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Phylogenetic relationships of *Dichotomaria* (Nemaliales, Rhodophyta) with the proposal of *Dichotomaria intermedia* (R.C.Y. Chou) comb. nov.

Abstract: Species delineation in the calcified red algal genus *Dichotomaria* is difficult due to the high degree of morphological similarity observed in these algae; thus, species descriptions are based on a limited set of morphological characters. In this study, we conducted phylogenetic analyses of plastid *rbcL* and mitochondrial *cox1* sequences and detailed morphological observations on 28 specimens of *Dichotomaria* from the Indo-Pacific, Bermuda, the Galápagos Islands, and the Lesser Antilles. As a consequence, we propose the new combination *Dichotomaria intermedia* (R.C.Y. Chou) comb. nov. based on *Galaxaura intermedia*. This species has flattened branches, three to four cortical cell layers, four sterile branches issuing from the hypogynous cell, and a pericarp formed from the basal cell, as in the genus *Dichotomaria*. In the *rbcL* tree, *D. intermedia* formed a group with *Dichotomaria tenera* from South Africa. Our results confirmed the occurrence of 11 species and one variety: *Dichotomaria apiculata*, *Dichotomaria australis*, *Dichotomaria diesingiana*, *Dichotomaria falcata*, *Dichotomaria hommersandii*, *Dichotomaria intermedia*, *Dichotomaria marginata*, *Dichotomaria obtusata*, *D. obtusata* var. *major*, *Dichotomaria papillata*, *Dichotomaria spathulata*, and *D. tenera*. Further study is required to address unidentified genetic groups that cannot currently be assigned to known species groups.

Keywords: biogeography; *cox1*; *Dichotomaria*; Galaxauraceae; *rbcL*; red algae.

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Introduction

Dichotomaria Lamarck (1816), one of four calcified genera in the family Galaxauraceae, was resurrected

by Huisman et al. (2004) on the basis of molecular and morphological evidence. The generitype is *Dichotomaria marginata* (J. Ellis et Solander) Lamarck from the Bahamas, with the basionym *Corallina marginata* J. Ellis et Solander (Ellis and Solander 1786). *Dichotomaria* is currently characterized by its life history with alternating isomorphic gametophytic and tetrasporophytic phases, the presence of a pericarp enclosing the cystocarp, a multinucleate fusion cell formed by incorporation of gonimoblast initials and inner gonimoblast cells, and cruciate tetrasporangia produced on stalk cells (Huisman et al. 2004, Wang et al. 2005, Huisman 2006, Kurihara and Huisman 2006). Subsequent analyses of plastid *rbcL* sequences strongly supported *Dichotomaria* as a distinct genus (Wang et al. 2005, Liu et al. 2013).

Dichotomaria currently includes 18 recognized species and three varieties that are distributed in tropical to warm temperate waters in the world (Guiry and Guiry 2013, Liu et al. 2013). The majority of species were transferred from *Galaxaura* (Huisman et al. 2004, Kurihara et al. 2005, Wynne 2005, 2011, Kurihara and Huisman 2006), while *Dichotomaria hommersandii* S.L. Liu et S.M. Lin was recently described from South Africa (Liu et al. 2013). *Galaxaura intermedia* R.C.Y. Chou occurs frequently in the lower littoral in the Galápagos Islands (Taylor 1945, Chou 1947). It is bushy with thickly shaggy stalks, up to 23 cm tall, dichotomously branched above. Despite its conspicuous habit, the taxonomy of *G. intermedia* has not been addressed since its description.

Recently, we have focused on the taxonomy of the galaxauracean red algae with the molecular tools, plastid *rbcL* and mitochondrial *cox1* sequences (Wiriyadamrikul et al. 2013a,b). Together with morphological observations, we employed both genes to provide a better understanding of genetic and species diversity in *Dichotomaria*. We also examined the isotype specimen of *G. intermedia* and analyzed *rbcL* and *cox1* sequences from the type. As a consequence of these studies, we herein propose the new combination *D. intermedia* (R.C.Y. Chou) J. Wiriyadamrikul, M.J. Wynne et S.M. Boo.

Materials and methods

Information on all specimens used in this study is given in Table 1. Specimens were pressed onto herbarium sheets, and tissue subsamples were dehydrated in silica gel for molecular work. The isotype specimen of *Galaxaura intermedia* R.C.Y. Chou and the holotype specimen of *Galaxaura obtusata* var. *major* W.R. Taylor, both in MICH (Thiers 2013), were studied. For morphological observation, distal branch portions were decalcified in 1% HCl solution (Sigma-Aldrich Corporation, Gyeonggi, Korea) and sectioned by hand using a razor blade. The sectioned preparations were stained with 1% aqueous aniline blue acidified with 1% diluted HCl and mounted in 70% glycerine (Sigma-Aldrich Corporation, Gyeonggi, Korea). Photographs were taken with Sony Alpha DSLR-A850 (Sony Corporation, Tokyo, Japan) and DP-71 Olympus cameras (Olympus Corporation, Tokyo, Japan) attached to Nikon Optiphot (Nikon Corporation, Tokyo, Japan) and BX 51 Olympus microscopes (Olympus Corporation, Tokyo, Japan), respectively. Voucher specimens are housed at the herbarium of Chungnam National University (CNUK), Daejeon, Korea.

DNA extraction, PCR amplification, and sequencing followed Geraldino et al. (2010). Specific primers for the amplification and sequencing of each gene were for *rbcl*, F43-R753 and F615-RrbcS start (Freshwater and Rueness 1994, Wiriadamrikul et al. 2013b); for *cox1*, *cox143F*-C880R (Geraldino et al. 2006, Yang et al. 2008).

Fifty-seven *rbcl* sequences (including 29 accessions from GenBank) and 28 *cox1* sequences (including eight previously published, Sherwood et al. 2010, Table 1) of *Dichotomaria* and outgroups were collated using the multiple-sequence editing program Se-Al v.2.0a11 (Rambaut 1996, available at <http://tree.bio.ed.ac.uk/software/seal/>) and aligned visually. Maximum-likelihood (ML) phylogenetic analyses were performed using RAxML software (Stamatakis 2006, The Exelixis Lab, Heidelberg, Germany) using the GTRGAMMA model. We used 200 independent tree inferences with the “number of run” option with default optimized SPR rearrangement and 25 distinct rate categories to identify the best tree. Statistical support for each branch was obtained from 1000 bootstrap replications using the same substitution model and RAxML program settings.

Bayesian analyses were performed for individual datasets with MrBayes v.3.2.1 (Ronquist et al. 2012, available at <http://mrbayes.sourceforge.net/download.php>) using Metropolis-coupled Markov Chain Monte Carlo (MCMC) with the GTR+ Γ +I model. For each matrix, 2 million generations of two independent runs were performed with four chains and by sampling trees every 100 generations. The burn-in period was identified graphically by tracking

the likelihoods at each generation to determine whether they reached a plateau. The 32,802 trees for *rbcl* and 32,802 trees for *cox1* sampled at stationary state were used to infer the Bayesian posterior probability.

Results

Characteristics of *rbcl* and *cox1* sequences

A total of 57 *rbcl* sequences (1371 bp), including 28 newly acquired accessions, were generated in the present study. Variable sites occurred at 409 positions (29.8%), and 306 positions (22.3%) were parsimony informative. Intraspecific pairwise divergence was high to 2.0% (27 bp difference) in *Dichotomaria obtusata* (J. Ellis et Solander) Lamarck. The average interspecific pairwise divergence ranged from 0.5% [7 bp difference between *Dichotomaria apiculata* (Kjellman) Kurihara et Masuda and *Dichotomaria falcata* (Kjellman) Kurihara et Masuda] to 7.7% (106 bp difference between *Dichotomaria hommersandii* and *Dichotomaria* sp. 2).

