On the genus *Rhodella*, the emended orders Dixonieiales and Rhodellales with a new order Glaucosphaerales (Rhodellophyceae, Rhodophyta)

Joe Scott¹, Eun Chan Yang², John A. West^{3,*}, Akiko Yokoyama⁴, Hee Jeong Kim², Susan Loiseaux de Goër⁵, Charles J. O'Kelly⁶, Evguenia Orlova¹, Su Yeon Kim⁷, Jeong Kwang Park⁷ and Hwan Su Yoon^{2,8,*}

¹Department of Biology, College of William & Mary, Williamsburg, VA 23187, USA

²Bigelow Laboratory for Ocean Sciences, 180 McKown Point Road, West Boothbay Harbor, ME 04575, USA

³School of Botany, University of Melbourne, Parkville, Victoria 3010, Australia

⁴Structural Biosciences, Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki Pref. 305-8572, Japan

⁵11 Rue des Moguerou, 29680 Roscoff, France

⁶Friday Harbor Laboratories, University of Washington, 620 University Road, Friday Harbor, WA 98250, USA

⁷Department of Biology, Chungnam National University, Daejeon 305-764, Korea

⁸Department of Biological Sciences, Sungkyunkwan University, Suwon 440-746, Korea

The marine unicellular red algal genus *Rhodella* was established in 1970 by L. V. Evans with a single species *R. maculata* based on nuclear projections into the pyrenoid. *Porphyridium violaceum* was described by P. Kornmann in 1965 and transferred to *Rhodella* by W. Wehrmeyer in 1971 based on plastid features and the non-parietal position of the nucleus. Molecular and fine structural evidences have now revealed that *Rhodella maculata* and *R. violacea* are one species, so *R. violacea* has nomenclatural priority and the correct name is *Rhodella violacea* (Kornmann) Wehrmeyer. The status of families within Rhodellophyceae was examined. The order Dixonieiales and family Dixonieiaceae are emended to include only *Dixonella* and *Neorhodella*. The order Rhodellales and family Rhodellaceae are emended to include *Rhodella* and *Corynoplastis*. *Glaucosphaera vacuolata* Korshikov and the Glaucosphaeraceae Skuja (1954) with an emended description are transferred to the Glaucosphaerales ord. nov.

Key Words: Dixonieiales; Glaucosphaerales ord. nov.; molecular phylogeny; Rhodellales; *Rhodella violacea*; ultrastructure; unicellular red algae

INTRODUCTION

The genus *Rhodella* was established by Evans (1970) to distinguish a unicellular red alga that possessed conspicuous ultrastructural features different from the other genera examined by transmission electron microscopy

(TEM) at that time, *Porphyridium* (e.g., Gantt and Conti 1965, Gantt et al. 1968) and *Rhodosorus* (Giraud 1962). *Rhodella maculata* (Evans 1970) has highly dissected chloroplast lobes attached to multiple sites of a central

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*Corresponding Author

E-mail: hsyoon2011@skku.edu, jwest@unimelb.edu.au
Tel: +82-31-290-5915, Fax: +82-31-290-7015

to eccentric naked pyrenoid. Pyrenoids are designated as being naked if they are not embedded in the chloroplast and instead border the cytoplasm, often partially surrounded by starch grains. The pyrenoid of *Rhodella* is unusual among red algal unicells in that the matrix does not contain thylakoids and it is invaded by one or two protrusions from the nucleus (Evans 1970, Patrone et al. 1991, Waller and McFadden 1995, Yokoyama et al. 2004). No other formally identified unicellular red algal genus has both of these subcellular features (Scott et al. 2008, Yang et al. 2010). In Wehrmeyer (1971) used TEM to characterize cells previously established as *Porphyridium violaceum* (Kornmann 1965) and determined that the cells bore a greater resemblance to *R. maculata* than to *Porphyridium*. Wehrmeyer transferred *P. violacea* to the genus *Rhodella* as *R. violacea* (Kornmann) Wehrmeyer. The two *Rhodella* species differed slightly from each other. The nuclear protrusions into the pyrenoid of *R. violacea* were not detected by Wehrmeyer (1971) but Patrone et al. (1991) noted their presence. In addition to having unique pyrenoids, both species lack a peripheral thylakoid in the chloroplast and Golgi bodies are associated only with endoplasmic reticulum (ER) and never with the nucleus or mitochondria (Scott et al. 2008, Yang et al. 2010, 2011).

