeny clearly showed that *E. australicus* is a distinct species, which is closely related to *E. coxiae*. The chemotaxonomically relevant and most abundant low molecular weight carbohydrate in *E. australicus* is floridoside with concentrations between 209 and 231 $\mu\text{mol g}^{-1}$ dry weight. Traces of digeneaside were also detected. These various approaches help to understand the taxonomic diversity of unicellular red algae.

Key Words: Australia; digeneaside; *Erythrolobus*; floridoside; molecular phylogeny; Porphyridiophyceae; ultrastructure; unicells

INTRODUCTION

The class Porphyridiophyceae of the division Rhodophyta forms a monophyletic group based on molecular data and is distinguished from other unicellular red algae by having specific ultrastructural features: Golgi bodies always associated with mitochondria and peripheral thylakoids absent in the plastid (Yang et al. 2010). Currently the class is comprised of four genera: *Porphyridium* with about 4 species [*P. purpureum* (Bory de

Saint-Vincent) K. M. Drew & R. Ross, *P. sordidum* Geitler, *P. aerugineum* Geitler and *P. marinum* Kylin] (Guiry and Guiry 2011); *Flintiella* with one species, *E. sanguinaria* Ott; *Erythrolobus* with two species, *E. coxiae* J. Scott et al. and *E. madagascarensis* E. Yang et al.; *Timspurckia*, with one species, *T. oligopyrenoides* E. Yang et al.

In *Flintiella* a pyrenoid is absent. In *Porphyridium* a single central pyrenoid is embedded within the plastid

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and the nucleus has a peripheral nucleolus. In *Erythrolobus* one or more 'naked' pyrenoids (bordering the cytoplasm) and a nucleus with a central or eccentric nucleolus are present. In *Timspurckia* 2-4 naked pyrenoids per cell and a nucleus with a peripheral nucleolus are present. In *Porphyridium* (Gantt and Conti 1965, Gantt et al. 1968, Schornstein and Scott 1982) the pyrenoid is traversed by thylakoids that appear to be flattened sheets (cisternae) while pyrenoids in *Erythrolobus* species and *Timspurckia* are traversed by tubular thylakoids (Yang et al. 2010). Molecular evidence clearly shows these genera to be in distinct clades (Yang et al. 2010).

The application of chemical characters has long been important in algal systematics. The distribution of low molecular weight carbohydrates (LMWCs) are useful chemotaxonomic characters particularly in red algae (Karsten et al. 1999, 2003). For an organic molecule to be useful as a chemotaxonomic character, it must be specific to a certain taxon or group of organisms. The distribution patterns of the main LMWC floridoside (α -D-galactopyranosyl-[1-2]-glycerol) and digeneaside (α -D-mannopyranosyl-[1-2]-glycerate), have been applied as chemotaxonomic characters in various red algae (Karsten et al. 1999, 2003, Eggert and Karsten 2010, Yang et al. 2010).

We have chosen the name *Erythrolobus australicus* sp. nov. for two marine unicellular red algal culture isolates from Queensland and New South Wales, Australia. They are closely related to *E. coxiae* and *E. madagascariensis* but *E. australicus* has 2-3 pyrenoids and arrays of electron dense bodies between the thylakoids forming a clade clearly distinguished from other lineages indicating it is a different species.

MATERIALS AND METHODS

Culture methods

Methods for collection, isolation and maintenance of cultures are presented in West and Zuccarello (1999) and West (2005). To promote growth of target unicellular red algae low nutrient levels were used initially (2 mL modified-Provasoli-media [MPM] enrichment per liter of sterile seawater). The seawater was adjusted to 30 psu with Milli-Q water (Millipore Corp., Billerica, MA, USA). Careful weekly observations with a dissecting stereomicroscope detected developing unicell colonies that were removed by a 100 μ L micro-pipet and subcultured in 50 $\times$ 70 mm crystallizing dishes containing 30 psu seawater

with 10 mL MPM enrichment per liter. Most cultures were maintained in 18-22°C, 5-20 μ mol photons $m^{-2} s^{-1}$ cool white fluorescent lighting with a 12 : 12 LD daily cycle. For low molecular weight carbohydrate analysis cultures were dried at 60°C and sent to UK.

Bright field microscopy

A small drop of cultured cells was placed on a glass slide, covered with a coverslip and sealed on the edges with VALAP (Pickett-Heaps et al. 2001). Motility of individual cells was followed for 5-10 min but no videomicroscopy was done. Images of live cells were taken on a Zeiss GFL microscope (Carl Zeiss, Thornwood, NY, USA) with a Canon G3 digital camera (Canon, Tokyo, Japan). Images were processed with Adobe Photoshop CS4 (Adobe Systems Incorporated, San Jose, CA, USA) for publication figures.