A total of 28 *cox1* sequences, including 20 newly generated sequences, were obtained for an alignment of 581 bp with 197 variable positions (33.2%) and 155 parsimoniously informative positions (26.1%). The genetic distance of *cox1* between species ranged from 4.4% [26 bp difference between *D. obtusata* and *D. obtusata* var. *major* (W.R. Taylor) M.J. Wynne] to 11.6% (69 bp difference between *D. falcata* and *Dichotomaria* sp. 5), and intraspecific divergences were also high up to 5.4% (32 bp difference) within *Dichotomaria marginata*.

A comparison for minimal pairwise distance of *Dichotomaria* species to nearest neighbor and intraspecific variations based on *rbcl* and *cox1* sequences is given in Table 2.

Phylogeny of *Dichotomaria* species

A ML tree of *rbcl* sequences provided evidence for the existence of seven groups within the genus *Dichotomaria* (marked as I–VII) (Figure 1) with strong bootstrap support. Group I consisted of six species groups: *Dichotomaria tenera* (Kjellman) Huisman, J.T. Harper et G.W. Saunders from South Africa, *Dichotomaria intermedia* (as *Galaxaura intermedia*) from the Galápagos Islands and Indonesia, *Dichotomaria marginata* from Guadeloupe, Oman, Papua New Guinea, the Philippines, and Thailand and more than three lineages, marked as sp. 1–sp. 3 that did not match identified

Table 1 Species, collection or publication information, and GenBank accession numbers of the *rbcl* and *cox1* sequences.

Species, collection site and date, collector or publication	Voucher	GenBank Acc. no.	
		<i>rbcl</i>	<i>cox1</i>
<i>Actinotrichia fragilis</i> (Forsskål) Børgesen			
Bandar Khayran, Oman; Liu and Wang (2009)		EU095253	–
Hawaii, USA; Sherwood et al. (2010)		–	HQ422678
<i>Actinotrichia robusta</i> Itono			
3 rd Nuclear Power Outlet, Kenting National Park, Taiwan; Wang et al. (2005)		AY688011	–
<i>Actinotrichia taiwanica</i> Liu et Wang			
Chiupeng, Kenting National Park, Taiwan; Liu and Wang (2009)		EU105470	–
<i>Dichotomaria apiculata</i> (Kjellman) Kurihara et Masuda			
Geomundo, Yeosu, Korea; 12 Oct. 2011; JW	CNU011781	KF752498	–
Maemuldo, Tongyoung, Korea; 24 Jul. 2011; THS	CNU011768	KF752499	–
Saeseom, Jeju, Korea; 6 Jul. 2011; YDK	CNU011710	KF752500	–
Sungsan, Jeju, Korea; 3 Dec. 2002; SMB	CNU032163	KF752501	KF752526
Amakusa, Kumamoto, Japan; Kurihara et al. (2005)		AB117623	–
<i>Dichotomaria australis</i> (Sonder) Huisman, Harper et Saunders			
Jervis Bay, New South Wales, Australia; Kurihara and Huisman (2006)		AB258440	–
Rye, Victoria, Australia; Kurihara and Huisman (2006)		AB258443	–
<i>Dichotomaria diesingiana</i> (Zanardini) Huisman, Harper et Saunders			
Port Alfred, Cape Province, South Africa; Wang et al. (2005)		AY688026	–
<i>Dichotomaria falcata</i> (Kjellman) Kurihara et Masuda			
Oura Bay, Shimoda, Japan; 31 Jul. 2009; KMK	CNU032192	KF752502	KF752527
Kijang, Busan, Korea; 15 May 2009; KMK	CNU032190	KF752503	KF752528
Saeseom, Jeju, Korea; 6 Jul. 2011; YDK	CNU011709	KF752504	–
Shinando, Wando, Korea; 13 Jul. 2007; SMB	CNU032196	KF752505	–
Shimoda, Shizuoka, Japan; Kurihara et al. (2005)		AB117629	–
<i>Dichotomaria hommersandii</i> Liu et Lin			
Port Alfred, Cape Province, South Africa; Liu et al. (2013)		JX072968	–
<i>Dichotomaria intermedia</i> (Chou) J. Wiryadamrikul, M.J. Wynne et S.M. Boo comb. nov.			
Isabela Island, Galapagos Island, Ecuador; WRT	WR Taylor 1934-164	KF752506	KF752529
Santa Maria Island, Galapagos Island, Ecuador; 17 Jan. 1934; WRT	Taylor 34-213	KF752507	KF752530
Santa Maria Island, Galapagos Island, Ecuador; WRT	WR Taylor 1934-246	KF752508	KF752531
Gerupuk, Lombok, Indonesia; 24 Oct. 2009; KMK	CNU032199	KF752509	KF752532
<i>Dichotomaria marginata</i> (Ellis et Solander) Lamarck			
Mirbat, Dhofar, Oman; 26 Sep. 2000; GR	Richards20002006	KF752510	–
Camotes Island, Cebu, Philippines; 14 Dec. 2003; ECV	CNU032166	KF752511	KF752533
Similan Islands, Phang Nga, Thailand; 5 Apr. 2010; JW	CNU032231	KF752512	KF752534
Surin Islands, Phang Nga, Thailand; 8 Apr. 2012; JW	CNU021563	KF752513	KF752535
Hachijo Island, Tokyo, Japan; Kurihara et al. (2005)		AB117630	–
Sulpa Island, Cebu, Philippines; Wang et al. (2005)		AY688017	–
Malang Lagoon, Papua New Guinea; Wang et al. (2005)		AY688018	–
St. Prorjono, Guadeloupe; Wang et al. (2005)		AY688019	–
Sail Rock, Kenting National Park, Taiwan; Wang et al. (2005)		AY688020	–
Tahsianglan, Taipei County, Taiwan; Wang et al. (2005)		AY688021	–
Sonora, Gulf of California, Mexico; Wang et al. (2005)		AY688022	–
Hawaii, USA; Sherwood et al. (2010)		–	HQ422611
Hawaii, USA; Sherwood et al. (2010)		–	HQ422632
Hawaii, USA; Sherwood et al. (2010)		–	HQ422899
<i>Dichotomaria obtusata</i> (Ellis et Solander) Lamarck			
Surin Islands, Phang Nga, Thailand; 8 Apr. 2012; JW	CNU021562	KF752514	KF752536
Itoman, Okinawa, Japan; Kurihara et al. (2005)		AB117632	–
Parakeet Bay, Rottneest Island, Australia; Kurihara and Huisman (2006)		AB258447	–
Tiaoshih, Kenting National Park, Taiwan; Wang et al. (2005)		AY688024	–
Palm Beach, Kwazulu Natal, South Africa; Wang et al. (2005)		AY688025	–
<i>D. obtusata</i> var. <i>major</i> (Taylor) M.J. Wynne			
Tucker's Town Bay, Hamilton I., Bermuda; 15 May 1949; WRT	WR Taylor 1949-288 (holotype)	KF752515	KF752537
Tucker's Town Bay, Hamilton, Bermuda; WRT	WR Taylor 1961-9	KF752516	KF752538

(Table 1 Continued)