In the 1980s, two more unicellular red algae were isolated, characterized by TEM and assigned to the genus *Rhodella*, *R. reticulata* (Deason et al. 1983) and *R. cyanea* (Billard and Fresnel 1986). The establishment of these two new algae as presumed species of *Rhodella* was again based on their ultrastructural dissimilarities to *Porphyridium*. But what was unexpected and left unexplained was the fact that these algae also did not bear any resemblance to either *R. maculata* or *R. violacea*. Furthermore, *R. reticulata* and *R. cyanea* clearly bore little resemblance to each other. Subsequent ultrastructural investigations resulted in transferring *R. reticulata* (= *R. grisea*, Fresnel et al. 1989) to a new genus *Dixoniella* as *D. grisea* (Scott et al. 1992) and *R. cyanea* was transferred to a new genus *Neorhodella* as *N. cyanea* (Scott et al. 2008). Recent molecular studies have confirmed the morphological work (Yokoyama et al. 2009) and *R. maculata* and *R. violacea* are currently assigned to the class Rhodellophyceae, Order Rhodellales, whereas *D. grisea* and *N. cyanea* are in the class Rhodellophyceae, Order Dixoniellales.

Yoon et al. (2006) established six classes of red algae in subphylum Rhodophytina, three of which include unicellular forms, the Porphyridiophyceae (*Erythrolobus*, *Flintiella*, *Porphyridium*, and *Timspurckia*), Stylonematoephyceae (*Rhodosorus* and *Rhodospira*), and Rhodellophyceae (*Corynoplastis*, *Dixoniella*, *Glaucosphaera*,

Neorhodella, and *Rhodella*). For details of the unicellular red algae systematics of utilizing microscopic, molecular, and biochemical methods see Yoon et al. (2006), Scott et al. (2008), Yokoyama et al. (2009), and Yang et al. (2010, 2011).

This current work presents molecular, confocal microscopy, and electron microscopy details on culture strains of a unicellular red alga identified as *Rhodella* sp. isolated from Bodega Bay, CA and Friday Harbor WA, USA. For the comparison, we added information on the type species, *R. maculata* CCMP 736. We have emended the previously established orders Dixoniellales and Rhodellales to correct the placement of the genera *Corynoplastis*, *Dixoniella*, *Neorhodella*, and *Rhodella* and established the new order *Glaucosphaerales* to accommodate a single genus, *Glaucosphaera*.

MATERIALS AND METHODS

Culture methods

Procedures for isolation and culture were as described in West (2005). Isolate JAW 2347 (CCMP 3129) was obtained from a mud sample in a *Salicornia* salt marsh at Bodega Bay, CA, USA on April 15, 1980. O'Kelly's isolates RV-FHLa & b (CCMP 3133, 3135) were obtained as epiphytes on the green alga *Capsosiphon* sp. on a seawall at Friday Harbor, WA, USA in March 2010. These three isolates are available from Bigelow Laboratory for Ocean Sciences, P. O. Box 475, 180 McKown Point Road, West Boothbay Harbor, ME 04575, USA.

Confocal and Nomarski microscopy

Fixation, staining, and observation methods were partially described in Yang et al. (2011). Cultured cells were transferred to 1.5 mL microtubes with 0.5 mL of culture medium, fixed with an equal volume of 3% paraformaldehyde in PHEM buffer [60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl₂ (6H₂O), pH 7.4] for 15 min. Ten μ L of fixed cells 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) at the University of Tsukuba, and an Axio Observer LSM 700 instrument at Bigelow

Laboratory for Ocean Sciences. Plastid autofluorescence and SYBR Green I fluorescence were detected with a 585 nm long pass filter and a 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 a single-track mode. Living cells were observed with a Nikon microscope (Eclipse E600) equipped with Nomarski interference optics (Nikon Co. Ltd., Tokyo, Japan).

Transmission electron microscopy

Cells from cultures (grown in window light in 15 psu von Stosch enriched seawater) were filtered onto poly-L-lysine coated 0.45 µm Millipore filters (Bedford, MA, USA) 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 2 h in the same buffer in 1% OsO₄ 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 22 h. Samples were then further dehydrated in a graded acetone series, infiltrated gradually, embedded in Embed 812 resin (Electron Microscopy Sciences, Hatfield, PA, USA), 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 for 1 min with lead acetate. A Zeiss EM 109 electron microscope was used for observation and photography.