Confocal microscopy

Isolates were cultured with 10 mL ESM medium (Okaichi et al. 1982) in glass culture tubes for two weeks before fixation. Cultured cells were then transferred to 1.5 mL microtubes with 0.5 mL of culture medium, fixed with equal volume of 3% paraformaldehyde in PHEM buffer [60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2(6H_2O)$, pH 7.4] for 15 min. Ten μ L of the fixative solution were placed on a glass slide coated with 0.1 w/v% poly-L-lysine and incubated for 10 min at room temperature. Then equal volumes of SlowFade® Antifade (Invitrogen, Carlsbad, CA, USA) and 1,000 $\times$ diluted SYBR® Green I (Invitrogen) were added and incubated for 15 min. Confocal images were taken with an inverted microscope Axiovert 100M with LSM 510 laser scanning equipment (Carl Zeiss AG, Jena, Germany). Plastid autofluorescence and SYBR Green I fluorescence were detected with a 585 nm long pass filter, 505 to 530 nm band pass filter, respectively, in the excitation line of a 488 nm argon laser and 543 nm He/Ne laser using single-track mode as described in Hirakawa et al. (2010).

Transmission electron microscopy

Cells from culture were filtered onto poly-L-lysine coated 0.45 μ m Millipore™ filters and fixed for 2 h at ambient temperature in 2% glutaraldehyde in a 0.1 M phosphate buffer solution (pH 6.8) with 0.15 M sucrose. Following buffer rinses samples were post-fixed 1 h in the same buffer with 1% OsO_4 at 4°C, rinsed thoroughly in

H₂O, left in 50% acetone for 30 min and stored in a 70% acetone-2% uranyl acetate solution at ambient temperature for 20 h. Samples were then further dehydrated in a graded acetone series, infiltrated gradually and embedded in Spurr's resin and polymerized at 70°C for 2-3 days. Thin sections were cut with an RMC MT6000-XL ultramicrotome (RMC Inc., Tucson, AZ, USA). Sections were stained 1 min with lead acetate. A Zeiss EM 109 electron microscope was used for observation and photography.

Molecular analyses

Genomic DNA was extracted from each culture strain using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), according to the manufacturers' instructions. PCR and sequencing were performed as described in Yang et al. (2010). Specific primer sets for two plastid genes were used: *psaA*130F-*psaA*1110R and *psaA*971F-*psaA*1760R for *psaA* and *psbA*F-*psbA*R2 for *psbA*. Amplified DNA was purified with the QIAquick PCR purification kit (Qiagen) and sent to a commercial sequencing company. The electropherogram output for each specimen was edited using the program Chromas version 1.45 (<http://www.technelysium.com.au/chromas.html>). Newly determined *psaA* and *psbA* sequences (JF440648-JF440651) were compared and aligned with published data by using program Se-Al version 2.0a11 (<http://tree.bio.ed.ac.uk/software/seal/>). The translated amino acid sequences were used for the DNA alignment. The introns of *psaA* (Yoon et al. 2006, 2010) were excluded in subsequent analyses.

Appropriate evolution models for DNA and protein data were selected by the program ModelGenerator v.0.85 (Keane et al. 2006). Based on Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC), general time reversible (GTR) substitution model for DNA and LG (Le and Gascuel 2008) for protein were selected as the best fit evolution models, respectively. Maximum likelihood (ML) analyses were performed using RAxML version 7.2.6 (Stamatakis 2006). Tree likelihoods were estimated using 100 independent replications using an unfixed GTR with rate heterogeneity among sites (gamma distribution, Γ) parameter. Automatically optimized Sub-tree Pruning and Reconnection (SPR) rearrangement with rapid hill climbing algorithm was used for the best tree search. Maximum parsimony (MP) analyses were conducted using the PAUP* version 4.0b10 (Swofford 2002). The heuristic search was carried out with 1,000 replicates, random sequences addition and Tree Bisection Reconnection (TBR) branch swapping. Bootstrap analyses were carried out with 1,000 replicates to test of

monophyly under the same ML (MLBt) and MP (MPBt) condition.

Bayesian analysis for DNA data was conducted with MrBayes version 3.2 (Ronquist and Huelsenbeck 2003) using GTR + Γ parameter, which was same evolution model in ML analyses. The GTR rates and Γ values were not fixed. Two independent runs of 2 million generations were performed with four chains and trees were sampled every 100 generations. The burn-in point was identified by the average of standard deviation from split frequencies (< 0.01) between two independent runs. Trees sampled after the burn-in point were used to infer the Bayesian posterior probability (BPP).