Species, collection site and date, collector or publication	Voucher	GenBank Acc. no.	
		<i>rbcl</i>	<i>cox1</i>
South east end of Kindley Air Base, Bermuda; AJB	AJB 50-439	KF752517	KF752539
<i>Dichotomaria papillata</i> (Kjellman) Kurihara et Masuda Amakusa, Kumamoto, Japan; Kurihara et al. (2005)		AB117631	–
<i>Dichotomaria spathulata</i> (Kjellman) Kurihara et Huisman Green Island, Rottneest Island, Australia; Kurihara and Huisman (2006)		AB258445	–
Green Island, Rottneest Island, Australia; Kurihara and Huisman (2006)		AB258446	–
<i>Dichotomaria tenera</i> (Kjellman) Huisman, Harper et Saunders Lala Neck, Kwazulu Natal, South Africa; 9 Aug. 2005; SMB	CNU032168	KF752518	–
Rocky Bay, Kwazulu Natal, South Africa; 17 Aug. 2005; SMB	CNU032161	KF752519	KF752540
Palm Beach, Kwazulu Natal, South Africa; Wang et al. (2005)		AY688023	–
<i>Dichotomaria</i> sp. 1 Lombok, Indonesia; 6 Jun. 2012; SMB	CNU023023	KF752520	KF752541
<i>Dichotomaria</i> sp. 2 Sand Bank Bay, St. Kitts, Lesser Antilles; 24 Nov. 1995; MJW	Wynne10514	KF752521	KF752542
<i>Dichotomaria</i> sp. 3 Pagudpud, Ilocos Norte, Philippines; 10 Mar. 2005; SMB	CNU032178	KF752522	KF752543
<i>Dichotomaria</i> sp. 4 Pangil, Ilocos Norte, Philippines; 11 Mar. 2005; SMB	CNU032180	KF752523	KF752544
<i>Dichotomaria</i> sp. 5 Rocky Bay, Kwazulu Natal, South Africa; 17 Aug. 2005; SMB	CNU032175	KF752524	KF752545
<i>Dichotomaria</i> sp. 6 Libong, Trang, Thailand; 18 Apr. 2004; KL	CNU021829	KF752525	–
<i>Galaxaura divaricata</i> (Linnaeus) Huisman et Townsend Bonin Islands, Tokyo, Japan; Kurihara et al. (2005)		AB117628	–
Hawaii, USA; Sherwood et al. (2010)		–	HQ422677
<i>Galaxaura filamentosa</i> Chou Hawaii, USA; Sherwood et al. (2010)		–	HQ422797
<i>Galaxaura pacifica</i> Tanaka Bonin Islands, Tokyo, Japan; Kurihara et al. (2005)		AB117638	–
<i>Galaxaura rugosa</i> (Ellis et Solander) Lamouroux Bulusan, Sorsogon, Philippines; Wang et al. (2005)		AY688000	–
Hawaii, USA; Sherwood et al. (2010)		–	HQ422631
<i>Tricleocarpa cylindrica</i> (Ellis et Solander) Huisman et Borowitzka Guymas, Gulf of California, Mexico; Wang et al. (2005)		AY688015	–
<i>Tricleocarpa fragilis</i> (Linnaeus) Huisman et Townsend Wanlidung, Kenting National Park, Taiwan; Wang et al. (2005)		AY688016	–
<i>Tricleocarpa</i> sp. Hawaii, USA; Sherwood et al. (2010)		–	HQ422640

AJB, A.J. Bernatowicz; ECY, Eun Chan Yang; GR, Glenn Richards; JW, Jutarat Wiriadamrikul; KL, Khanjanapaj Lewmanomont; KMK, Kyeong Mi Kim; MJW, Michael J. Wynne; SMB, Sung Min Boo; THS, Tae Ho Seo; WRT, William Randolph Taylor; YDK, Yong Deok Ko.

Sequences generated in the present study are in bold.

sequences in GenBank. Group II consisted of *Dichotomaria spathulata* (Kjellman) Kurihara et Huisman from Western Australia, and Group III represented the *Dichotomaria* sp. 4 from the Philippines. Group IV comprised *Dichotomaria obtusata* complexes in the Indo-Pacific Ocean, including *D. obtusata* var. *major* from Bermuda. Two taxa, sp. 5 and sp. 6, occurred in South Africa and Thailand, respectively. Group V comprised three species from Japan and Korea: *Dichotomaria apiculata*, *Dichotomaria falcata*, and

Dichotomaria papillata (Kjellman) Kurihara et Masuda. Group VI contained *D. australis* (Sonder) Huisman, J.T. Harper et G.W. Saunders from Australia and *Dichotomaria diesingiana* (Zanardini) Huisman, J.T. Harper et G.W. Saunders from South Africa, but two sequences of *Dichotomaria australis* differed by 1.3%. The last group (Group VII) represented *Dichotomaria hommersandii* from South Africa.

Four groups (I, III, IV, V) discerned in the *rbcl* tree were also identified in the *cox1* phylogeny (Figure 2).

Table 2 Minimal pairwise distances between *Dichotomaria* species and nearest neighbors and intraspecific variations based on *rbcl* and *cox1* sequences.

Species	Nearest neighbor and minimal distance		Intraspecific variation	
	<i>rbcl</i>	<i>cox1</i>	<i>rbcl</i>	<i>cox1</i>
<i>Dichotomaria australis</i>	<i>Dichotomaria diesingiana</i> , 3.2%		1.3%	
<i>Dichotomaria falcata</i>	<i>Dichotomaria apiculata</i> , 0.5%	<i>D. apiculata</i> , 4.5%	0%	0.7%
<i>Dichotomaria hommersandii</i>	<i>D. australis</i> , 6.4%			
<i>Dichotomaria intermedia</i>	<i>Dichotomaria tenera</i> , 0.9%	<i>Dichotomaria</i> sp. 1, 4.7–5.1%	0.5%	3.4%
<i>Dichotomaria marginata</i>	<i>D. "marginata"</i> , AY688021, 1.5%	<i>Dichotomaria</i> sp. 3, 4.5–5.1%	0.9%	0.2–5.4%
<i>D. "marginata"</i> AY688020	<i>Dichotomaria</i> sp. 3, 2.0%			
<i>D. "marginata"</i> AY688021	<i>Dichotomaria</i> sp. 3, 1.2%			
<i>Dichotomaria obtusata</i>	<i>D. obtusata</i> var. <i>major</i> , 1.3%	<i>D. obtusata</i> var. <i>major</i> , 4.4%	0.6–2.0%	
<i>Dichotomaria papillata</i>	<i>D. falcata</i> , 1.8%			
<i>Dichotomaria spathulata</i>	<i>D. "marginata"</i> , AY688021, 2.3%		0.4%	
<i>D. tenera</i>	<i>D. intermedia</i> , 0.9%	<i>D. marginata</i> , 4.9–7.2%	0.3%	
<i>Dichotomaria</i> sp. 1	<i>D. intermedia</i> , 1.1%	<i>D. intermedia</i> , 4.7–5.1%		
<i>Dichotomaria</i> sp. 2	<i>D. tenera</i> , 1.0%	<i>D. marginata</i> , 5.1–6.6%	0.6%	
<i>Dichotomaria</i> sp. 3	<i>D. "marginata"</i> , AY688021, 1.2%	<i>D. marginata</i> , 4.5–6.1%	0.4%	
<i>Dichotomaria</i> sp. 4	<i>D. spathulata</i> , 2.6%	<i>D. marginata</i> , 5.7–6.9%		
<i>Dichotomaria</i> sp. 5	<i>D. obtusata</i> var. <i>major</i> , 2.0%	<i>D. obtusata</i> , 7.7%	0.6%	
<i>Dichotomaria</i> sp. 6	<i>Dichotomaria</i> sp. 5, 2.2%			

However, Groups II, VI, and VII in *rbcl* were not available for *cox1* analysis.