DNA extraction, amplification and sequencing

Genomic DNA was extracted from each culture strain using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. Polymerase chain reaction (PCR) and sequencing were performed with published and newly designed specific primer sets for each gene; *psaA*130F-*psaA*1110R, *psaA*870F-*psaA*1600R and *psaA*971F-*psaA*1760R for *psaA* (Yoon et al. 2002, Yang and Boo 2004), *rbcL*43F (5'-CGT-TAYGAATCTGGTGTAAATYCC-3')-*rbcL*1414R (5'-TCAGCTG-TATCTGTAGAAGTATA-3') for *rbcL*. PCR amplification was performed in a total volume of 25 µL, containing 0.02 unit of Phusion High-Fidelity DNA polymerase (Finnzymes OY, Espoo, Finland), 5 µL of the 5× Phusion HF Buffer (contain 1.5 mM MgCl₂), 200 µM of each dNTPs, 10 µM of each primer and 1-20 ng of template DNA. PCR was carried out with an initial denaturation at 98°C for 30 s, followed by 30 main amplification cycles of denaturation at 94°C for 10 s, annealing at 50-55°C for 30 s, extension

at 72°C for 1 min, and a final extension at 72°C for 7 min. Amplified DNA was purified with the QIAquick PCR Purification kit (Qiagen) and sent to a commercial sequencing company. Electropherogram outputs for each specimen were edited using the program Chromas v.1.45 (<http://www.techneleysium.com.au/chromas.html>). Newly determined sequences were deposited in the GenBank databases (<http://www.ncbi.nlm.nih.gov>) under the accession numbers JN934872-JN934889 (see detail in Table 1).

Phylogenetic analyses

Published sequences were obtained from GenBank and aligned with newly determined sequences using ClustalW implemented SeaView version 4.2.5 (Gouy et al. 2010) and manually refined using Se-AI version 2.0a11 (<http://tree.bio.ed.ac.uk/software/seai/>). We excluded insertions of the nuclear small subunit rRNA (SSU rRNA) (*Rhodella* sp. MBIC10593, AB183619: #538-1045; *Thorea violacea* SAG 51.94, AF342744: #1356-1479) and introns of plastid *psaA* (*Rhodella maculata* CCMP 736, DQ308448: #79-858; *Rhosospora sordida* UTEX 2621, DQ308453: #226-723). There were no indel in *psbA* and *rbcL*. We used two data sets for tree search, (i) DNA and protein mixed data (total 3,070 characters; 1,881 base pairs of nuclear SSU rRNA + 1,189 amino acid sequences from plastid *psaA*, *psbA*, and *rbcL*) and (ii) DNA data (total 5,448 bp; 1,881 bp SSU rRNA + 3,567 bp plastid genes).

The evolutionary model for individual genes was chosen using the weighted Akaike information criterion (AIC) implemented in ModelGenerator version 0.85 (Keane et al. 2006). The selected best fitting models were the general time reversible (GTR) substitution with the gamma distributed rate heterogeneity (G) for DNA data; the LG substitution (Le and Gascuel 2008) with empirical amino acid frequencies (F) and G (LG + F + G model) for protein data. We used an independent model for each partition of the concatenated data. For example, it was used GTR + G model for SSU part and separate LG + F + G for *psaA*, *psbA* and *rbcL* parts of mixed data; separate GTR + G for individual gene of DNA data.

Maximum likelihood (ML) analyses were performed using RAxML version 7.2.8 (Stamatakis 2006). Tree likelihoods were estimated using 200 independent replications, each with a random starting point. The separate site-specific model was used for partitioned data and the automatically optimized SPR branch rearrangements were used during the rapid hill climbing tree search for each replication. Bootstrap analyses (MLB) were conducted using 1,000 replications with the same evolu-