Low molecular weight carbohydrates (LMWC) analyses

Both heterosides (floridoside and digeneaside) were separated and quantified by high-performance liquid chromatography (HPLC). For the chromatographic analyses, 10-15 mg dry weight (DW) of red algal samples were extracted in 1 mL of 70% (v/v) ethanol and heated for 3 h in a waterbath at 70°C. After centrifugation for 5 min at 6,200 $\times$ g, 700 mL of the supernatant were evaporated to dryness under vacuum (Speed Vac Concentrator SVC 100H; Savant, Farmingdale, NY, USA). Dried extracts were redissolved in 700 mL of distilled water, sonicated for 1 min and vortexed for 30 s. After centrifugation at 16,000 $\times$ g, the supernatants were analysed with an isocratic Agilent HPLC system equipped with a differential refractometer. The LMWCs were separated (floridoside and digeneaside) using a resin-based Phenomenex (Rezex ROA-Organic Acid, 300 $\times$ 7.8 mm O.D.) column protected with a Phenomenex Carbo-H+ guard cartridge (4 $\times$ 3 mm I.D.) and quantified with an isocratic Agilent HPLC system equipped with a differential refractometer (Karsten et al. 2005). On this column both heterosides were eluted with 5 mM H₂SO₄ at a flow rate of 0.4 mL min⁻¹ at 75°C. Peaks on both columns were identified by comparison of the respective retention times with those of standard compounds and quantified by peak areas. The concentrations were expressed in μ mol g⁻¹ DW.

RESULTS

Diagnosis

Erythrolobus australicus sp. nov. E. C. Yang, J. L. Scott et J. A. West. Cellulae sphaericae 10-20 μ m diametro, ma-

trice extracellulari < 1 µm crassa, roseo-rubrae vel rubrae, phototacticae. Plastidus singulis lobis multis extensis a pyrenoidibus multis per totam cellulam distributis; pyrenoidibus thylacoides tubulares numerosas continentibus, cytoplasmati expositis, plerumque granulis amyli circumcinctis; thylacoidibus peripheralibus nullis. Conso-cies globulorum parvorum (30-60 nm) uniformium electronibus opacorum inter thylacoides saepe visae. Omne corpus Golgi uno mitochondrio semper conso-ciatum et prope peripheriam cellulae positum. Nucleus peripheralis nucleolo singulo centrali vel excentrico. Carbonii hydrata parvi ponderis floridosidum et digeneasidum.

Cells spherical, 10-20 µm in diameter, extracellular matrix (< 1 µm thick), rose-red to red, phototactic. Single plastid with numerous lobes extending from multiple pyrenoids located throughout the cell. Pyrenoids containing numerous tubular thylakoids border the cytoplasm and usually surrounded by starch granules. Peripheral thylakoids are absent. Arrays of small (30-60 nanometers) uniform electron dense globules often occur between thylakoids. Each Golgi body always associated with a mitochondrion and located near the cell periphery. The nucleus peripheral with a single central to eccentric nucleolus. LMWCs are floridoside and digeneaside. Holotype: Cryopreserved culture - CCMP 3123 (JAW3904). Paratype: Cryopreserved culture - CCMP 3124 (JAW4188). Bigelow Laboratory for Ocean Sciences, P.O. Box 475, 180 McKown Point Road, West Boothbay Harbor, ME 04575, USA.

Iconotype: Figs 1-3 of this paper.

Live culture specimens: holotype, CCAP 1393/7 (JAW 3904); paratype, CCAP 1380/8 (JAW4188). Culture Collection of Algae and Protozoa, Scottish Association for Marine Science, Scottish Marine Institute, Dunstaffnage Marine Laboratory, Dunbeg, Oban, Argyll, PA37 1QA, UK. Holotype locality: Culture 3904 was isolated from a cultured specimen of *Caloglossa vieillardii* (Kützinger) W. Setchell collected by G. C. Zuccarello and J. A. West on 25 July 1998, at Tewantin, Queensland, Australia (26°23' S 153°02' E).

Paratype locality: Culture 4188 was isolated from a cultured specimen of *Bostrychia moritziana* (Sonder ex Kützinger) J. Agardh collected by G. C. Zuccarello on 1 October 2001 at Woolooware Bay, New South Wales, Australia (34°01' S 151°09' E).

Bright field microscopy

The cell diameter of both isolates varied from 10-20

µm in diameter. The cellular details were difficult to discern with conventional brightfield microscopy. Even with a careful through-focus it was hard to see more than one pyrenoid in many cells but when the cell preparation was fully flattened by the weight of the coverslip, pyrenoids (Fig. 1A & E) and the nucleus (Fig. 1E) were more visible. The dark refractive outline of starch grains around the pyrenoid aided in their identity. The narrow fissures between plastid lobes were especially visible when focused at the outermost level (Fig. 1F). During observations on cell motility the cells remained spherical and rotated slowly while gliding without substrate contact. Polysaccharide tails visible on some cells (Fig. 1B & G) did not appear to be involved in movement.