Morphological observations

Dichotomaria apiculata (Kjellman) Kurihara et Masuda

Dichotomaria apiculata (Figure 3A) was usually attached to hard substrata in the subtidal zone in Jeju, Korea. Thalli were up to 10 cm in height, dichotomously branched, with branching intervals 0.5–1.5 cm long; branches were flattened, glabrous, 1–3 mm in diameter (Figure 3B). The margins of the flattened branches were always ridged, especially noticeable on dried specimens (Figure 3B,C). The structure of the cortical layer differed considerably between gametophytes and tetrasporophytes. In gametophytes, the cortex was composed of four to five cell layers. The inner layers were composed of colorless, irregular cells (20–40×35–75 μm). The outer layers consisted of two pigmented cell layers: a sub-epidermal layer and an epidermal layer. The cells in the sub-epidermal layer were cup- or lens-shaped in cross section, tightly arranged (15–25 μm high), whereas epidermal cells were obovoid or clavate, widest in the upper third or quarter, with an apiculate apex (rarely with a round apex), measuring 20–40×15–25 μm (Figure 3D).

The gametophytes were dioecious. Spermatangia (5–8×3–5 μm) were formed in hemispherical conceptacles of 250–350 μm in diameter (Figure 3E). Carpogonial branches consisted of three cells (basal cell, hypogynous

cell, and distal carpogonium) and arose near the apex of the plant in place of a normal vegetative filament (Figure 3F). Cystocarps were hemispherical, 350–550 μm in diameter. Carposporangia were ovoid to obovoid and 25–50×15–25 μm in size. The multinucleate fusion cell was formed by the incorporation of several inner gonimoblast cells, whereas the hypogynous and basal cell remained distinct throughout cystocarp development (Figure 3G).

In tetrasporophytes, the cortex consisted of four to five cell layers. The inner layer cells were as in gametophytes. The outer layers consisted of two pigmented cell layers: sub-epidermal layer of elongated stalk-like cell (13–30×10–20 μm) and epidermal layer of obovoid cells, with an apiculate apex, measuring 35–50×20–35 μm (Figure 3H). Tetrasporangial initials were cut off laterally from the sub-epidermal cells. Mature tetrasporangia were cruciately divided, spherical to ovoid in shape, and 35–45 μm in diameter (Figure 3I).

Dichotomaria falcata (Kjellman) Kurihara et Masuda

Dichotomaria falcata occurred in Korea and Japan. Plants were dichotomously branched (Figure 4A,B). Branches were flattened with raised margins, 1–2 mm in diameter (Figure 4C). The epidermal cells were cylindrical, without an apiculate apex (rarely with a sharp end), measuring 30–40 μm in diameter (Figure 4D,H). Reproductive structures (Figure 4E,G,I) were identical to those of *Dichotomaria apiculata*.

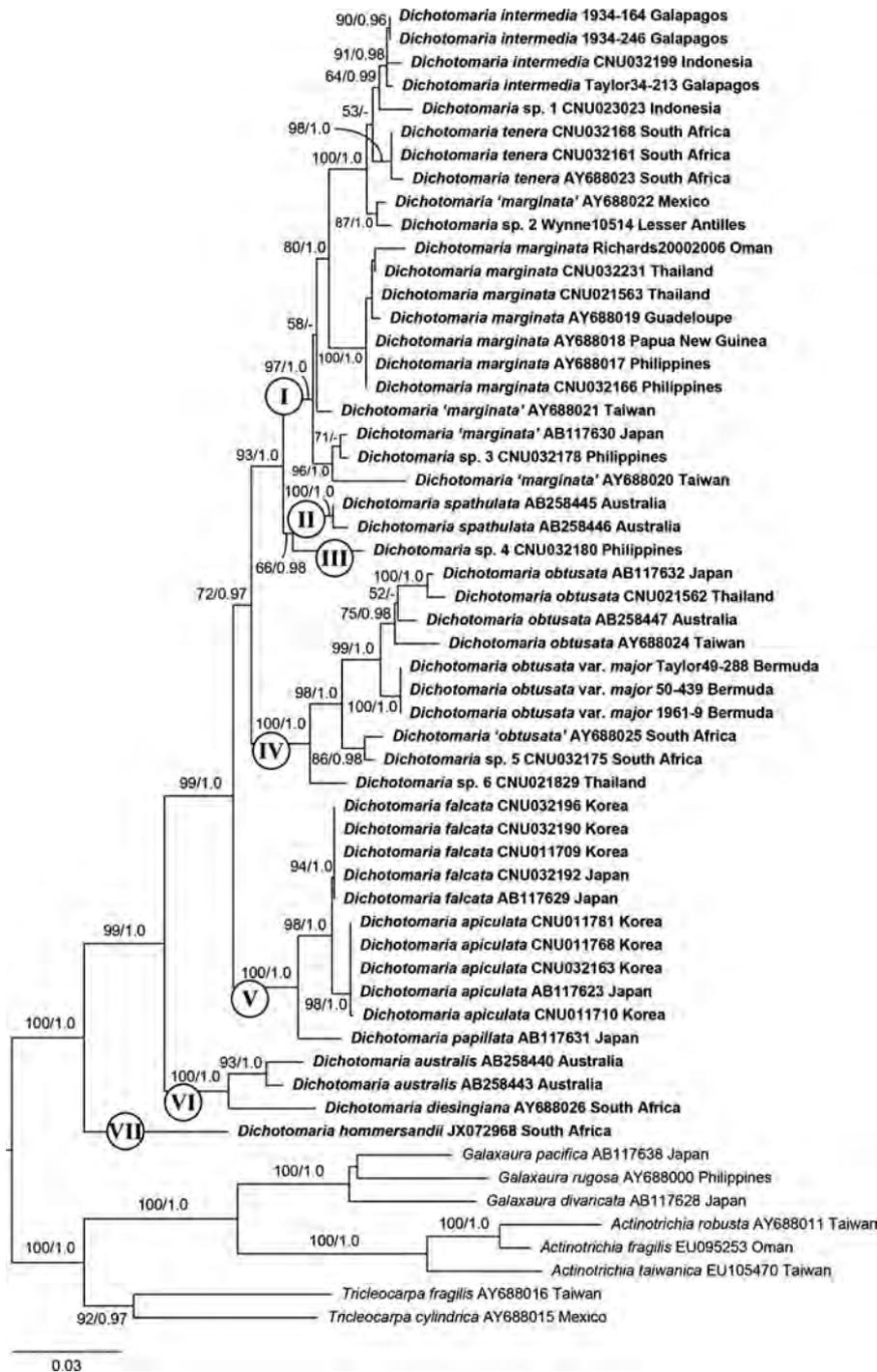


Figure 1 Maximum likelihood tree of *Dichotomaria* inferred from the phylogenetic analysis of *rbcL* sequences. Values shown near branches are bootstrap values (1000 iterations) and Bayesian posterior probabilities. Only bootstrap values >50% and Bayesian posterior probabilities >0.95 are shown. Roman numbers in circles denote clades as described in the text.

***Dichotomaria intermedia* (R.C.Y. Chou) J. Wiryadamrikul, M.J. Wynne et S.M. Boo comb. nov.**

Basionym

Galaxaura intermedia R.C.Y. Chou in Taylor 1945. Pacific marine algae of the Allan Hancock Expeditions to the Galápagos Islands. *Allan Hancock Pac. Exped.* 12: 144–145.

Type

W.R. Taylor, No. 34-213, Black Beach Anchorage, Isla Santa Maria, Galápagos Islands, Ecuador; 17 Jan. 1934 (Taylor 1945: 144–145). Holotype is now in UC; isotype in MICH.

It is worth pointing out that Taylor (1945: iv) stated the “technical types” of his new species were then being deposited in the Allan Hancock Foundation (AHFH).