Table 1. Taxa list used in present study

Taxa	Strain or locality	Nuclear	Plastid		
		SSU	<i>psaA</i>	<i>psbA</i>	<i>rbcL</i>
Bangiophyceae					
<i>Bangia fuscopurpurea</i>	SAG 59.81	AF342745	AY119699	AY119735	AY119771
<i>Porphyra leucosticta</i>	SAG 55.88	AF342746	DQ308442	-	DQ308424
<i>Porphyra purpurea</i>	NHA 61946	AF362363	-	-	-
	Avonport	-	U38804	U38804	U38804
Compsopogonophyceae					
<i>Compsopogon caeruleus</i>	USA, Louisiana, Whiskey Chitto	AF087128	-	-	AF087116
	USA, Alabama	-	DQ308445	-	-
<i>Compsopogon caeruleus</i>	SAG B 36.94	AF342748	AY119701	AY119737	-
<i>Rhodochaete parvula</i>	UTEX 2715	AJ880420	AY119707	AY119743	AY119777
Cyanidiophyceae					
<i>Cyanidium caldarium</i>	DBV 182 JAVA	-	AY541282	AY541290	AY541298
<i>Galdieria daedala</i>	IPPAS P508; Russia, Kunashira Island	AF441362	AY541283	AY541291	AY541302
<i>Galdieria maxima</i>	IPPAS P507; Russia, Kunashira Island	AF441367	AY391364	AY391367	AY391370
Florideophyceae					
<i>Chondrus crispus</i>	EMBLCH307012	DQ316999	-	-	-
	Canada, Nova Scotia	-	AY119710	AY119746	-
	Northern Ireland, Co. Antrim, Bally Castle	-	-	-	U02984
<i>Palmaria palmata</i>	n/a	Z14142	-	-	U28421
	P244	-	DQ787599	DQ787639	-
<i>Thorea violacea</i>	SAG 51.94	AF342744	AY119712	AY119747	AF506271
Prophyridiophyceae					
<i>Flintiella sanguinaria</i>	SAG 40.94	AF342749	AY119704	AY119740	AY119774
<i>Porphyridium aerugineum</i>	SAG 1380-2	-	AY119705	AY119741	AY119775
Rhodellophyceae					
<i>Corynoplatis japonica</i>	CCMP 3089	JN934872	JN934874	JN934879	JN934884
<i>Corynoplatis japonica</i>	TB4-T2	AB045605	-	-	-
<i>Dixoniella grisea</i>	SAG 39.94	AB045581	AY119702	AY119738	AY119773
<i>Dixoniella grisea</i>	MF-G2	AB045596	-	-	-
<i>Glaucosphaera vacuolata</i>	UTEX 1662	AB045583	DQ308447	-	DQ308427
<i>Neorhodella cyanea</i>	RHO4	FJ595494	-	-	-
<i>Neorhodella cyanea</i>	CCMP 3235 (= CCAP 1346/1, CCMP 3083, JAW 4072)	JN934873	JN934875	-	JN934885
<i>Rhodella maculata</i>	CCAP 1388/6; Sweden, Öland Island (Baltic Sea)	GU191839	-	-	-
<i>Rhodella maculata</i>	CCMP 736; UK, England, Essex, Southend-on-Sea (North Atlantic)	AJ880419	DQ308448	-	DQ308428
<i>Rhodella maculata</i>	Japan, Kagoshima, Amami Island (North Pacific)	AB045608	-	-	-
<i>Rhodella</i> sp.	BC73C	AB045594	-	-	-
<i>Rhodella</i> sp.	GA6-T4 (North Pacific)	AB045591	-	-	-
<i>Rhodella</i> sp.	MBIC10593	AB183619	-	-	-
<i>Rhodella</i> sp.	MBIC10825	AB183648	-	-	-
<i>Rhodella</i> sp.	MBIC11021	AB183652	-	-	-
<i>Rhodella</i> sp.	Nagura1; Japan, Okinawa, Ishigaki Island, Nagura River (North Pacific)	AB045598	-	-	-
<i>Rhodella</i> sp.	CCMP 3129 (= JAW 2347); USA, CA, Bodega Marine Lab (North Pacific)	-	JN934876	JN934880	JN934886
<i>Rhodella</i> sp.	CCMP 3133 (= RV-FHLA, Friday Harbor Lab strain A)	-	JN934877	JN934881	JN934887
<i>Rhodella</i> sp.	CCMP 3135 (= RV-FHLb, Friday harbor Lab strain B)	-	JN934878	JN934882	JN934888
<i>Rhodella violacea</i>	BRW; USA, Alaska, BRW Elson Lag (North Pacific)	AB045604	-	-	-
<i>Rhodella violacea</i>	SAG 115.79; Germany	EU861395	AY119706	AY119742	AY119776
<i>Rhodella violacea</i>	SAG B 115.79	AB045580	-	-	-
<i>Rhodella violacea</i>	UTEX 2427 (= CCMP 3229, JAW 3621, NEPCC-14, NIBB-2010; North Sea)	AF168624	-	JN934883	JN934889

Table 1. Continued

Taxa	Strain or locality	Nuclear	Plastid		
		SSU	<i>psaA</i>	<i>psbA</i>	<i>rbcl</i>
Stylonematophyceae					
<i>Bangiopsis subsimplex</i>	PR21	AF168627	AY119700	AY119736	AY119772
<i>Chroodactylon ornatum</i>	SAG 103.79	AF342751	DQ308449	-	DQ308429
<i>Chrootheca mobilis</i>	FD Ott O1363	-	DQ308450	-	DQ308430
<i>Rhodosorus marinus</i>	SAG 116.79	AF342750	AY119708	AY119744	AY119778
<i>Rhodosorus</i> sp.	CCMP 1530	AF168626	DQ308454	-	DQ308434
<i>Rhodospira sordida</i>	UTEX 2616	FJ595495	DQ308453	-	DQ308433
<i>Stylonema alsidii</i>	UTEX 1424	AF168633	-	-	-
	SAG 2.94	-	AY119709	AY119745	AY119779

GenBank accessions for new sequences are in bold.

tionary model setting as that used for the best topology search.