Confocal microscopy

Late stages of cell division were observed in cells of 3904 (Fig. 1C). The nucleus was 3.2-3.5 µm in diameter and positioned near the perimeter between plastid lobes. The plastid divided after the nucleus. Plastid genophores were spherical to elongate up to 0.37-0.55 µm long. As many as 15 genophores were visible in a single optical section but it was not possible to determine if they were connected. In isolate 4188 3 pyrenoids (Fig. 1D) were visible as dark areas (2.4-2.8 µm in diameter) when a series of focal levels were photographed.

Transmission electron microscopy

Since both isolates of *Erythrolobus australicus* sp. nov. were collected approximately 1,000 km from each other, it was of interest to determine if cellular details of each isolate were also indistinguishable. Therefore, ultrastructural features of each isolate are presented separately.

Isolate 3904 - Queensland. Most transmission electron microscopy (TEM) observations revealed cells showing either a single pyrenoid and / or nucleus. However, examination of numerous thin sections and hundreds of cells provided views of cells with a medially sectioned nucleus and one or more pyrenoids (Fig. 2A). Occasionally three pyrenoids were seen in a single non-dividing cell (Fig. 2B). The extracellular sheath is faintly visible and approximately 0.5 µm thick. A peripheral endoplasmic reticulum (ER) system was just beneath the cell membrane (Fig. 2B) but short tubules that interconnected this system with the cell membrane, as seen in many red algal unicells (Scott et al. 2006), were not detected. The 2.5-3.0 µm nuclei (Fig. 2A) contained a single central to eccentric nucleolus and were usually near the cell periphery but

Fig. 1. *Erythrolobus australicus* sp. nov. Bright field microscopy (A, B & E-G). Confocal microscopy (C & D). Fluorescent SYBR[®] Green showing the nucleus (green), plastid genophores (yellow) and plastid autofluorescence (red). Isolate 3904 in A-C, and isolate 4188 in D-G. (A) Most plastids with only one pyrenoid visible, some show two or three (black arrows). Bright field images do not show the pyrenoids as clearly as confocal images. (B) Some cells with a polysaccharide tail extending from the extracellular matrix (hollow arrows). (C) These views show the peripheral nucleus (green) and plastid genophores (yellow) in 3 stages of cell division. (D) Three pyrenoids (white arrowheads) are visible at different focal levels in same cell. Nucleus is peripheral on the left. (E) Bright field microscopy of various cells shows 1-3 pyrenoids (black arrows) per cell and the single nucleus (black arrowheads). (F) The narrow fissures of the multilobed plastids are distinct in cells focused at the outer surface of the cells. (G) Polysaccharide tails (hollow arrows) similar to those of 3904 in Fig. 1B are visible on some cells. Scale bars represent: A-G, 12 μ m.

Fig. 2. *Erythrolobus australicus* sp. nov. (3904) ultrastructure. (A) Low magnification electron micrograph of a medially sectioned cell. Two electron dense pyrenoids containing tubular thylakoids are bounded by starch grains (arrows). The nucleus (N) contains an eccentric nucleolus. Thylakoids within plastid lobes (white asterisks) are not clearly resolved. The cell is enveloped by a thin extracellular matrix. (B) Low magnification electron micrograph of a medially sectioned cell. Three pyrenoids can be seen extending from plastid lobes into a centralized vacuole region (V). A peripheral endoplasmic reticulum system is beneath the cell membrane (arrowheads). Several random arrays of electron dense globules are present between the thylakoids (arrows) and a peripheral thylakoid is absent (asterisks). (C) High magnification electron micrograph of a Golgi body (asterisk) associated with a mitochondrion (M). A peripheral thylakoid is absent as seen in termini of plastid lobes (arrows). Scale bars represent: A, 1.8 µm; B, 1.5 µm; C, 0.5 µm.

interior to other cellular components, mainly the plastid. Electron dense heterochromatin was never seen.

The plastid occupied the majority of the cell interior and was quite variable in shape (Fig. 2A & B). It was obvious that plastid morphology was strongly influenced by pyrenoid numbers since plastid lobes extended from naked pyrenoids that projected into the cytoplasm. We could not determine if all cells contained multiple pyrenoids since serial thin-sectioning was not attempted. Pyrenoids appeared variable in size in thin sections, the largest being about the same size as nuclei. Many pyrenoids showed two or more connections to the plastid lobes (Fig. 2A & B). The pyrenoids were electron dense with numerous tubular electron transparent thylakoids extending from the plastid lobes (Fig. 2A & B). Cytoplasmic starch grains commonly were associated with the pyrenoids but were also found in other cell regions. Linear arrays of electron dense globules, 40-60 nm in diameter and of unknown function, were frequently seen in random locations between the thylakoids in plastids (Fig. 2B). Peripheral thylakoids were absent (Fig. 2B & C).

