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Taxonomic transfer of the red algae *Chrysymenia enteromorpha* and *C. wrightii* to the genus *Botryocladia* (Rhodymeniaceae, Rhodymeniales)

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Abstract

Genera in the Rhodymeniaceae Harvey that have a hollow thallus lacking diaphragms have been placed in *Chrysymenia* J. Agardh 1842 (including *Gloiosaccion* Harvey 1859), *Botryocladia* (J. Agardh) Kylin 1931*, Irvinea* Guiry in Saunders *et al.* 1999, and *Cresia* C. Lozada-Troche, D.L. Ballantine & H. Ruíz 2010. *Chrysymenia* has traditionally been defined by a lack of internal rhizoids and with the only solid portion of the thallus limited to the stipe. *Botryocladia* has been differentiated from *Chrysymenia* by the presence of larger, solid axes. Our results suggest that on the basis of vegetative characters alone, the true *Chrysymenia* clade contains specimens that exclusively produce gland cells directly on unmodified medullary cells, whereas *Botryocladia* and *Cresia* may or may not cut off gland cells from modified gland-supporting medullary cells. On the basis of comparative *rbc*L and SSU sequence analyses and morphological data, the hollow, gelatinous species *Chrysymenia enteromorpha* Harvey from the Gulf of Mexico and Caribbean Sea, and *Chrysymenia wrightii* Harvey described from the northwest Pacific Ocean are newly transferred to the genus *Botryocladia* as *Botryocladia enteromorpha* (Harvey) comb. nov. and *B. wrightii* (Harvey) comb. nov., respectively.

Keywords: algae, biodiversity, *Botryocladia*, *Chrysymenia*, Gulf of Mexico, marine, new combination, morphology, phylogeny, Puerto Rico, *rbc*L, Rhodophyta, Rhodymeniaceae, seaweeds, SSU rDNA

Introduction

The hollow genera in the Rhodymeniaceae that lack diaphragms, *i.e*. *Chrysymenia* J. Agardh 1842 (including *Gloiosaccion* Harvey 1859), *Botryocladia* (J.Agardh) Kylin 1931, *Irvinea* Guiry (in Saunders *et al.* 1999) and *Cresia* C. Lozada-Troche, D.L. Balantine & H. Ruiz 2010, are taxa that are most commonly distributed in warm-temperate to tropical environments (Abbott and Littler 1969, Brodie and Guiry 1988). These genera are currently circumscribed by underdefined and overlapping features. For example, *Chrysymenia* has been historically characterized by an almost completely hollow thallus that lacks diaphragms and internal rhizoids, and in which the only solid portion is limited to the stipe. A similar thallus organization, but with internal rhizoids, is characteristic of *Cryptarachne* (Harvey) Kylin (1931), a genus that was previously considered a subgenus of *Chrysymenia* by Harvey (1859a). Most phycologists today have followed Okamura (1936) in disagreeing with the taxonomic value of such rhizoidal filaments and consider the latter two genera congeneric (Wynne 2005).

In J. Agardh's (1842) original concept, *Chrysymenia* contained a section, *Botryocladia,* that was later elevated to generic rank by Kylin (1931). *Botryocladia* is currently the second largest genus in the Rhodymeniaceae in terms of number of species and is circumscribed by possessing one to several hollow vesicles that may or may not be branched, lack internal diaphragms, and are borne on simple or solid ramisympodial axes (Gavio and Fredericq 2003, 2005; Afonso-Carillo *et al*. 2006). The presence of larger, solid axes currently differentiates *Botryocladia* from *Chrysymenia*

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(Feldmann 1945, Kylin 1956, Norris 1989, Norris and Ballantine 1995). *Botryocladia* was split into two sections, *B.* sect. Microphyseae that includes small-sized species bearing a small number of vesicles that may or may not be determinate in growth, and *B. sect*. Botryoideae that includes larger taxa comprised of many grape-like vesicles (see Afonso-Carrillo 2006).

Irvinea shares with *Botryocladia* vesicles borne on solid axes (Brodie and Guiry 1988) and with some species of *Botryocladia* the presence of strongly protruding cystocarps, rosettes of cortical cells, and gland (secretory) cells borne on modified medullary cells – the glandiferous or gland-supporting cells; however, the main rationale for the erection of *Irvinea* came from gene-sequence analysis. The morphological characters that delineate *Irvinea* have also been observed in some species that are currently still placed in *Botryocladia* (Schneider and Lane 2008). *Gloiosaccion*, a genus limited to the west and south Australian coast, was recently merged with *Chrysymenia* on the basis of comparative morphology and multi-marker gene phylogenies (Schmidt *et al*. 2016). Recent molecular data have revealed that many of the hollow members that lack diaphragms are not natural groupings and that the generic relationships within the Rhodymeniaceae still require further analyses (Saunders *et al*. 1999, Wilkes *et al*. 2006). We here examine two common species referred to as *Chrysymenia enteromorpha* Harvey and *C. wrightii* (Harvey) Yamada.