RESULTS

Confocal observations

The following observations were made with the culture isolate JAW 2347 (CCMP 3129). SYBR Green stain gave a green fluorescence to nuclear DNA as well as chloroplast and mitochondria genophores, whereas the whole chloroplast showed a red autofluorescence (Fig. 1). The nucleus of the interphase stage was always eccentric and associated with a pyrenoid (Fig. 1A-C). The single chloroplast had its pyrenoid centrally located and had many lobes positioned at the cell periphery. Plastid genophores were spherical to subspherical (about 0.25 μ m diameter) and present in the peripheral lobes. Two nuclei were found in some dividing cells (Fig. 1D). All observed characters were also found in *R. maculata* CCMP 736 (Fig. 1E & F), without any features distinguishing it from JAW 2347 (CCMP 3129).

Transmission electron microscopy

The cells were 8-12 μ m in diameter and were coated by thin fibrillar material (Fig. 2). A discontinuous layer of ER was present beneath the cell membrane and appeared interconnected with the cell membrane by short tubules (Figs 2 & 3D), as seen by Patrone et al. (1991) in *Rhodella violacea*, *R. maculata* and many other unicellular red algae. The spherical nucleus was approximately 3 μ m in diameter, occupying a peripheral location and was always closely associated with the more central pyrenoid (Fig. 2).

The nucleus contained one mostly central nucleolus (Fig. 2). In favorable sections usually one but occasionally two nuclear protrusions could be seen penetrating the pyrenoid as deep as 0.5-1 μ m (Figs 2 & 3A). The protrusions always contained an electron dense, fibrillar material, not well seen in Figs 2 & 3A. In the examination of several hundred cells, nuclear protrusions were detected in over 20.

Usually one pyrenoid was found in each cell but a few cells contained two pyrenoids. The pyrenoids were commonly larger than nuclei and were often bordered by starch that was also seen elsewhere in the cell, frequently near nuclei (Fig. 2). The moderately electron dense pyrenoid matrix was devoid of thylakoids (Figs 2 & 3A). As observed in two-dimensional sections, 1-4 chloroplast lobes extended from the pyrenoid throughout the cytoplasm. Thylakoids were abundant and maintained consistent spacing from each other (Figs 2, 3B & C), but phycobilisomes were not usually clearly seen. Peripheral thylakoids were absent (Figs 2 & 3C). Electron dense clusters of spherical globules (plastoglobuli) were commonly seen at the outermost periphery of chloroplast lobes (Fig. 3C). Profiles of mitochondria were found throughout the cell (Figs 2, 3B & C) as well as small electron transparent vacuoles (Figs 2 & 3B). Golgi bodies were somewhat sparse and usually located at peripheral cell regions. All observed Golgi bodies had fused or closely appressed cisternae (Fig. 3B).

Four-gene phylogeny

Newly determined rhodellophycean nuclear SSU rRNA and three plastid protein-coding *psaA*, *psbA*, and *rbcl* genes were aligned and used for the phylogenetic analysis. ML trees based on DNA + protein mixed data and

Fig. 1. Morphological characters from *Rhodella violacea* (JAW 2347 / CCMP 3129, A-D) and *R. maculata* (CCMP 736, E-F). (A) Nomarski differential interference microscopic image shows a single pyrenoid filled with uniform material and surrounded by starch. Plastid globules (yellowish orange) are scattered at the cell surfaces. A single eccentric nucleus is situated beside the pyrenoid. (B) Confocal microscopic image from single focal level. Fluorescent SYBR Green staining show a single nucleus with single nucleolus and chloroplast genophores as small particles in the lobes at the cell perimeter. (C) Combined image of SYBR Green staining and plastid autofluorescence (red). A pyrenoid region is the central dark space. Plastid genophores (greenish-yellow) are located in the central areas of chloroplast lobes. (D) Dividing cell with two nuclei. (E) Confocal microscopy of CCMP 736 shows a single nucleus and chloroplast genophores similar to JAW 2347. (F) Combined images of SYBR Green staining and plastid autofluorescence from CCMP 736. Scale bars represent: A-F, 2.0 μ m.