Golgi bodies were found usually at the cell periphery, always in close association with a mitochondrion (Fig. 2C). Unlike most red algal unicells (Broadwater and Scott 1994), a lobed vacuolar region occurs in most cells (Fig. 2B).

Isolate 4188 - New South Wales. Cell ultrastructure of this isolate (Fig. 3) was virtually identical to that of isolate 3904. The peripheral ER system was seen in some cells but was not well preserved. Although many sectioned cells were observed, none was seen with more than two pyrenoids. The naked pyrenoids were electron dense and filled with numerous thylakoids. Some pyrenoids (Fig. 3A & B) were associated with fewer starch grains than others (Fig. 3C). Peripheral thylakoids were not seen but hemi-discoidal phycobilisomes were more obvious (Fig. 3D) than in 3904. The nucleus was near the periphery with a single central to eccentric nucleolus (Fig. 3A, B & C) and no visible heterochromatin. Linear arrays of electron dense globules were present between thylakoids and a vacuolar region was conspicuous. Golgi bodies associated with a mitochondrion were near the cell periphery.

Molecular analyses

Newly determined sequences were aligned with 27 sequences from Genbank, which included seven red algal classes. The *psaA* gene was aligned to 1,365 base pairs including 719 variable positions (52.7%). The *psbA* gene was aligned to 873 bp including 374 variable positions (42.8%). Within the Porphyridiophyceae, sequence di-

vergence of *psaA* ranged from 11.2% (153 nucleotide [nt]) between *E. australicus* and *E. coxiae*; to 22.3% (305 nt) between *T. oligopyrenoides* and *P. aerugineum*. The *psbA* gene varied by 3.8% (33 nt) between *E. australicus* and *E. coxiae*; to 9.7% (85 nt) between *T. oligopyrenoides* and *P. aerugineum*.

ML phylogeny using concatenate *psaA* + *psbA* data supports the monophyly of the Porphyridiophyceae (96% MLBt, 75% MPBt and 1.0 BPP for DNA; 64% MLBt for protein data). The monophyly of the genus *Erythrolobus* was strongly supported (100% MLBt, 99% MPBt and 1.0 BPP for DNA; 100% MLBt for protein). Within the *Erythrolobus* clade, *E. australicus* was clearly separated from other *Erythrolobus* species but showed a sister-group relationship with the type species, *E. coxiae* with strong statistical supports (100% MLBt, 99% MPBt and 1.0 BPP for DNA; 73 MLBt for protein) (Fig. 4).

LMWC analyses

The LMWC data clearly indicate that members of the Porphyridiophyceae typically contain floridoside as their main LMWC along with digeneaside as a minor, sometimes even as a trace, component except in both *E. coxiae* isolates where the amount of digeneaside was relatively high (Table 1). Both *E. australicus* strains (3904, 4188) exhibited floridoside levels between 209 and 231 $\mu\text{mol g}^{-1}$ DW and very low concentrations of digeneaside (1-1.5 $\mu\text{mol g}^{-1}$ DW). Since the HPLC methodology for digeneaside detection and quantification exists only since 2005 (Karsten et al. 2005), earlier samples could not be analysed for this heteroside.

DISCUSSION

Bright field microscopy

Although video microscopy of cell motility was not done for either 3904 or 4188 the spherical cells showed slow gliding motility. Some cells showed long polysaccharide tails. These movements were similar to those seen in the other porphyridiophycean algae, *Erythrolobus* sp. (3797) from Fiji, *E. madagascarensis* (4329) from Madagascar, *T. oligopyrenoides* (3827), from Victoria, and *Porphyridium purpureum* (3630), an endosymbiont of peneroplid foraminifera, that were videoed and described in (Pickett-Heaps et al. 2001, Yang et al. 2010). No amoeboid motility or pseudopodia were evident in any of these.

Fig. 3. *Erythrolobus australicus* sp. nov. (4188) ultrastructure. (A) Low magnification electron micrograph of a medially sectioned cell revealing a predominant plastid. Random arrays of electron dense globules (arrows) are present within the plastid lobes (white asterisks). A portion of the vacuolar region (V) is next to the nucleus (N), which contains a slightly eccentric nucleolus. The central, electron dense pyrenoid is not closely associated with starch grains. (B) Low magnification electron micrograph of a medially sectioned cell. Two pyrenoids are visible, one interconnecting plastid lobes (white asterisks) and the other appearing as a plastid lobe protrusion bordered by starch grains (arrow); Vacuolar regions (V). (C) Low magnification electron micrograph of a cell sectioned through a region revealing only a small portion of the plastid and a single pyrenoid. Nucleus (N) with eccentric nucleolus; Vacuolar regions (V); Golgi mitochondrion association (asterisk). (D) High magnification electron micrograph of a Golgi body (asterisk) associated with a mitochondrion (M). Thylakoids terminate against plastid envelope (arrow) due to absence of a peripheral thylakoid. Scale bars represent: A, 2.3 μm ; B, 1.25 μm ; C, 1.4 μm ; D, 0.6 μm .