Material and methods

Samples of Rhodymeniaceae were investigated from the Gulf of Mexico, Puerto Rico and other locations worldwide (Table 1). Vouchers were preserved upon collection in 5% Formalin/seawater for morphological studies, in silica gel for molecular studies, and pressed on herbarium sheets for archival preservation. The vouchers are deposited in the Algal Herbarium of the University of Louisiana at Lafayette (LAF) and the Algal Collection of the U.S. National Herbarium, National Museum of Natural History, Smithsonian Institution (US).

Morphological Analysis. Light-microscope observations were made on specimens preserved using different methods, in 5% Formalin/seawater, silica gel or as herbarium-pressed material. Silica gel-dried samples and herbarium pressed vouchers were rehydrated in distilled water for 20 min prior to observation. Recently collected *Chrysymenia* and *Botryocladia* samples were rinsed with seawater to remove excess mucilage. Cross and longitudinal sections through vegetative and reproductive structures were made manually using stainless steel razor blades. Longitudinal, cross and oblique sections were stained with 1% aqueous aniline blue (Hommersand *et al*. 1992) acidified with 1% diluted acetic acid and mounted with 50% Karo Syrup/water solution with phenol to retard microbial growth. Photomicrographs were taken with either a Polaroid DMC le Digital camera (Polaroid, Cambridge, MA) or a Canon Rebel EOS T2G attached to an Olympus BX60 microscope. The habit of herbarium-pressed specimens was scanned using an all-in-one Epson Artisan 835 inkjet printer.

Molecular Data Acquisition. Total DNA extractions were performed on the silica gel-dried specimens or herbarium material using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. PCR and sequencing of the chloroplast-encoded ribulose bisphosphate carboxylase/oxygenase gene (*rbc*L) was conducted using the methods and primers described in Lin *et al*. (2012) and Schmidt *et al*. (2016). The ribulose bisphosphate carboxylase/oxygenase (*rbc*L) primers used for amplification and sequencing reactions were as follows: F7, F57, F577, F993, R376, R753, R1150, and R*rbc*S start (Gavio and Fredericq 2002); F64, F645 (Lin *et al*. 2001); F481 (AATTTGGTCGTCCGTTCTTA), R935, R1015 (CCTCTAATCATTAAAGGATCACC), R1434, R1466 (TATACATTCGCTGTTGGAGTTTC) (Schmidt *et al*. 2016: primer combinations F7-R753, F7-R376, F57-R753, F64-R935, F64-R1015, F481-R935, F481-R1150, F577-R935, F577-R1015, F577-R1150, F645-R1434, F645-R*rbc*S, F993-R*rbc*S, F993-R1434, F993-R1466).

PCR products were gel-purified and sequenced in-house in both directions using the BigDye^{tm} Terminator v. 3.1 (Life Technologies Grand Island NY, USA) and run on an ABI 3130xl Genetic Analyzer. All extracts are deposited in the Seaweed Lab at the University of Louisiana at Lafayette.

Sequence Alignment. A 1381 bp portion of the *rbc*L gene for 64 specimens in the Rhodymeniales was assembled with Sequencher v. 5.2 (Gene Codes Corporation). Additional *rbc*L sequences were downloaded from the public NCBI database GenBank and added to the data set (Table 1). The newly generated *rbc*L sequences, in addition to downloaded *rbc*L sequences from GenBank previously generated from our and other labs, were then manually aligned in Mega v. 6 (Tamura *et al*. 2013). The initial SSU alignment consisted of 1762 base pairs (bp). A final yield of 1698 bp for SSU phylogenetic analysis was generated after removing regions ambiguously aligned, missing data, and gaps.

TABLE 1. Specimens used in *rbc*L analysis. Accession numbers in bold represent new sequences in this study.