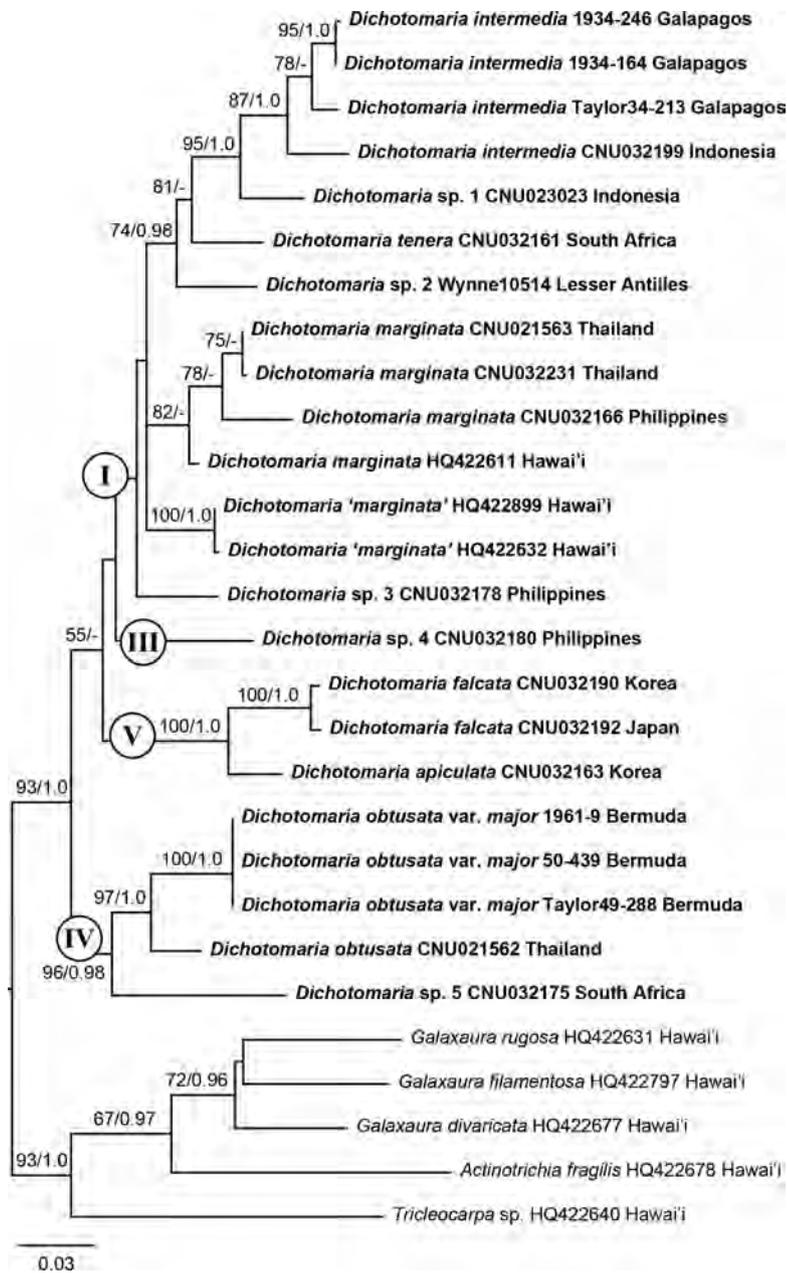


Figure 2 Maximum likelihood tree of *Dichotomaria* inferred from the phylogenetic analysis of *cox1* sequences. Values shown near branches are bootstrap values (1000 iterations) and Bayesian posterior probabilities. Only bootstrap values >50% and Bayesian posterior probabilities >0.95 are shown. Roman numerals in circles denote clades as described in the text, except for II, VI, and VII, which were not available for *cox1* analysis.

Those AHFH algal holdings were later transferred to LAM, and subsequently, the algal holdings of LAM were transferred to UC. Taylor (1945) deposited a second set of types (the isotypes) of his new species in the University of Michigan Herbarium (MICH).

The isotype specimen in MICH was studied. This isotype was depicted by Chou (1947, pl. XIII) in a publication subsequent to her description of this species in

Taylor (1945). It was up to 23 cm in height, dichotomously branched, with branching intervals 1.0–2.5 cm long. Branches were flattened, glabrous, 1–2 mm in diameter. The margins of the branches were neither involute nor much thickened (Figure 5A). The cortex was composed of three cortical cell layers throughout the thallus. The inner layers were composed of colorless, subglobose, or angular cells, often laterally fused, measuring 28–40×50–70 μm.

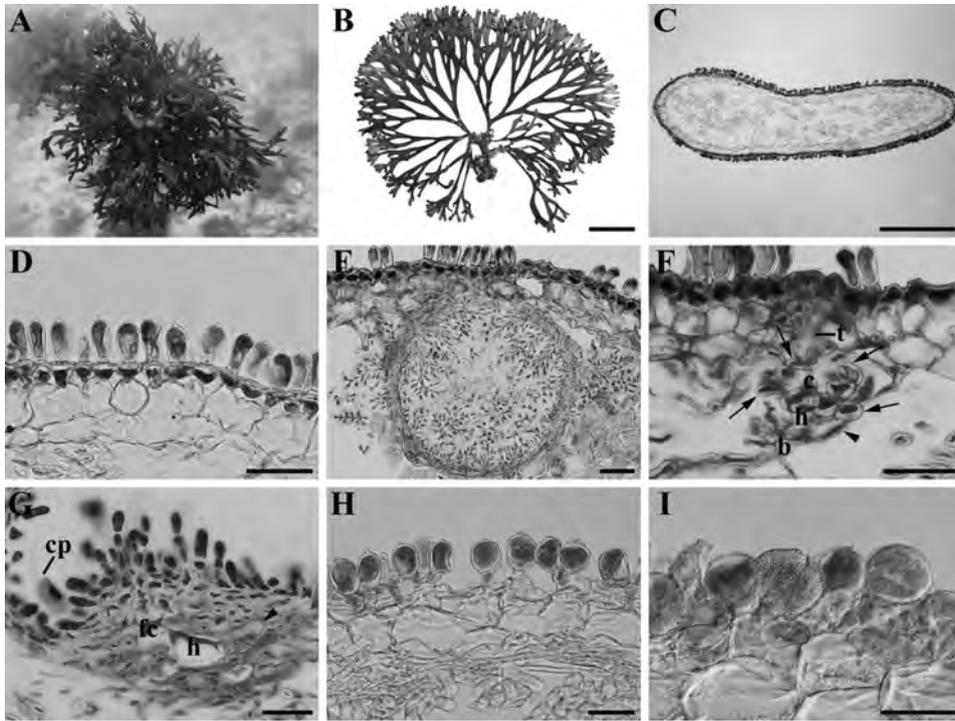


Figure 3 *Dichotomaria apiculata*: (A) A thallus attached to rock in Jeju Island, Korea. (B) Cystocarpic plant. (C) Section of flattened branch. (D) Section of gametophyte cortex showing apiculate epidermal cells. (E) Spermatangial conceptacle. (F) Young carpogonial branch showing carpogonium with trichogyne, hypogynous cell with sterile branches (arrows), and basal cell bearing sterile branches (arrowhead). (G) Section of cystocarp showing carposporangia, fusion cell, and distinct hypogynous cell with its sterile branches (arrowhead). (H) Tetrasporophyte cortex showing apiculate outer cortical cells with distinct stalk cell in middle cortex. (I) Tetrasporangia on stalk cells. b, basal cell; c, carpogonium; cp, carposporangia; fc, fusion cell; h, hypogynous cell; t, trichogyne. Scale bars: (B) 2 cm; (C) 500 μm ; (D–I) 50 μm .

The outer layer consisted of pigmented cells that were cup- or lens-shaped in cross section, tightly arranged, measuring $10\text{--}18 \times 16\text{--}24 \mu\text{m}$, without the spinulose cells (Figure 5B).