Fig. 2. *Rhodella violacea* (JAW 2347 / CCMP 3129) ultrastructure. Low magnification electron micrograph of a medially sectioned cell. The nucleus (N) is peripheral with a centrally located nucleolus (NL). A nuclear extension (arrow) projects into the adjacent pyrenoid (P), which is surrounded by starch grains (S). The pyrenoid lacks thylakoids and is seen connected to one of the chloroplast lobes (asterisk). A peripheral thylakoid is absent, as detected in several regions of the chloroplast lobes (arrowheads). A peripheral endoplasmic reticulum (ER) system (PER) is visible in some areas just inside of the cell membrane. Scale bar represents: 1.0 μ m.

Fig. 3. *Rhodella violacea* (JAW 2347 / CCMP 3129) ultrastructure. (A) The nuclear protrusion contains electron dense material. N, nucleus; P, pyrenoid; S, starch. (B) A Golgi body (G) is near the cell membrane (arrow). An expanded portion of endoplasmic reticulum (ER) is seen between the cell membrane and the Golgi cis-region. Golgi cisternae are closely appressed in the mid-region. C, chloroplast lobe. (C) Electron dense globules (plastoglobuli, arrows) are consistently located at the periphery of chloroplast lobes. M, mitochondrion. (D) Tubules of the peripheral ER system (arrows) are seen in cross section. Scale bars represent: A-C, 0.5 μ m; D, 0.1 μ m.

DNA data were consistent with each other, except for the position of Porphyridiophyceae (Fig. 4). The Porphyridiophyceae grouped with a Bangiophyceae / Florideophyceae / Rhodellophyceae clade in the DNA + protein mixed data, whereas it grouped together with the Compsopogonophyceae / Stylonematophyceae clade in the DNA-only tree. However, this incongruence was not supported statistically (less than 50% bootstrap value in each phylogeny).

Multigene trees supported the monophyly of Rhodellophyceae strongly (MLB and mixed and DNA 100%). Within the rhodellophycean monophyly, two subclades were identified: i) the Rhodellales clade (MLB mixed and DNA 100%) including *Rhodella* and *Corynoplastis*, ii) the Glaucosphaerales and Dixonellales clade (MLB mixed 97% and DNA 100%), including *Glaucosphaera*, *Dixonella*, and *Neorhodella*. Within Rhodellales, all 16 *Rhodella* isolates were strongly grouped together (MLB mixed and DNA 100%). However, they did not make monophyletic groups for each species of *R. violacea* (BRW, UTEX 2427, SAG 115.79) and *R. maculata* (CCMP 736, CCAP 1388/6, Japan: Amami). *Rhodella* sp. BC73C was separated from the rest of the isolates (MLB mixed 91% and DNA 94%). Four isolates (MBIC11021, MBIC10825, MBIC10593 and Japan: Amani) were grouped together (MLB mixed 79% and DNA 77%).

Sequence divergences within *Rhodella* isolates ranged from 0 to 37 bp (2% p distance; between MBIC 10593 and GA6-T4) in SSU rRNA, up to 18 bp (1.5%; between JAW 2347 and UTEX 2427) in *rbcL*, up to 6 bp (0.7%; between RV-FHLA and SAG 115.79) in *psbA*, and up to 2 bp (0.1%; between CCMP 736 and SAG 115.79) in *psaA*. Sequence divergences between *Rhodella* sp. BC73C and the rest of the isolates were clearly higher, ranging from 68 (3.6%, with *R. violacea* UTEX 2427) to 78 bp (4.1%, with *R. maculata* Japan: Amani isolate) in the SSU rRNA sequence.

DISCUSSION

Our ultrastructural examination of JAW 2347 revealed two cell characters found in no other unicellular red algae except *R. maculata* and *R. violacea* (Evans 1970, Wehrmeyer 1971, Patrone et al. 1991): the pyrenoid matrix lacked thylakoids and was penetrated by one or more protrusions from the closely associated nucleus. Also present in *Rhodella* were Golgi bodies associated only with ER, a chloroplast lacking a peripheral encircling thylakoid, and accumulations of electron dense globules (plastoglobuli) in the outer chloroplast lobes at the cell periphery.

The only other member of the Rhodellophyceae possessing Golgi bodies associated with ER is *Corynoplastis*. Golgi bodies in *Dixonella*, *Glaucosphaera*, and *Neorhodella* are consistently situated near the outer membrane of the nuclear envelope. No other red alga has this peculiar Golgi association. However, the fact that the ER and the outer membrane of the nuclear envelope are both functional equivalents is what unifies Golgi differences in the Rhodellophyceae (see discussion in Scott et al. 2008). In contrast, Golgi bodies in all members of the Porphyridiophyceae, Bangiophyceae, and Florideophyceae are always closely associated with mitochondria. Only the relatively few genera in the Stylonematophyceae and Compsopogonophyceae have an ER-Golgi alignment as is routinely found in most other eukaryotes. An additional Golgi attribute found only in the other rhodellophycean unicells and sporangia of certain multicellular red algae (Scott et al. 2008) is fusion or apposition of Golgi cisternae. The significance of this unusual trait is unknown.

