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A second species of *Rhodachlya* (Rhodachlyales, Rhodophyta) from Hawaii, with a description of *R. hawaiiana* sp. nov.

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Abstract – *Rhodachlya hawaiiana* Kurihara, J.A. West, Conklin *et* A.R. Sherwood sp. nov. is described based on cultured material from the island of Hawaii as the second member of the genus *Rhodachlya*. The pit plug ultrastructure of this alga is characterized by the presence of inner and outer cap layers but lacking a cap membrane between the layers, which is a diagnostic feature of the order Rhodachlyales and the genus *Rhodachlya*. Morphologically, *R. hawaiiana* is distinctive from *R. madagascarensis* in vegetative cell dimensions, frequency of colorless hair cell formation, and spore germination pattern. *Rhodachlya hawaiiana* and *R. madagascarensis* are also distinguishable by their nuclear SSU rRNA gene sequence divergence (*p*-distance = 0.0064), which is comparable to other species-level divergences among members of the Nemaliophycidae.

Hawaii / *Rhodachlya hawaiiana* / Rhodachlyales / Rhodophyta / SSU rRNA / taxonomy

Résumé – *Rhodachlya hawaiiana* Kurihara, J.A. West, Conklin *et* Sherwood sp. nov., décrite à partir de materiel cultivé provenant de l'île d'Hawaii, est la seconde espèce appartenant au genre *Rhodachlya*. L'ultrastructure des synapses de cette algue montre la présence d'une couche interne et d'une couche externe recouvrant le bouchon synaptique, sans membrane de revêtement entre les couches, ce qui est une des caractéristiques de l'ordre des Rhodachlyales. Morphologiquement *R. hawaiiana* diffère de *R. madagascarensis* par la taille de ses cellules végétatives, par la fréquence des cellules formant des poils hyalins et par le mode de germination des spores. Ils diffèrent aussi par la séquence de leur SSU rRNA avec une divergence (p-distance=0.0064) comparable à d'autres divergences entre espèces parmi les Nemaliophycidae.

Hawaii / *Rhodachlya hawaiiana* / Rhodachlyales / Rhodophyta / SSU rRNA / taxonomie

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INTRODUCTION

Members of the red algal orders Acrochaetiales and Colaconematales are uniaxial, simple branched filaments with prostrate and/or erect thalli in the vegetative stage (*i.e.* "acrochaetioids"). Acrochaetioid thalli are also known in the Nemaliales as microscopic tetrasporophytes, and in the recently established order, the Rhodachlyales. Although all acrochaetioids appear similar, the Rhodachlyales are distinctive among marine forms in their pit plug ultrastructure (West *et al.*, 2008). All members of the Nemaliophycidae, which are currently classified in eight orders, are characterized by two-layered plug caps [although the ultrastructure of pit plugs has yet to be observed in the Balliales (Choi *et al.*, 2000)], with ordinal level distinctions based on the presence or absence of cap membranes and the shape of the outer cap layers. Plate-shaped outer cap layers and a lack of cap membranes characterize the pit plugs of the Rhodachlyales (West *et al.*, 2008), Thoreales (Müller *et al*., 2002) and *Batrachospermum macrosporum* (as *Audouinella macrospora*; Pueschel *et al*., 2000); the latter two represent freshwater red algae. A distinctive phylogenetic position based on DNA sequence analysis also supported the independence of the Rhodachlyales from other recognized orders and the description of the first species within the order, *Rhodachlya madagascarensis* J.A. West, J.L. Scott, K.A. West, U. Karsten, S.L. Clayden *et* G.W. Saunders (West *et al.*, 2008).

As part of the Hawaiian Rhodophyta Biodiversity Survey (Sherwood *et al*., 2010a,b) we isolated an acrochaetioid alga from a Hawaiian sample of *Gelidium* sp. Observations of pit plug ultrastructure and nuclear small subunit (SSU) rRNA gene sequence analyses indicated that this alga is a close relative of *R. madagascarensis*, which is currently considered to be endemic to Madagascar (West *et al.*, 2008). Here we report and describe the second member of the genus *Rhodachlya* and the order Rhodachlyales.

MATERIALS AND METHODS

Samples & culture – The sample used in this study (Sherwood Lab accession 03778) was isolated from a culture of *Gelidium* sp. (Sherwood Lab accession 04140), which was collected from the shallow subtidal (< 1 m depth) at Whittington Beach Park, Hawaii Island, Hawaii (leg. *A. Kurihara & K.Y. Conklin*; 24 January 2008). The host thallus was brought to the laboratory on Oahu for culturing. After rinsing the thallus with autoclaved, filtered seawater, several branch tips were cut off and cleaned of epiphytes and placed in petri dishes. To prevent diatom contamination, GeO_2 solution at a final concentration of 1 mg/L was used (West, 2005). Filamentous thalli grew in the petri dish and were isolated by Pasteur pipette, placed in new petri dishes and grown in PES medium (Provasoli, 1968) at 20-25°C under a photon flux of 5-15 μmol m^{-2} s^{-1}.