Only a female gametophytic plant was observed in this study. Carpogonial branches were three-celled, consisting of a carpogonium with trichogyne, a hypogynous cell that cut off four sterile branches, and a basal cell with four to five involucrel filaments that ultimately formed the pericarp (Figure 5C). Mature cystocarps were hemispherical, $400\text{--}550 \mu\text{m}$ in diameter. Ovoid to obovoid carposporangia ($30\text{--}45 \times 15\text{--}30 \mu\text{m}$) were produced singly and terminally from the gonimoblast filaments along the inner surface of the cystocarp (Figure 5D). The multinucleate fusion cell was formed by the incorporation of several inner gonimoblast cells, whereas the hypogynous and basal cell remained distinct throughout cystocarp development (Figure 5E).

Dichotomaria marginata (J. Ellis et Solander) Lamarck

Plants were usually found growing on dead coral in the intertidal to 15 m (Figure 6A). Thalli were up to 15 cm in

height, subdichotomously branched, with branching interval 0.5–1.5 cm long. Branches were flattened with raised margins, 1–5 mm in diameter (Figure 6B,C). The gametophyte cortex was composed of four to five cell layers. The apiculate epidermal cells of $30\text{--}35 \times 10\text{--}15 \mu\text{m}$ were observed only at the margin of the branch (Figure 6C,D). Gametophytes were dioecious. Male conceptacle and cystocarp development (Figure 6E–H) were identical to those of *Dichotomaria apiculata*.

In tetrasporophytes, the cortex consisted of four to five cell layers, with ellipsoidal or subglobose, apiculate epidermal cells, measuring $30\text{--}50 \times 25\text{--}35 \mu\text{m}$ (Figure 6I). Tetrasporangia were not observed in the present study.

Dichotomaria obtusata (J. Ellis et Solander) Lamarck

Thalli usually occurred on reef rock at 1–16 m depths and were up to 8 cm in height with subdichotomous branches (Figure 7A). Branches were terete, glabrous, and articulate, 1.5–2.5 mm in diameter (Figure 7B). The cortex was composed of three cell layers throughout (Figure 7C,D).

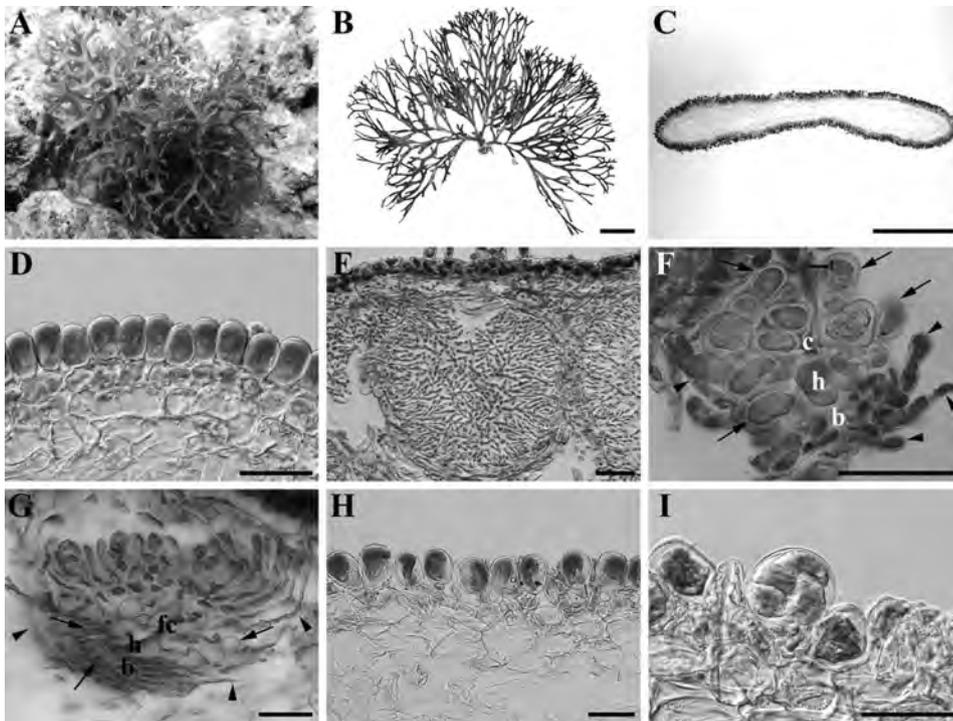


Figure 4 *Dichotomaria falcata*: (A) A thallus growing on rock in Jeju Island, Korea. (B) Cystocarpic plant. (C) Section of flattened branch. (D) Cortex of gametophyte showing subspherical epidermal cells. (E) Spermatangial conceptacle. (F) Young carpogonial branch showing carpogonium, hypogynous cell with sterile branches (arrows), and basal cell bearing sterile branches (arrowheads). (G) Section of cystocarp showing carposporangia and fusion cell. Hypogynous cell with sterile branches (arrows), and basal cell bearing sterile branches (arrowheads) remain distinct. (H) Cortex of tetrasporophyte showing subspherical epidermal cells on stalk cells. (I) Tetrasporangia on stalk cells. b, basal cell; c, carpogonium; fc, fusion cell; h, hypogynous cell; t, trichogyne. Scale bars: (B) 2 cm; (C) 500 μm ; (D–I) 50 μm .

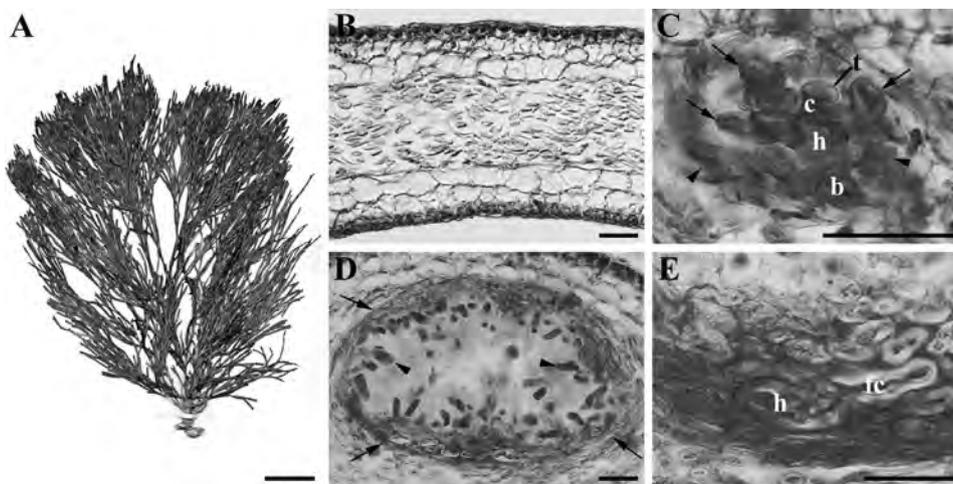


Figure 5 *Dichotomaria intermedia* comb. nov.: (A) Isotype specimen in MICH. (B) Section of flattened branch of gametophyte showing tristromatic cortical cell layer and medulla. (C) Tristromatic cortical cell layer of gametophyte. Young carpogonial branch showing carpogonium with trichogyne, hypogynous cell with sterile branches (arrows), and basal cell bearing sterile branches (arrowheads). (D) Mature cystocarp showing carposporangia (arrowheads) and pericarp (arrows). (E) Detail of cystocarp showing the fusion cell and distinct hypogynous cell. b, basal cell; c, carpogonium; fc, fusion cell; h, hypogynous cell; t, trichogyne. Scale bars: (A) 3 cm; (B–E) 50 μm .