Fig. 4. The most likelihood phylogeny of *Erythrolobus australicus* based on *psaA* + *psbA* data (-lnL = 25252.028). Support values shown on the branch are maximum likelihood (MLBt), maximum parsimony (MPBt), and Bayesian posterior probability (BPP) from DNA data and under the branch is MLBt from protein data. Two GenBank accession numbers in parenthesis indicate *psaA* and *psbA*, respectively. One accession number indicates plastid genome data. Five major ultrastructural characters of the Porphyradiophyceae were summarized.

Confocal microscopy

Plastid genophores of *E. coxiae* as seen in confocal fluorescence microscopy and in TEM were spherical to subspherical and measured about 0.25 μm diameter (Scott et al. 2006). By contrast those of *E. australicus* appear to be more variable in size and shape, spherical to elongate, localized mostly in the central plastid, not in the peripheral lobes, and perhaps up to 15 per plastid. The confocal images indicate these are individual not continuous genophores. Coleman (1985) summarizes comparative information about red algal genophores.

Transmission electron microscopy

TEM has significantly enhanced our knowledge about red algal unicells. Several new genera and species have been described since the middle of the 20th century and have been shown to differ, often subtly but sometimes dramatically, in their subcellular architecture. These differences, of course, have been a part of the basis for describing new taxa. More recently, molecular techniques

have greatly contributed to unicellular red algal systematics (e.g., Yoon et al. 2006, Yang et al. 2010). Biochemistry of LMWCs also is important (Karsten et al. 1999, 2003, Scott et al. 2008, Yokoyama et al. 2009, Yang et al. 2010). These three approaches have worked in concert to establish a useful and rational explanation of unicellular red algal systematics.

Currently red algae are placed in two subphyla of the phylum Rhodophyta, the Cyanidiophytina and Rhodophytina. The three rather unusual genera, *Cyanidium*, *Cyanidioschyzon* and *Galdieria*, constituting the former subphylum (Yoon et al. 2010) will not be considered in our discussion. The Rhodophytina consists of six classes, two are represented only by unicells (Rhodellophyceae and Porphyridiophyceae) whereas one other class, the Stylonematophyceae, is comprised of unicells and small filamentous and pseudofilamentous multicellular algae. Numerous attempts to prepare the two unicellular genera (*Rhodosorus* and *Rhodospira*) in the Stylonematophyceae for ultrastructural studies have been mostly or completely inadequate to describe their subcellular organization (Scott personal observations).

Table 1. Low molecular weight carbohydrates (LMWCs) of Porphyridiophyceae in culture

Taxonomic assignment	Culture No.	Date	LMWCs ($\mu\text{mol g}^{-1}$ dry weight)	
			Floridoside	Digeneaside
<i>Erythrolobus australicus</i> sp. nov.	4188	Dec 2009	209.1 $\pm$ 10.6	< 1.0
	3904	Dec 2009	231.0 $\pm$ 11.9	1.5 $\pm$ 0.1
<i>Erythrolobus coxiae</i>	4299	Jun 2003	372.1 $\pm$ 41.2	154.3 $\pm$ 11.1
	4300	Jun 2003	329.3 $\pm$ 19.7	160.8 $\pm$ 17.2
<i>Erythrolobus madagascarensis</i>	4329	Dec 2009	138.6 $\pm$ 11.9	19.3 $\pm$ 2.3
<i>Erythrolobus</i> sp.	3797	2003	280.9 $\pm$ 10.1	n.a.
		Dec 2009	169.5 $\pm$ 0.1	18.4 $\pm$ 3.0
	4696	Dec 2009	< 1	16.0 $\pm$ 1.3
	4721	Dec 2009	224.7 $\pm$ 22.9	30.9 $\pm$ 4.9
	4777	Sep 2010	59.9 $\pm$ 25.3	< 1.0
<i>Porphyridium marinum</i>	2223	1999	191.0 $\pm$ 17.0	n.a.
		Mar 2010	112.2 $\pm$ 15.0	5.3 $\pm$ 0.8
<i>Porphyridium purpureum</i>	3630	1999	65.0 $\pm$ 12.0	n.a.
	4126	Mar 2010	127.1 $\pm$ 20.3	4.4 $\pm$ 0.6
	4719	Dec 2010	142.3 $\pm$ 32.5	18.2 $\pm$ 4.7
<i>Porphyridium sordidum</i>	4778	Sep 2010	38.8 $\pm$ 5.7	n.t.
<i>Timspurckia oligopyrenoides</i>	3827	2003	111.8 $\pm$ 5.2	n.a.
		2010	178.8-346.7	32.3-38.5
	4318	Dec 2009	304.2 $\pm$ 12.2	3.2 $\pm$ 0.2

Shown are the mean values of 3-4 replicate samples ($\pm$ standard deviation).

n.a., not analysed; n.t., no trace.