Light microscopy – Live specimens were examined for thallus structure and pyrenoid morphology. Subsequently, thalli were fixed in 4% formalin in seawater and stained with 0.5% aniline blue in a lactic acid/phenol/glycerol/water (1:1:1:1) solution and mounted in 30% corn syrup on microscope slides as voucher specimens.

Transmission Electron Microscopy – Samples were fixed in Karnovsky's fixative (Karnovsky, 1965). Fixed samples were processed as described in Cole & Sheath (1980) and thin sections were viewed with a Zeiss LEO 912 Energy-Filtering Transmission Electron Microscope at 100 kV.

DNA extraction, PCR amplification & Sequencing – Total genomic DNA was extracted using 5% Chelex 100 resin solution as a modified Walsh *et al.* (1991) procedure [*i.e.* samples were incubated at 70°C for 20 min and 99°C for 20 min in an Eppendorf Mastercycler ep gradient S thermal cycler (Eppendorf Hamburg, Germany), and then placed on ice for at least 30 min]. Methods of PCR amplification and direct sequencing followed Conklin *et al.* (2009) except for the primers and purification method. Primer sets used for PCR amplification and sequencing of the nuclear SSU rRNA gene were as follows: SR1 and SR5 (Nakayama *et al.*, 1986) or SR5N (5'-AACACTCTAATTTNTTCACAGTAAA-3'), SR4 (Nakayama *et al.*, 1986) and SR9N (5'-AACTAAGAACGGCCAT-GCAC-3'), and SR8N2 (5'-GAAAYCAAAGTNTTTGCTTTCYGGG-3') and G07 (Saunders & Kraft, 1994). The PCR amplification conditions included an initial denaturation of 94°C for 1 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension of 72°C for 5 min. PCR products were purified using ExoSAP-IT® (USB Corp., Ohio, U.S.A.) according to the manufacturer's instructions.

Molecular phylogenetic analyses – The new SSU rRNA gene sequence was aligned with 24 sequences representing orders within the Nemaliophycidae (see Appendix 1) using MEGA4 (Tamura *et al.*, 2007) with the default values. All insertions and deletions were removed from the dataset prior to phylogenetic analyses. Molecular phylogenetic trees were constructed using maximum likelihood (ML) and Bayesian methods. For the ML analysis, the best-fit evolutionary model was calculated by ModelTest 3.7 (Posada & Crandall, 1998), and the TrN + I + Γ model (A = 0.2460, C = 0.2096, G = 0.2863, T = 0.2581, r_{AC} = 1.0000, r_{AG} = 3.8417, r_{AT} = 1.0000, r_{CG} = 1.0000, r_{CT} = 6.7412, r_{GT} = 1.0000, gamma distribution shape parameter = 0.6940, proportion of invariable sites = 0.7393) was selected by the Akaike Information Criterion (AIC). The ML tree was heuristically searched using TBR branch swapping and 100 random-sequence-additions. Bayesian phylogenetic analysis was performed with MrBayes v. 3.1.2 (Huelsenbeck & Ronquist, 2001). The most appropriate parameter values and substitution models for the Bayesian analysis were determined using the AIC approach implemented in MrModeltest v. 2.2 (Nylander, 2006). The optimal model was identified as GTR + I + Γ and the default parameters were used. Two million generations were run with sampling every 100 generations and four Markov chains, and a burn-in value of 5,000 trees. The remaining 15,000 trees were sampled to construct an unrooted consensus tree. Statistical tests of internal branches were calculated by bootstrap tests in NJ (5,000 replications with ML corrected distances), parsimony (5,000 replications with SPR branch swapping and 10 random-addition-sequence replicates) and ML methods (500 replications with SPR branch swapping and 10 random-addition-sequence replicates) and by posterior probabilities in the Bayesian method (2,000,000 generations); however, only the posterior probabilities and ML bootstrap values are shown. The *p*-distances were calculated using the pairwise-deletion option of the map/missing data function implemented in MEGA4.

RESULTS & DISCUSSION

All observations were made on specimens grown in unialgal culture.

Rhodachlya hawaiiana **A. Kurihara, J.A. West, K.Y. Conklin *et* A.R. Sherwood sp. nov.**
Rhodophyta parva marina filis ramousis basalis erectisque. Basalis filis patente repenia patente adhaerente, 25-40 µm longus and 13-18 µm latus. Cellula filamentorum erecta elongate, 29-50 µm longus, 8-18 µm latus. Pilis elongatis unicellularibus in extremitate superiore. Omni cellula vegetative et spora chlomatophoro parietali, alte lobato, qui pyrenoide una. Sporangia sphaericae, 10-11 (20) µm in diametro. Obturamentis fovearum pileis externalibus internalisubsque patelliformibus instructis. Rhodachlya hawaiiana *nucleo SSU rRNA genis ordines est dissimilior aliae* R. madacascarensis*, cum 0.65% differentia*.