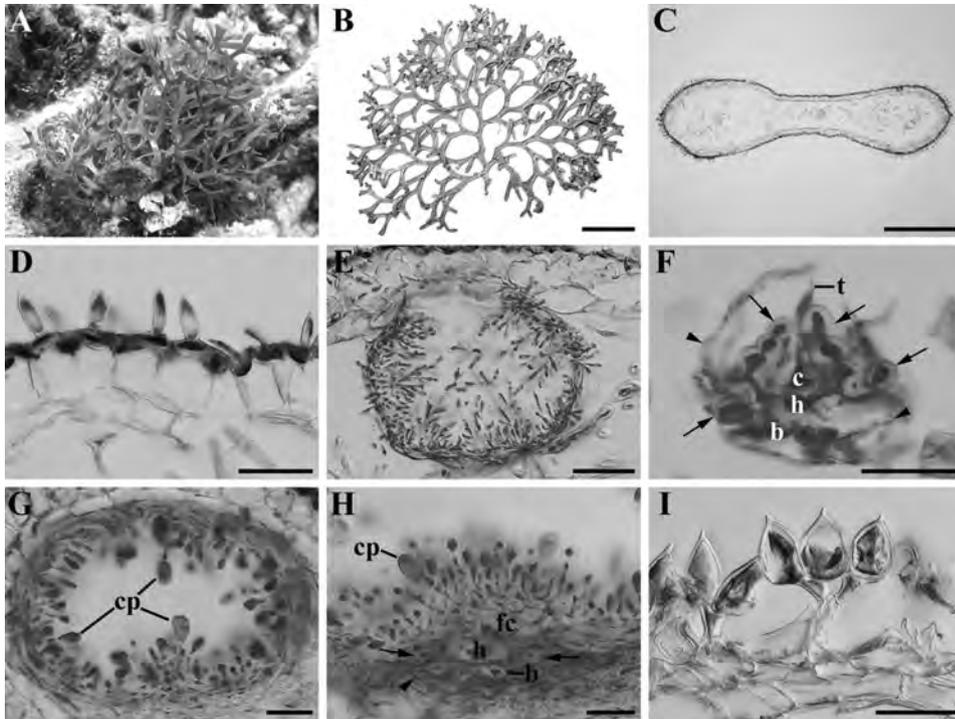


Figure 6 *Dichotomaria marginata*: (A) Habitat in Surin Islands, Thailand. (B) Gametophytic plant. (C) Section of flattened branch. (D) Section of gametophyte cortex showing apiculate epidermal cells. (E) Spermatangial conceptacle. (F) Young carpegonial branch showing carpogonium, hypogynous cell with sterile branches (arrows), and basal cell bearing sterile branches (arrowheads). (G) Mature cystocarp. (H) Detail of cystocarp showing carposporangia and fusion cell. Hypogynous cell with sterile branches (arrows) and basal cell bearing sterile branches (arrowheads) remain distinct. (I) Tetrasporophyte cortex showing apiculate epidermis on stalk cells. b, basal cell; c, carpogonium; cp, carposporangia; fc, fusion cell; h, hypogynous cell; t, trichogyne. Scale bars: (B) 3 cm; (C) 500 μm , (D–F) 50 μm .

Spermatangia ($7\text{--}9 \times 4\text{--}7 \mu\text{m}$) were produced in hemispherical conceptacles of 250–350 μm in diameter (Figure 7E). Cystocarps were not observed in this study.

The cortex of tetrasporophytes differed from that of gametophytes in that the sub-epidermis consisted of a layer of elongated stalk-like cells (Figure 7F), whereas

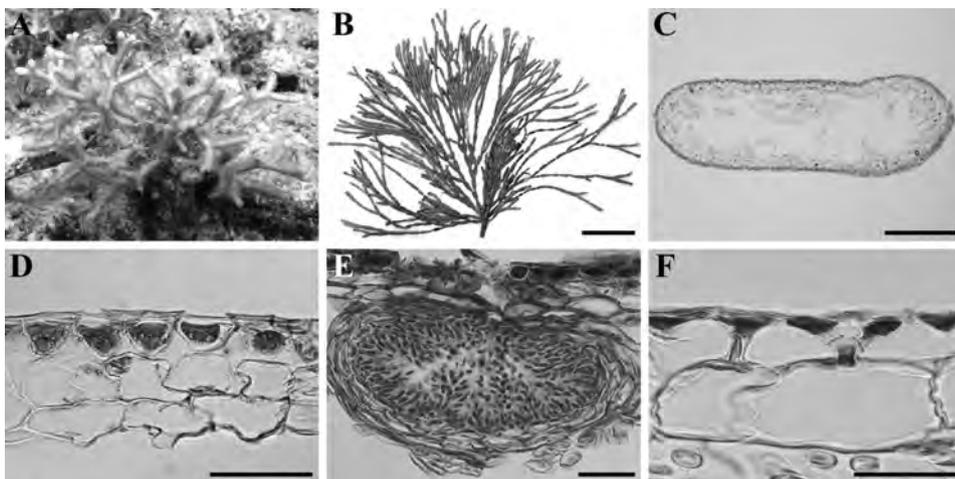


Figure 7 *Dichotomaria obtusata*: (A) Habitat in Surin Islands, Thailand. (B) Gametophytic plant. (C) Section of terete branch. (D) Section of gametophyte cortex. (E) Spermatangial conceptacle. (F) Tetrasporophyte cortex. Scale bars: (B) 2 cm; (C) 500 μm ; (D–F) 50 μm .

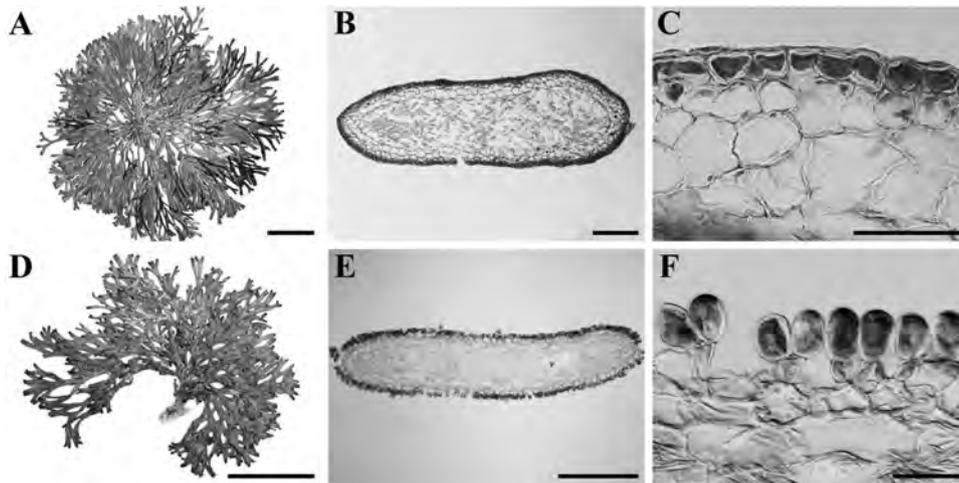


Figure 8 *Dichotomaria obtusata* var. *major* (A–C) and *Dichotomaria tenera* (D–F): (A) Dichotomous branches from holotype specimen. (B) Section of terete branch. (C) Tristromatic cortical cell layer of gametophyte. (D) Tetrasporic plant. (E) Section of flattened branch. (F) Cortex of tetrasporophyte showing ellipsoidal outer cortical cells. Scale bars: (A and D) 5 cm; (B and E) 500 μ m; (C and F) 50 μ m.

the sub-epidermal cells in gametophytes were tightly arranged (Figure 7D).

Dichotomaria obtusata var. *major* (W.R. Taylor)

M.J. Wynne

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Galaxaura obtusata var. *major* W.R. Taylor in *Marine algae of the eastern tropical and subtropical coasts of the Americas*, 1960: 342, 343, 633, 750, 751, pl. 44: fig. 5.