The presence or absence of a peripheral thylakoid is variable in the Rhodellophyceae. *Dixonella*, *Glaucosphaera*, and *Corynoplastis* possess a peripheral thylakoid, whereas *Rhodella* and *Neorhodella* do not. The four genera of Porphyridiophyceae, *Erythrolobus*, *Flintella*, *Porphyridium*, and *Timspurckia* all lack this feature. Within the Stylonematophyceae, Compsopogonophyceae, Bangiophyceae, and Florideophyceae only the sporophyte (conchocelis) stages of *Bangia* and *Porphyra* (Pueschel 1990) and vegetative cells of *Rhodachlya* (Rhodachlyales, Florideophyceae) (West et al. 2008) lack a peripheral thylakoid. The phylogenetic and functional significance of the presence or absence of this thylakoid is unknown. Electron dense globules or plastoglobuli are fairly commonly seen in red algal chloroplasts (Pueschel 1990). Plastoglobuli clusters located in the peripheral lobes of chloroplasts are present in all members of Rhodellophyceae but are absent in other unicellular red algae (Scott et al. 2008, Yokoyama et al. 2009). See Deason et al. (1983) for a detailed discussion of plastoglobuli (stigmata = eyespots) in chloroplasts of unicellular red algae.

It is obvious that the presence or absence of starch grains closely bordering the naked pyrenoids of *Rhodella* is a variable. Some cells of JAW 2347 isolate (CCMP 3129) had pyrenoids free of starch, whereas other cells showed a close association. Pyrenoids in *R. violacea* cells observed by Wehrmeyer (1971) were surrounded by starch, whereas cells of the same culture strain studied by Patrone et al. (1991) lacked starch in the region adjacent to the nucleus where the nuclear protrusions are located. However, as Wehrmeyer (1971) did not find any nuclear protrusions

Fig. 4. The maximum likelihood phylogeny of *Rhodella violacea* is based on nuclear small subunit (SSU) rRNA + plastid protein *psaA*, *psbA*, and *rbcL* (-lnL = 26232.95). Likelihood is estimated under the general time reversible (GTR) + G model for the DNA part and separate LG + G + F model for the individual protein gene part. The maximum likelihood (ML) bootstrap support values are shown on the branches (from DNA + protein mixed data) and under branch (from DNA data).

in his study, it is possible that starch could likewise be absent in the protrusion zone of the isolate of *R. violacea* that he examined. All regions of pyrenoids in *R. maculata* are bordered by starch grains, even between the pyrenoids and nuclei in the protrusion zone (Evans 1970, Patrone et al. 1991). However, in *R. maculata* cells studied by Waller and McFadden (1995), several micrographs showed pyrenoids heavily bordered by starch, whereas one micrograph showed a starch-free pyrenoid. Most likely these observed variations in starch content and localization are more likely dependent upon differences in culture conditions used or cell cycle stage studied rather than reliable indicators to discriminate taxa.

The nuclear protrusions in strain JAW 2347 (CCMP 3129), *R. violacea* (Patrone et al. 1991), and those in *R. maculata* observed by Evans (1970), Patrone et al. (1991), and Waller and McFadden (1995) were variable length but all contained an electron dense material. Waller and McFadden (1995) determined that the material was non-ribosomal RNA and could possibly serve a structural role. Two protrusions were occasionally seen in a single cell of *R. violacea* (Patrone et al. 1991) and, two protrusions were commonly found by Waller and McFadden (1995) in *R. maculata* cells by serial sectioning.

The low molecular weight carbohydrate (LMWC) mannitol is present in all Rhodellophyceae including JAW 2347 (CCMP 3129) (Karsten et al. 1999, 2003, Scott et al. 2008, Nitschke et al. 2010).

JAW 2347 (CCMP 3129) is readily recognized as a species of *Rhodella* based on electron microscopic and molecular evidence. As seen in thin sections: (1) Golgi bodies were consistently associated with ER and no other organelles (e.g., mitochondria or the nucleus), (2) the chloroplast is highly dissected with thylakoid-filled lobes containing peripheral plastoglobuli and no peripheral thylakoid, (3) the matrix of the central to eccentric pyrenoid is devoid of thylakoids but penetrated by one or two unique digitate protrusions arising from the adjacent nucleus. No other unicellular red algal genus has this combination of ultra-structural characters (Scott et al. 2008).