Description translation: Small marine red alga with filamentous, branched basal and erect filaments. Basal filaments creeping, spreading, adherent; cells irregularly shape, 25-40 µm long and 13-18 µm broad. Cells of erect filaments elongated, 29-50 µm long and 8-18 µm broad; irregularly branched. Unicellular hairs on upper end of intercalary cells. Each vegetative cell and spore provided with a parietal, H-shaped or lobed plastid, containing a pyrenoid. Sporangia spherical, 10-11 (20) µm in diameter on terminal apical cells. Pit plugs with plate-like outer and inner caps and lacking a cap membrane. Nuclear SSU rRNA gene sequence of *Rhodachlya hawaiiana* differs from that of *R. madagascarensis* by 0.65%.

Holotype: BISH 751561 (Bernice Pauahi Bishop Museum, Honolulu, Hawaii 96817, U.S.A.).
Isotype: BISH 751562; US 217875 (Smithsonian Institution, NMNH), Botany Department, MRC 0166, P.B. Box 37012, Washington, DC, 20013-0920, U.S.A.).
Type Culture: KU-MACC KU-2984 (Kobe University Macroalgal Culture Collection), Rokkodai 1-1, Nadaku, Kobe 657-8501, Japan).
Cryopreserved Culture: KU-MACC (Kobe University Macroalgal Culture Collection), Rokkodai 1-1, Nadaku, Kobe 657-8501, Japan.
Etymology: Named for the type locality.
Type DNA Sequences: GenBank accessions GQ327930 (nuclear SSU gene), HQ422149 (nuclear LSU partial gene; Sherwood *et al.*, 2010a), HQ421178 (plastid UPA marker; Sherwood *et al.*, 2010a).

Thalli consist of filamentous prostrate and erect systems (Fig. 1). The basal filaments are creeping, sinuous and irregularly branched (Fig. 2). Cells of basal filaments are fusiform when not issuing branches, 25-40 µm (mean = 30 µm) long and 13-18 µm (mean = 15 µm) broad (Fig. 3), but have a T-shape or triangular shape when branching (Figs 4-5). Erect filaments are composed of cells slightly constricted at both ends, becoming terete or barrel-shaped (or elongated pear-shaped), 29-50 µm (mean = 40 µm) long and 8-18 µm (mean = 11 µm) broad (Fig. 6). Colorless unicellular hair cells are infrequently produced from the upper part of intercalary cells, and 2.5 µm broad when present. Each cell of the creeping and erect filaments has one axial plastid and a single pyrenoid, positioned at