Several ultrastructural features have proven to be of greater value than others in distinguishing the Rhodellophyceae and Porphyridiophyceae. A very simple, straight forward character is that Golgi bodies are always closely associated with a mitochondrion in the Porphyridiophyceae while they are not in the Rhodellophyceae. In the Rhodellophyceae Golgi bodies are associated either with ER or with the functionally equivalent outer membrane of the nuclear envelope (Scott et al. 2008, Yokoyama et al. 2009). Also, peripheral thylakoids are absent in plastids of all members of the Porphyridiophyceae while they are present in *Dixoniella* and *Glaucosphaera* but absent in *Corynoplastis*, *Neorhodella*, and *Rhodella* of the Rhodellophyceae (Scott et al. 2008). Therefore, the Porphyridiophyceae appears to be a class with the most uniform ultrastructure (Table 2). Only one other assemblage of red algae, the gametophyte (macro) stages of bangiophycean genera (Pueschel 1990), consistently shares the combination of 1) Golgi bodies associated with mitochondria and 2) the absence of a peripheral thylakoid. However, it is unlikely that these cellular features found in these two groups are phylogenetically significant since all recent accounts of red algal molecular phylogeny (Saunders and Hommersand 2004, Yoon et al. 2006, 2010) indicate a rather distant interrelationship.

Two genera of the four relegated to this class, *Porphyridium* and *Flintiella*, stand apart from the three species of *Erythrolobus* and *Timspurckia* by virtue of pyrenoid characters. *Porphyridium* has a pyrenoid embedded in a stellate plastid and is not in contact with the cytoplasm; *Flintiella* lacks a pyrenoid (Yang et al. 2010). However, the three *Erythrolobus* species and *Timspurckia* all have naked pyrenoids usually in contact with closely appressed starch grains located in the cytoplasm. *Erythrolobus coxiae* and *E. madagascarensis* differ from each other in that the nucleus is peripheral and the pyrenoid is central in *E. coxiae* while both the nucleus and pyrenoid are eccentric in *E. madagascarensis*. Additionally, *E. madagascarensis* uniquely contains numerous, conspicuous fibrous vesicles at all times (Yang et al. 2010). The similarities and differences in the previously described Porphyridiophycean species are discussed in Table 2 and Yang et al. (2010).

While evolutionarily distant from each other based on molecular analysis, at first appearance *E. australicus* closely resembles *T. oligopyrenoides*. Both species routinely possess multiple pyrenoids and their overall plastid morphology is more complex and less uniform than in *E. coxiae* and *E. madagascarensis*. However, there is a distinct and persistent dissimilarity between *E. australicus* and *T. oligopyrenoides*. The nucleolus in *E. australicus*,

similar to other *Erythrolobus* species, is central or eccentric in the nucleus. The nucleolus in *T. oligopyrenoides* is always located at the periphery of the nucleus, as it is in *Porphyridium* (Yang et al. 2010). The function of this unusual feature is not understood but it is remarkable in that once again the molecular relationships parallel ultrastructural differences in the Porphyridiophyceae. So far as is known, each species can be recognized by its own unique ultrastructure, except possibly for *Porphyridium* where only two of the four reported species, *P. purpureum* (Gantt and Conti 1965) and *P. aeruginum* (Gantt et al. 1968), have been carefully described with TEM. A dichotomous key of the Porphyridiophyceae taxa based on ultrastructural characters is in Appendix A.

Molecular phylogeny

The topology of *psaA* + *psbA* phylogeny was congruent with the seven-class system based on multigene data reported in previous studies (Yoon et al. 2006, Yang et al. 2010). The monophyly of the Porphyridiophyceae was strongly supported, although inter-class relationships were not fully resolved. Within the Porphyridiophyceae interrelationships among the four genera were not well resolved. Additional sequence data are needed to resolve the generic relationships within the Porphyridiophyceae.

The monophyly of the genus *Erythrolobus* and species level relationships within the genus were strongly supported. *Erythrolobus madagascarensis* was positioned sister to *E. coxiae* and *E. australicus*. The new isolates (JAW 3904 = CCAP 1380/7 = CCMP 3123, JAW 4188 = CCAP 1380/8 = CCMP 3124) were clearly separated from *E. coxiae* and *E. madagascarensis*. This strongly suggests that the two new isolates should be recognized as a novel species.

Low molecular weight carbohydrates

In all the Porphyridiophyceae isolates tested so far (Table 1) floridoside is the primary LMWC varying from 38 to 330 $\mu\text{mol g}^{-1}$ DW and digeneaside ranging from < 1.0 to 154 $\mu\text{mol g}^{-1}$ DW with the exception of *Erythrolobus* sp. (4696) in which the digeneaside level is much greater (16 $\mu\text{mol g}^{-1}$ DW) than floridoside (1 $\mu\text{mol g}^{-1}$ DW). Further investigation is required to understand the carbohydrate physiology of this isolate.