The concatenated phylogeny (nuclear SSU rRNA + plastid *psaA*, *psbA*, and *rbcL*) is congruent with the seven-class system of Rhodophyta and the monophyly of Rhodellophyceae (Yoon et al. 2006, 2010). In the present study, as rhodellophycean ingroup taxa increased, there was greater ML bootstrap support for the sister relationship of Rhodellophyceae to the Bangio-Florideophyceae clade than that of previous studies, i.e., >50% from SSU rRNA tree in Yokoyama et al. (2009); 53% from a nine-gene tree in Yoon et al. (2006); 58% from a two-gene tree

in Yang et al. (2010). These results support the idea that broad taxa sampling increased robustness of the tree (Rokas and Carroll 2005, Parfrey et al. 2010). Our phylogeny suggest that the Rhodellophyceae is the sister class of the Bangiophyceae-Florideophyceae clade. Within the Rhodellophyceae, the Glaucosphaerales and Dixonellales are sister group taxa separated from the Rhodellales.

In the *Rhodella* phylogeny, all *Rhodella* isolates (except isolate BC73C) were grouped together, and neither *R. violacea* nor *R. maculata* were a monophyletic group in the genus. Most isolates were strongly grouped together in the tree. The results showed low sequence divergences in SSU rRNA (2%) and three plastid genes (< 2%), which were significantly lower than those of other unicellular red algae (e.g., 11-15% divergence in *psaA* of *Erythrolobus* spp.) (Yang et al. 2010). However, isolate BC73C is genetically distinct within *Rhodella* and may be a distinct species, but further investigation is required.

Redefinition of the Rhodellophyceae orders and families

Wynne and Schneider (2010) indicated that the family Dixonellaceae established by Yokoyama et al. (2009) was invalid because the family Glaucosphaeraceae, which originally included only *Glaucosphaera*, was established by Skuja (1954) and had precedence. However, based on the structural characters, cell activity (i.e., continuous Golgi vesicle formation and extrusion), and molecular evidence, we have placed the genus *Glaucosphaera* in the separate new order Glaucosphaerales Yang, Scott, Yoon and West in which the family Glaucosphaeraceae Skuja is now placed. The Glaucosphaerales is phylogenetically closely related to the Dixonellales.

Glaucosphaerales ord. nov., Yang, Scott, Yoon and West. The order, family and genus are defined as a unicellular freshwater red alga, containing a blue green chloroplast with a peripheral thylakoid, plastoglobuli clusters, no pyrenoid, active perinuclear Golgi bodies continuously producing and rapidly ejecting small vesicles from the cell, cells larger than most unicellular reds (to 25 µm diameter) (Broadwater et al. 1995, Pickett-Heaps et al. 2001, Wilson et al. 2006), LMWCs unknown.

Glaucosphaeraceae Skuja (1954). Same characters as the order. The only taxon at present is *Glaucosphaera vacuolata* Korshikov (1930), known as the original type specimen from a large pond in a meadow at the outskirts of the city Charkova (Kharkov), Ukraine, in August 1929 and a single collection and culture obtained by Richard Starr in May 1968 in soil from a horse pond near Elletts-

ville, IN, USA.

Dixoniellales and Dixoniellaceae Yokoyama et al. (2009). Emended ordinal and family description: single chloroplast with a single or multiple pyrenoids, plastoglobuli at chloroplast periphery, Golgi bodies perinuclear, mannitol as the only LMWC. *Dixoniella* and *Neorhodella* are now the only genera in this order and family.

Rhodellales Yoon et al. (2006). As originally defined by Yoon et al. (2006) this order includes *Rhodella*, *Dixoniella* and *Glaucosphaera*. Yokoyama et al. (2009) placed *Dixoniella* and *Neorhodella* in the Dixoniellales Yokoyama et al. (2009).

Emended ordinal description: unicellular red algae, a single highly lobed plastid with single or multiple pyrenoids, plastoglobuli at plastid periphery, scattered Golgi associated with ER, contain mannitol as a LMWC.

Rhodellaceae Yoon et al. (2006) now contains only two genera *Rhodella* and *Corynoplatis* (Yokoyama et al. 2009). The genus description of *Rhodella* is emended to specify that digitate nuclear intrusions occur in the pyrenoid and the pyrenoid matrix is devoid of thylakoids. The two currently recognized species [*R. maculata* Evans and *R. violacea* (Kornmann) Wehrmeyer] are merged as one, *R. violaceum* (Kornmann) Wehrmeyer. *Rhodella violacea* is used because it is the basionym *Porphyridium violaceum* Kornmann (1965), and the name with priority.

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