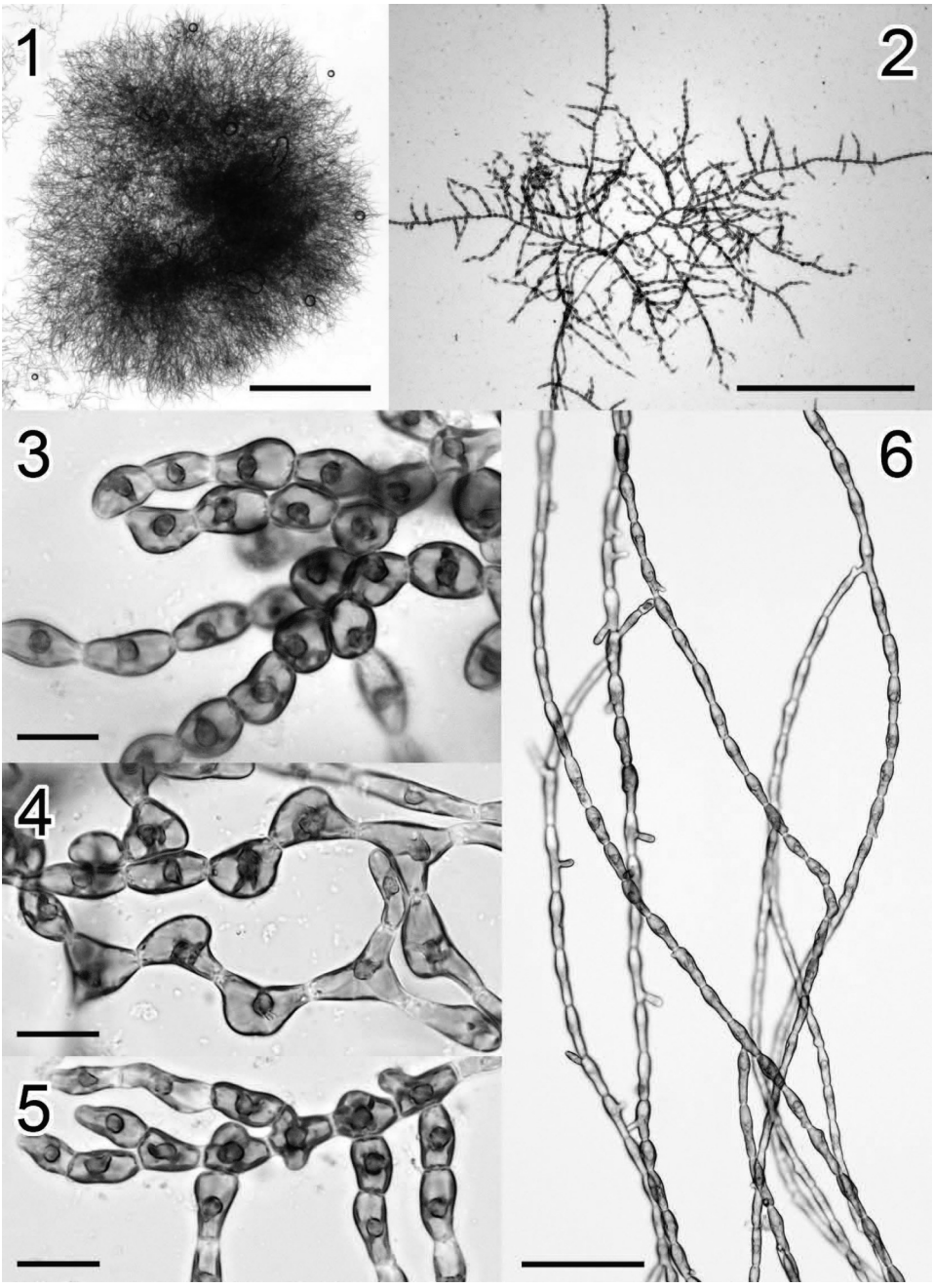
the center of each vegetative cell (Figs 3-5, 7-9). Parietal, H-shaped plastids (Fig. 7) or highly-lobed stellate plastids (Figs 8, 9) are present. Spores are spherical, 10-11 µm (20 µm maximum) when discharged (Fig. 10). Spores germinate with retention of spore contents (Fig. 11). Pit plugs are composed of the plug core, inner cap layer, and outer cap layer (Fig. 12); the outer cap layer has a plate-like structure. A cap membrane was not observed between the inner and outer cap layers.

Molecular Phylogenetic Analyses – 1,696 nucleotides of the SSU gene [which correspond to positions 20-1759 of *Cyanidioschyzon merolae* P. De Luca, R. Taddei *et* L. Varano (CMQ305R, Matsuzaki *et al.*, 2004)] were used in the alignment. Eleven nucleotide differences (eight transitional and three transversional substitutions) were found between *R. hawaiiana* and *R. madagascarensis*. To determine outgroup taxa for our analysis, we first used a dataset including *Ballia callitricha* (C. Agardh) Kützing (AF236790), a member of the Nemaliophycidae, three species of Corallinales [*Corallina officinalis* Linnaeus (L26184), *Lithothamnion glaciale* Kjellman (U60738), and *Metagoniolithon chara* (Lamarck) Ducker (U60743)] and one species of Rhodogorgonales [*Rhodogorgon carriebowensis* R.E. Norris *et* Bucher (AF006089)], and found that the members of Corallinales and Rhodogorgonales were the deepest lineage and *B. callitricha* was sister to the rest of the taxa but on an extremely long branch (data not shown). We did not include *B. callitricha* in the final dataset because of the possibility of long-branch artefacts.

Rhodachlya hawaiiana resolved as sister to *R. madagascarensis* with high support from all analyses (Fig. 13). The closest lineage to the genus *Rhodachlya* was not resolved in the analyses, although each order was strongly supported, as has been previously demonstrated (Verbruggen *et al*., 2010).

Remarks – The diagnostic feature of this new taxon segregating typical acrochaetioid algae is possession of two-layered pit plugs without cap membranes; a pit plug structure that has only been known previously in *R. madagascarensis* among marine acrochaetioids (West *et al.*, 2008); however, this type of pit plug is known from members of the freshwater red algal order Thoreales (Müller *et al*., 2002) as well as *Batrachospermum macrosporum* (as *Audouinella macrospora*; Pueschel *et al*., 2000). This pit plug morphology, in combination with our molecular and morphological data, supports this new alga as a member of the Rhodachlyales. The Hawaiian isolate also possesses plastids characterized by a lack of peripheral encircling thylakoids, which is considered a distinctive trait for the genus (West *et al.*, 2008). Morphological differences between *R. hawaiiana* and *R. madagascarensis* include a larger vegetative cell size in *R. hawaiiana*, rarity of colorless hair cell formation in *R. hawaiiana* and differing spore germination patterns (spore contents are retained in germination in *R. hawaiiana*).