Taxa	Collection information / voucher	*rbc*L GenBank Accession No.	Reference
Botryocladia ballantinei Gavio & Fredericq	Offshore Louisiana, USA	HQ400569	Gavio & Fredericq 2005
Botryocladia ballantinei Gavio & Fredericq	Bermuda	EU977499	Schneider & Lane 2008
Botryocladia bermudana C.W.Schneider & C.E.Lane	Bermuda	EU977500	Schneider *et al*. 2008
Botryocladia bermudana C.W.Schneider & C.E.Lane	Bermuda	EU977501	Schneider *et al*. 2009
Botryocladia botryoides (Wulfen) Feldmann	Adriatic Sea	AY444169	Wilkes *et al*. 2006
Botryocladia canariensis Afonso-Carrillo & Sobrino	Canary Islands, Spain	AY444172	Wilkes *et al*. 2006
Botryocladia canariensis Afonso-Carrillo & Sobrino	N/A	EU977498	Schneider & Lane 2008
Botryocladia enteromorpha (Harvey) W.E. Schmidt, Lozada-Troche, D.L.Ballantine & Fredericq *comb. nov.*	Campeche Banks, Mexico (WES33)	**KT154688**	**This study**
Botryocladia enteromorpha (Harvey) W.E. Schmidt, Lozada-Troche, D.L.Ballantine & Fredericq *comb. nov.*	Campeche Banks, Mexico (WES24)	**KT154689**	**This study**
Botryocladia exquisita C.W.Schneider & C.E.Lane	Bermuda	EU977496	Schneider & Lane 2008
Botryocladia exquisita C.W.Schneider & C.E.Lane	Bermuda	EU977497	Schneider & Lane 2009
Botryocladia flookii C.W.Schneider & C.E.Lane	Bermuda	EU977492	Schneider & Lane 2010
Botryocladia flookii C.W.Schneider & C.E.Lane	Bermuda	EU977493	Schneider & Lane 2011
Botryocladia flookii C.W.Schneider & C.E.Lane	Bermuda	EU977494	Schneider & Lane 2012
Botryocladia leptopoda (J. Agardh) Kylin	Australia (WES77)	**HQ400597**	**This study**
Botryocladia leptopoda (J. Agardh) Kylin	Bulusan, Philippines	KU726723	Filloramo & Saunders 2016
Botryocladia madagascariensis G.Feldmann	Canary Islands, Spain	AY444168	Wilkes *et al*. 2006
Botryocladia monoica Schnetter	Caribbean Panama (WES 220)	**HQ400596**	**This study**
Botryocladia monoica Schnetter	Offshore LouisianaTexas, USA	AY168666	Gavio & Fredericq 2003
Botryocladia neushulii E.Y. Dawson	Baja California, Mexico	KU873090	unpublished, submitted by Filloramo & Saunders
Botryocladia occidentalis (Børgesen) Kylin	Offshore Louisiana, USA	AY168660	Gavio & Fredericq 2003
Botryocladia occidentalis (Børgesen) Kylin	Campeche Banks, Mexico	KT154690	Schmidt *et al*. (2016)
Botryocladia occidentalis (Børgesen) Kylin	Campeche Banks, Mexico	**HQ400594**	**This study**
Botryocladia papenfussiana Ganesan & Lemus	Cape Verde	AY444173	Wilkes *et al*. (2006)
Botryocladia pyriformis (Børgesen) Kylin	Offshore Louisiana, USA	KT154746	Schmidt *et al.* (2016)
Botryocladia pyriformis (Børgesen) Kylin	Offshore Louisiana, USA	KT154691	Schmidt *et al.* (2016)
Botryocladia pyriformis (Børgesen) Kylin	Offshore Louisiana, USA	**HQ400595**	**This study**
Botryocladia pseudodichotoma (Farlow)Kylin	Point Loma, California, USA	KU687846	unpublished, submitted by Filloramo & Saunders
Botryocladia shanksii E.Y.Dawson	Isla Colon, Caribbean Panama	AY168662	Gavio & Fredericq 2003
Botryocladia skottsbergii (Børgesen) Levring	Taiwan (WES310)	**KT154692**	**This study**
Botryocladia spinulifera W.R.Taylor & I.A.Abbott	Cape Verde	AY444174	Wilkes *et al*. 2006
Botryoclada uvarioides E.Y.Dawson	San Diego CA, USA	AY168663	Gavio & Fredericq 2003
Botryocladia wrightii (Harvey) W.E. Schmidt, D.L.Ballantine & Fredericq *comb. nov*.[1]	Spain	EU916715	Barbara *et al.* 2008
Botryocladia wrightii (Harvey) W.E. Schmidt, D.L.Ballantine & Fredericq *comb. nov*.[1]	Korea	EU916717	Barbara *et al.* 2008
Chrysymenia agardhii Harvey	La Parguera, Puerto Rico	EU715133	Ballantine *et al.* 2010