Floridoside is the most common LMWC and photo-assimilatory product in almost all red algal orders. In contrast, members of the Ceramiales (Florideophyceae) generally synthesize and accumulate digeneaside in-

Table 2. Principal ultrastructural features of species in the Porphyridiophyceae: *Erythrolobus*, *Flintiella*, *Porphyridium* and *Tinspurckia*

	<i>Porphyridium</i> spp.	<i>Flintiella</i> <i>sanguinaria</i>	<i>Erythrolobus</i> <i>coxiæ</i>	<i>Erythrolobus</i> <i>madagascariensis</i>	<i>Erythrolobus</i> <i>australicus</i> sp.nov.	<i>Tinspurckia</i> <i>oligopyrenoides</i>
Golgi association	Mitochondrion-ER	Mitochondrion-ER	Mitochondrion-ER	Mitochondrion-ER	Mitochondrion-ER	Mitochondrion-ER
Plastid with peripheral thylakoid	(-)	(-)	(-)	(-)	(-)	(-)
Pyrenoid	(+)	(-)	(+)	(+)	(+)	(+)
Pyrenoid numbers	1	0	1; 2 or more in old cells	1	2 or more	2 or more
Pyrenoid / Location	Central, embedded ^a	NA	Central, naked ^b	Eccentric, naked	Random locations, naked	Random locations, naked
Nucleus location	Peripheral	Peripheral	Peripheral	Eccentric	Peripheral	Peripheral
Nucleolus position	Nucleus periphery ^c	Nucleus center	Nucleus center	Nucleus center	Nucleus center / eccentric	Nucleus periphery ^d
Persistent fibrous vesicles	(-)	(-)	(-)	(+)	(-)	(-)

ER, endoplasmic reticulum; NA, not applicable.

^aEmbedded in center of chloroplast, not exposed to cytoplasm / starch.^b'Naked', exposed to cytoplasm / starch sheath.^cFacing cell center.^dFacing everywhere except cell periphery.

stead of floridoside, and hence the latter heteroside was proposed as chemotaxonomic marker for the Ceramiales (Kremer 1978, Eggert and Karsten 2010). However, it has been demonstrated that the ceramialean genera *Laurencia* and *Osmundea* produce and accumulate floridoside instead of digeneaside (Barrow et al. 1995). In addition, members of other red algal orders such as the Gigartinales (Florideophyceae) contain floridoside along with digeneaside (Karsten et al. 2007). The same is true for the investigated porphyridiophyceae strains of the present study. Consequently, the use of digeneaside as a chemotaxonomic marker in red algae has to be taken with great care.

Nevertheless, LMWC patterns are useful in defining each of the three classes containing unicellular red algae. Digeneaside and the sugar alcohol sorbitol (*Rhodossorus*) or sorbitol and dulcitol (*Rhodospira*) are in the two unicells of the Stylonematophyceae whereas the polyol mannitol is present in all examined members of the Rhodellophyceae (Karsten et al. 1999, 2003). Scott et al. (2006) demonstrated for the first time the occurrence of both floridoside and digeneaside in *E. coxiae*. Unfortunately, the presence of digeneaside in *Erythrolobus* was overlooked in Scott et al. (2008), who stated that all members of the Porphyridiophyceae (*Erythrolobus*, *Porphyridium*, and *Flintiella*) contain only floridoside. In a recent paper by Yang et al. (2010), however, *T. oligopyrenoides* and *E. madagascarensis* were reported to contain high amounts of floridoside and low to intermediate levels of digeneaside. This LMWC pattern is confirmed by this new species, indicating that the ultrastructural and molecular characters are well supported by this chemical information.

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Appendix A. Key to Porphyridiophyceae species using ultrastructural features

1. Nucleolus appressed to nuclear envelope2
1. Nucleolus in nucleus center4
2. Multiple pyrenoids bordering cytoplasm*Timspurckia oligopyrenoides*
2. Single pyrenoid embedded in plastid3
3. Phycobilisomes hemispherically shaped*Porphyridium purpureum*
3. Phycobilisomes hemisdiscoidally shaped*Porphyridium aeruginum*
4. Pyrenoids absent*Flintiella sanguinaria*
4. Pyrenoids present5
5. Predominantly multiple pyrenoids bordering cytoplasm.....*Erythrolobus australicus*
5. Predominantly single pyrenoid bordering cytoplasm6
6. Cytoplasm with abundant, persistent fibrous vesicles*Erythrolobus madagascariensis*
6. Cytoplasm without abundant, persistent fibrous vesicles*Erythrolobus coxiae*