The possibility that frequency of hair cell formation and cell size for the two taxa was affected by culture conditions (*e.g.* temperature, light intensities, or nutrient conditions; West, 1972; O'Connor & West, 1991) was examined in more detail. Conditions in the present study and those in West *et al.* (2008) were not identical; seawater in the present study was collected from a well, originating 14 m below the Waikiki Aquarium, Honolulu, Hawaii, and is known to be nutrient rich (Atkinson *et al.*, 1995). Temperatures were also slightly different: our cultures were maintained between 20-25°C, versus 20-22°C for West *et al.* (2008). However, an additional comparative culture experiment with *R. hawaiiana*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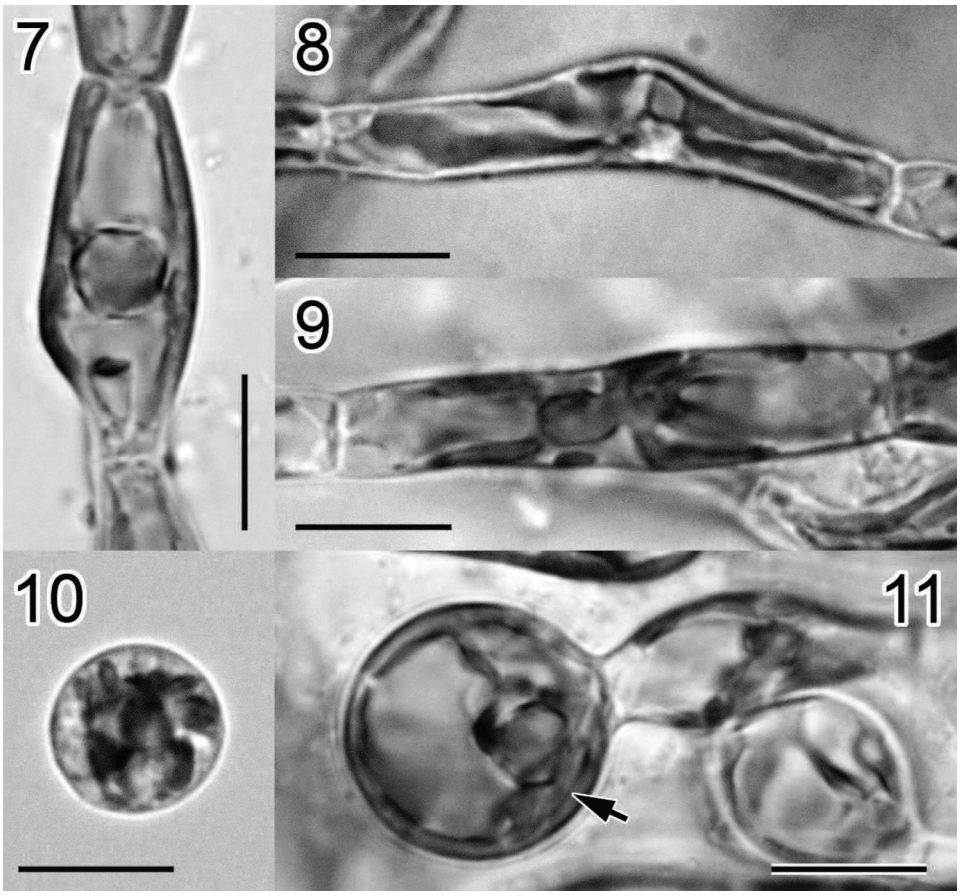

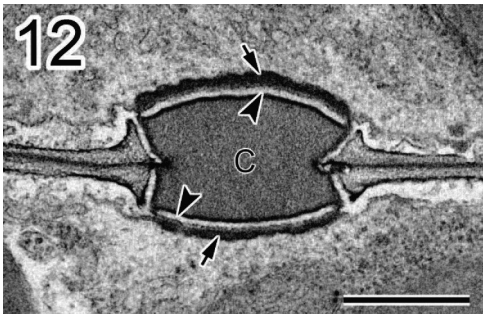

Figs 1-12. *Rhodachlya hawaiiana* A. Kurihara, J.A. West, K.Y. Conklin *et* A.R. Sherwood *sp. nov.* **1.** Basal and erect filaments (Scale bar = 2 mm). **2.** Basal filaments (Scale bar = 500 µm). **3-5.** Cells of basal filaments (Scale bars = 20 µm). **6.** Cells of erect filaments (Scale bar = 100 µm). **7.** Parietal, H-shaped plastid in a vegetative cell (Scale bar = 10 µm). **8, 9.** Highly lobed plastids in vegetative cells (Scale bars = 10 µm). **10.** Released spore (Scale bar = 10 µm). **11.** Spore germination in which protoplast is retained in spore (arrow) after formation of germ tube (Scale bar = 10 µm). **12.** Ultrastructure of pit plug. The pit plug consists of a plug core (C), inner plug cap layers (lighter portions; arrowheads) and plate-shaped outer plug core layers (darker portions; arrows). No cap membrane is visible between the inner and outer cap layers (Scale bar = 300 nm).