The holotype specimen in MICH was up to 17 cm in height, subdichotomously branched. Branches were flattened, glabrous, and articulate, 2.5–3.0 mm in diameter (Figure 8A). Thalli were composed of a longitudinally branched filamentous medulla and three to four cortical cell layers of tightly arranged cells (Figure 8B,C).

Dichotomaria tenera (Kjellman) Huisman, J.T. Harper

et G.W. Saunders

Thalli were up to 6 cm in height and consisted of many subdichotomous branches, with branching intervals 0.4–1.0 cm long (Figure 8D). Branches were flattened with flat or slightly raised margins (Figure 8E). The cortex consisted of two to three inner layers, and a sub-epidermal layer of elongated stalk-like cells (17–27 \times 10–15 μ m), bearing pairs of ellipsoidal epidermal cells of 30–40 \times 25–30 μ m (Figure 8F).

Discussion

Fortunately, the extraction of DNA and PCR amplifications of both *rbcl* and *cox1* were successful from the small fragments of the type specimen of *Galaxaura intermedia*, which was collected by W.R. Taylor in the Galápagos Islands, Ecuador (Taylor 1945), and *Galaxaura obtusata* var. *major*, which was collected by W.R. Taylor in Bermuda (Taylor 1960). Tree topologies of 57 galaxauracean *rbcl* sequences including *G. intermedia* are largely consistent with those obtained in previous studies (Kurihara et al. 2005, Wang et al. 2005, Huisman 2006, Liu et al. 2013). *G. intermedia* was distinct and placed within the *D. tenera* group. One specimen collected from Lombok, Indonesia, was grouped with *G. intermedia*.

Vegetative and reproductive structures of the Indonesian specimens were consistent with those of the isotype specimen of *G. intermedia* lodged in the Herbarium of the University of Michigan. The Indonesian collections also agree well with the protologue of *G. intermedia* that includes a description by Chou in Taylor (1945): “plant bushy, below the axis caulescent and villous; above dichotomous, the lower segment subterete and villous, the upper ones complanate, glabrous, 1.0–2.5 cm long, 1–2 mm wide, the margins neither involute nor much thickened, and cortex 3–4 stromatic.” Our fresh collections contained cystocarps, and we also found several cystocarps on the isotype fragment of *G. intermedia* that were not included in the original description (see Chou in Taylor 1945). The female structures and development of cystocarps of the

Indonesian specimens as well as the isotype specimen clearly place it in the genus *Dichotomaria*.

Carposporophyte development is an important diagnostic character for distinguishing genera within the family Galaxauraceae (Huisman and Borowitzka 1990, Wang et al. 2005, Huisman 2006, Liu et al. 2013). *Dichotomaria* has a three-celled carpogonial branch, consisting of a carpogonium, a hypogynous cell that cuts off four sterile branches, and a basal cell with four to five involucreal filaments that ultimately form the pericarp (Wang et al. 2005, Liu et al. 2013). A morphological comparison of *Dichotomaria intermedia* and similar species is given in Table 3. It is, therefore, concluded that *G. intermedia* should be moved to the genus *Dichotomaria* based on reproductive structures as well as the *rbcL* and *cox1* phylogenies. We also found that the specimens, collected in the Galápagos Islands by Taylor (1945) and identified as “*Galaxaura spathulata*” by Ruth Chou (in Taylor 1945) (Chou 1945), grouped with *D. intermedia* in both *rbcL* and *cox1* sequences; they were distantly related to *Dichotomaria spathulata* from western Australia, the type locality (Huisman and Kurihara 2006). This is the first document to report *D. intermedia* outside the Galápagos Islands, its distribution being extended to Indonesia.

Dichotomaria obtusata is morphologically variable and occurs very widely in warm waters of the world (see Guiry and Guiry 2013). High genetic divergence is found within *D. obtusata*, consisting of at least two groups in *rbcL* and *cox1*; the first from Australia, Japan, Taiwan, and Thailand and the second from South Africa. Although they are very similar in morphology, the genetic divergence is very high, up to 2.0% for *rbcL* (see Table 2), indicating that these two groups may represent distinct species. Because we could not analyze material from the type locality, Bahamas, West Indies (Huisman and Womersley 1994), we cannot decide which group is true *D. obtusata*, and simultaneously, the other group needs to be described as a distinct species. In addition, analyses of specimens from Africa (Silva et al. 1996), Australasia (Huisman 2006), Europe (Haroun et al. 2002), North and South America (Wynne 2011), and the Pacific Islands (Skelton and South 2007) are needed. *D. obtusata* var. *major* was described as *G. obtusata* var. *major* based on specimens collected in Tuckers Town Bay, Bermuda (Taylor 1960, Wynne 2005, 2011). Analyses of *rbcL* and *cox1* sequences from three Bermudan specimens of this variety indicate that it is clearly separate from two groups of *D. obtusata*. It may be raised to the rank of species; however, this is beyond resolution in the present study.

Our analysis of *rbcL* and *cox1* sequences from numerous specimens of *Dichotomaria* from the Indo-Pacific,

Table 3 Comparison of morphological characters of *Dichotomaria intermedia* and similar species.

	<i>Dichotomaria intermedia</i>	<i>Dichotomaria marginata</i>	<i>Dichotomaria obtusata</i>	<i>D. obtusata</i> var. <i>major</i>	<i>Dichotomaria tenera</i>
Plant height (cm)	Up to 23	Up to 15	Up to 8	Up to 17	Up to 6
Branch shape	Flattened	Flattened to terete	Terete	Terete	Flattened
Branch length (cm)	1.0–2.5	0.5–1.5	0.5–1.0	3–9	0.4–1.0
Branch width (mm)	1.0–2.0	1.0–5.0	1.5–2.5	2.5–4	1.0–2.0
Cortical cell layers					
Gametophyte	Three to four	Four to five	Three to four	Three to four	–
Tetrasporophyte	Unknown	Four to five with stalk cells	Three to four with stalk cells	Three to four with stalk cells	Four to five with stalk cells
Cystocarp diam. (µm)	400–550	500–600	300–600	–	–
Cystocarp shape	Hemispherical	Spherical to slightly flattened	Hemispherical	–	–
Habitat	Lower intertidal zones	Intertidal to subtidal zones	Subtidal zones	Subtidal zones	Intertidal zones
Reference	Taylor 1945 This study	Huisman and Borowitzka (1990), Huisman (2006) This study	Huisman and Womersley (1994), Wang et al. (2005) This study	Taylor (1960) This study	Huisman et al. (2004) This study

Bermuda, the Galápagos Islands, and the Lesser Antilles reveal high genetic diversity within the genus. The *rbcL* sequences provided sufficient resolution to confirm 10 species and one variety: *Dichotomaria apiculata*, *Dichotomaria australis*, *Dichotomaria diesingiana*, *Dichotomaria falcata*, *Dichotomaria hommersandii*, *Dichotomaria marginata*, *D. obtusata*, *D. obtusata* var. *major*, *Dichotomaria papillata*, *D. spathulata*, and *Dichotomaria tenera*, as seen in previous studies (Kurihara et al. 2005, Wang et al. 2005, Liu et al. 2013). However, the *rbcL* tree revealed six remaining taxa that, despite being genetically distinct, cannot be assigned to species because of the similarity of vegetative morphology and the lack of reproductive structures. The pairwise divergences (1.4–4.9%) between these unidentified taxa were higher than those (0.5%) between *D. apiculata* and *D. falcata* in the present study (Table 2) and in other red algae, for example, 0.2–2% between *Pterocladia capillacea* (S.G. Gmelin) Santelices et Hommersand and *P. tenuis* (Okamura) Shimada, Horiguchi et Masuda (Boo et al. 2010). All these unidentified taxa, except sp. 6 that was not amplified for unknown reasons, were consistently supported by *cox1*. Further sampling of these taxa is necessary to determine which taxa will need to be described as distinct species.

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