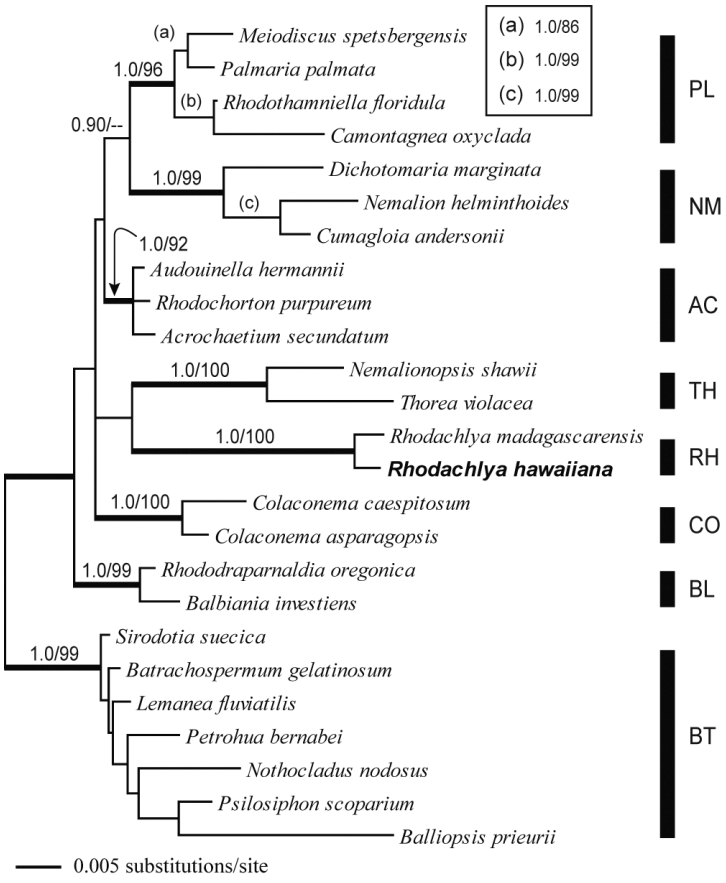

Fig. 13. Maximum likelihood tree (*ln*L = -4783.68714) inferred from 24 sequences of SSU rRNA gene for the Nemaliophycidae. Numbers at nodes indicate support values (posterior probabilities and ML bootstrap values, respectively). (a) 1.00/86, (b) 1.00/99, and (c) 1.00/99. Only values greater than 70% (0.7) are shown. Each thick branch corresponds to a clade at the ordinal level. AC, Acrochaetiales; BL, Balbianiales; BT, Batrachospermales; CO, Colaconematales; NM, Nemaliales; PL, Palmariales; RH, Rhodachlyales; and TH, Thoreales. Scale bar = substitutions per site.

material under the same conditions as those of West *et al.* (2008) reproduced the morphological characteristics (size of vegetative cells, frequency of colorless hair cells, and spore germination pattern) reported here for *R. hawaiiana*.

Our results also indicate that spore germination pattern can differ for species of *Rhodachlya*. In Type I germination (West, 1972), which is found in *R. madagascarensis* (West *et al.*, 2008), the entire contents of the spore move into the germ tube and a transverse wall is formed. In Type II germination (sensu West, 1972), which was observed in *R. hawaiiana*, the spore retains a protoplast after germ tube formation. However, it should be noted that spore germination pattern is not necessarily consistent for red algal species. For example, sometimes only one type occurs in carpospores and tetraspores of a single species (*e.g. Colaconema asparagopsis* Chemin; Abdel-Rahman & Magne, 1981), while, in contrast, germination patterns can differ in tetraspores/monospores from tetrasporophytes

Table 1. Sequence divergence comparisons of the nuclear SSU rRNA gene for members of the Nemaliophycidae. GenBank accession numbers are listed below. Gray shading represents comparisons where *p*-distances are equal to or less than 0.0064

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*Acrochaetium arcuatum*	—													
2	*Ac. secundatum*	0.0029	—												
3	*Audouinella hermannii*	0.0058	0.0041	—											
4	*Colaconema amphiroae*	0.0193	0.0175	0.0169	—										
5	*C. asparagopsis*	0.0216	0.0198	0.0169	0.0047	—									
6	*C. caespitosus*	0.0222	0.0216	0.0199	0.0076	0.0099	—								
7	*C. dasyae*	0.0204	0.0198	0.0181	0.0058	0.0082	0.0076	—							
8	*C. daviesii*	0.0193	0.0187	0.0169	0.0023	0.0047	0.0064	0.0047	—						
9	*C. pectinatum*	0.0216	0.0210	0.0193	0.0058	0.0058	0.0099	0.0082	0.0047	—					
10	*C. proskaueri*	0.0204	0.0198	0.0181	0.0047	0.0047	0.0088	0.0070	0.0035	0.0012	—				
11	*C. rhizoidea*	0.0216	0.0198	0.0181	0.0058	0.0082	0.0035	0.0070	0.0058	0.0093	0.0082	—			
12	*C. tetrasporum*	0.0199	0.0193	0.0175	0.0053	0.0070	0.0070	0.0006	0.0041	0.0076	0.0064	0.0064	—		
13	*Rhodochorton purpureum*	0.0064	0.0047	0.0029	0.0152	0.0163	0.0193	0.0175	0.0152	0.0175	0.0163	0.0175	0.0169	—	
14	*R. tenue*	0.0053	0.0047	0.0029	0.0163	0.0175	0.0193	0.0175	0.0152	0.0175	0.0163	0.0187	0.0169	0.0012	—

1. *Acrochaetium arcuatum* (AF079786, as *Audouinella arcuata*), 2. *Ac. secundatum* (AF079784, as *Au. secundata*), 3. *Au. hermannii* (AF026040), 4. *Colaconema amphiroae* (K.M. Drew) P.W. Gabrielson (AF079785, as *Au. amphiroae*), 5. *C. asparagopsis* (AF079795, as *Au. asparagopsis*), 6. *C. caespitosum* (AF079787, as *Au. caespitosa*), 7. *Colaconema dasyae* (F.S. Collins) Stegenga, I. Mol., Prud'homme van Reine *et* Lokhorst (L26181, *Au. dasyae*), 8. *Colaconema daviesii* (Dillwyn) Stegenga (AF079788, as *Au. daviesii*), 9. *Colaconema pectinatum* (Kylin) J.T. Harper *et* G.W. Saunders (AF079790, as *Au. pectinata*), 10. *C. proskaueri* (AF079791, as *Au. proskaueri*), 11. *Colaconema rhizoideum* (K.M. Drew) P.W. Gabrielson (AF079792, as *Au. rhizoidea*), 12. *Colaconema tetrasporum* (Garbary *et* Rueness) Athanasiadis (AF079793, as *Au. tetraspora*), 13. *Rhodochorton purpureum* (U23816), 14. *Rhodochorton tenue* Kylin (AF079796, as *Au. tenue*).

Table 1. Sequence divergence comparisons of the nuclear SSU rRNA gene for members of the Nemaliophycidae. GenBank accession numbers are listed below. Gray shading represents comparisons where *p*-distances are equal to or less than 0.0064 *(continued)*

		15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
15	*Devaleraea ramentacea*	—															
16	*Halosaccion glandiforme*	0.0029	—														
17	*Rhodophysema elegans*	0.0035	0.0041	—													
18	*Palmaria palmata*	0.0029	0.0047	0.0041	—												
19	*P. palmata*	0.0029	0.0047	0.0041	0.0012	—											
20	*Batrachospermum boryanum*	0.0305	0.0299	0.0310	0.0305	0.0305	—										
21	*B. gelatinosum*	0.0293	0.0293	0.0305	0.0293	0.0293	0.0047	—									
22	*Lemanea fluviatilis*	0.0281	0.0287	0.0299	0.0281	0.0282	0.0041	0.0035	—								
23	*Paralemanea catenata*	0.0299	0.0293	0.0305	0.0299	0.0299	0.0047	0.0035	0.0029	—							
24	*Sirodotia huillensis*	0.0287	0.0293	0.0305	0.0275	0.0276	0.0064	0.0035	0.0035	0.0053	—						
25	*S. suecica*	0.0293	0.0299	0.0310	0.0293	0.0293	0.0059	0.0029	0.0041	0.0059	0.0018	—					
26	*Tuomeya americana*	0.0299	0.0305	0.0316	0.0299	0.0299	0.0059	0.0053	0.0053	0.0070	0.0064	0.0059	—				
27	*Balbiania investiens*	0.0216	0.0222	0.0222	0.0204	0.0205	0.0264	0.0258	0.0264	0.0269	0.0234	0.0252	0.0293	—			
28	*Rhododraparnaldia oregonica*	0.0193	0.0210	0.0210	0.0181	0.0181	0.0275	0.0252	0.0252	0.0269	0.0223	0.0240	0.0275	0.0058	—		
29	*Nemalionopsis shawii*	0.0362	0.0380	0.0368	0.0362	0.0363	0.0381	0.0381	0.0381	0.0387	0.0375	0.0370	0.0387	0.0392	0.0362	—	
30	*N. tortuosa*	0.0345	0.0362	0.0350	0.0344	0.0345	0.0363	0.0363	0.0363	0.0369	0.0358	0.0352	0.0369	0.0374	0.0345	0.0029	—

15. *Devaleraea ramentacea* (Linnaeus) Guiry (L26186), 16. *Halosaccion glandiforme* (S.G. Gmelin) Ruprecht (L26193), 17. *Palmaria palmata* (Z14142), 18. *P. palmata* (X53500), 19. *Rhodophysema elegans* (J. Agardh) Dixon (U23817), 20. *Batrachospermum boryanum* Sirodot (AF026044), 21. *B. gelatinosum* (AF026045), 22. *Lemanea fluviatilis* (AF026051), 23. *Paralemanea catenata* (Kützing) M.L. Vis *et* R.G. Sheath (AF026052), 24. *Sirodotia huillensis* (W. West *et* G.S. West) Skuja (AF026954), 25. *S. suecica* (AF026053), 26. *Tuomeya americana* (Kützing) M.J. Wynne (AF026055), 27. *Balbiania investiens* (AF132294), 28. *Rhododraparnaldia oregonica* (AF026043), 29. *Nemalionopsis shawii* (AF506272), 30. *Nemalionopsis tortuosa* Yoneda *et* Yagi (AF342743)

and carpospores [*e.g. Acrochaetium parvulum* (Kylin) Hoyt; Abdel-Rahman, 1984]. Additionally, both germination types are found in carpospores and tetraspores [*e.g. Grania efflorescens* (J. Agardh) Kylin; Clayden & Saunders, 2008; *Colaconema proskaueri* (J.A. West) P.W. Gabrielson; West, 1972]. Germination patterns also appear to be affected by the age of spores (Guiry, 1990).

Two kinds of plastid morphologies were observed in *R. hawaiiana* (parietal, H-shaped, or axial, lobed, stellate; Figs 7-9). Plastid morphology is often used as an important character at the ordinal level in red algae (*e.g.* Harper & Saunders, 2002); however, it can be variable even within a single thallus (West, 1968). Light intensities, nutrient levels or angle of viewing may underlie reported variation in plastid morphology.

Another difference between the two species is the sequence divergence of the SSU rRNA gene (11 nucleotide substitutions; *p*-distance = 0.0064). This sequence divergence is large enough to separate the two species based on a comparison of divergences between other members of the Nemaliophycidae (Table 1).

Neither sexual reproduction nor evidence of the filamentous stages of *R. hawaiiana* and *R. madagascarensis* corresponding to gametophytic or tetrasporophytic generations has been observed thus far. Morphological features of spermatangial and female organs or postfertilization development may be elucidated in the future as further diagnostic characters separating the two *Rhodachlya* species.

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Appendix 1.

GenBank accession numbers used for molecular phylogenetic analyses

[SSU rRNA gene] *Acrochaetium secundatum* (Lyngbye) Nägeli (AF079784, as *Audouinella secundata*); *Audouinella hermannii* (Roth) Duby (AF026040); *Balbiania investiens* (Kützing) Sirodot (AF132294); *Balliopsis prieurii* (Kützing) G.W. Saunders *et* Necchi (AF419245); *Batrachospermum gelatinosum* (Linnaeus) De Candolle (AF026045); *Camontagnea oxyclada* (Montagne) Pujals (AF079794); *Colaconema asparagopsis* Chemin (AF079795); *Colaconema caespitosum* (J. Agardh) Jackelman, Stegenga *et* J.J. Bolton (AF079787); *Cumagloia andersonii* (Farlow) Setchell *et* N.L. Gardner (DQ343669), *Dichotomaria marginata* (Ellis *et* Solander) Lamarck (AF006090, as *Galaxaura marginata*); *Lemanea fluviatilis* (Linnaeus) C. Agardh (AF026051); *Meiodiscus spetsbergensis* (Kjellman) G.W. Saunders *et* McLachlan (U23814); *Nemalion helminthoides* (Velley) Batters (L26196); *Nemalionopsis shawii* Skuja (AF506272); *Nothocladus nodosus* Skuja (U23815); *Palmaria palmata* (Linnaeus) Kuntze (Z14142); *Petrohua bernabei* G.W. Saunders (EF033583); *Psilosiphon scoparium* Entwisle (AF026041); *Rhodachlya madagascarensis* (EU262260); *Rhodochorton purpureum* (Lightfoot) Rosenvinge (U23816); *Rhododraparnaldia oregonica* R.G. Sheath A. Whittick *et* K.M. Cole (AF026043); *Rhodothamniella floridula* (Dillwyn) Feldmann (U23818); *Sirodotia suecica* Kylin (AF026053); *Thorea violacea* Bory de Saint-Vincent (AF026042).