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TABLE 1. (Continued)

Taxa	Collection information / voucher	*rbc*L GenBank Accession No.	Reference
Chrysymenia brownii (Harvey) De Toni	Port McDonnell, SA, Australia	HQ400575	Schmidt *et al.* (2016)
Chrysymenia coriacea (Harvey) W.E.Schmidt, Fredericq & J.N.Norris *comb. nov.*	Tarcoola Beach, WA, Australia	HQ400574	Schmidt *et al.* (2016)
Chrysymenia halymenioides Harvey	Campeche Banks, Mexico	HQ400584	Schmidt *et al.* (2016)
Chrysymenia halymenioides Harvey	Offshore Louisiana, USA	HQ424470	Schmidt *et al.* (2016)
Chrysymenia littleriana J.N. Norris & Ballantine	Diamond Rock, Martinique, F.W.I.	HQ400583	Schmidt *et al.* (2016)
Chrysymenia nodulosa J.N. Norris & Ballantine	Isla de Culebra, Puerto Rico	HQ400578	Schmidt *et al.* (2016)
Chrysymenia ornata Kylin	Jervis Bay, Australia	HQ400587	Schmidt *et al.* (2016)
Chrysymenia planifrons (Melvill) J. Agardh	Long Bay Point, Panama	HQ400580	Schmidt *et al.* (2016)
Chrysymenia pseudoventricosa W.E. Schmidt, Gurgel & Fredericq.	Campeche Banks, Mexico	HQ400581	Schmidt *et al.* (2016)
Chrysymenia pumila (J. Agardh) Weber-van Bosse	Yorke Peninsula, SA, Australia	HQ400576	Schmidt *et al.* (2016)
Chrysymenia sp.	Florida Middle Grounds, Florida, USA	HQ400588	Schmidt *et al.* (2016)
Chrysymenia ventricosa (J.V. Lamouroux) J. Agardh	Côte des Albères, France, 17.vii.84, 24m	KT154745	Schmidt *et al.* (2016)
Coelarthrum cliftonii (Harvey) Kylin	Offshore Louisiana, USA (WES316)	**KT154693**	**This study**
Halopeltis adnata (Okamura) G.W.Saunders & C.W.Schneider	Jeju I., South Korea	JQ907552	Schneider *et al.* (2012)
Halopeltis australis (J. Agardh) G.W.Saunders	Tarcoola Beach, WA, Australia (WES235)	**HQ400599**	**This study**
Halopeltis australis (J. Agardh) G.W.Saunders	Kangaroo Island, Australia (WES131)	**HQ400598**	**This study**
Halopeltis pellucida C.W.Schneider & G.W.Saunders	Bermuda	JQ907555	Schneider *et al.* (2012)
Halopeltis prostrata G.W.Saunders	Victoria, Australia	JQ828280	Schneider *et al.* (2012)
Halopeltis willisii Freshwater & G.W.Saunders	Offshore Louisiana, USA	**KT154696**	**This study**
Halopeltis willisii Freshwater & G.W.Saunders	North Carolina, USA	JQ907553	Schneider *et al.* (2012)
Irvinea ardreana (J. Brodie & Guiry) Guiry	Portugal	AY444177	Wilkes *et al.* (2005)
Irvinea boergesenii (Feldmann) R.J.Wilkes *et al.*	Corsica	AY444176	Wilkes *et al.* (2005)
Rhodymenia californica Kylin	Mosquito Pass, Washington, USA	KT154747	Schmidt *et al.* (2016)
Rhodymenia corallina (Bory) Greville	Coquimbo, Chile	AY168657	Gavio & Fredericq (2003)
Rhodymenia pseudopalmata (Lamouroux) Silva	Port Aransas, Texas, USA	AY168656	Gavio & Fredericq (2003)
Sparlingia pertusa (Postels & Ruprecht) G.W.Saunders *et al.*	Sunshine Cove, Alaska (WES100)	**KT154697**	**This study**
Sparlingia pertusa (Postels & Ruprecht) G.W.Saunders *et al.*	British Columbia, Canada	JQ907561	Schneider *et al.* (2012)
Outgroup taxa:			
Champia parvula var. prostrata L.G.Williams	Offshore LA, USA	KT154691	Schmidt *et al.* (2016)
Fryeella gardneri (Setchell) Kylin	Moss Landing, California, USA	**KT154695**	**This study**
Webervanbossea tasmanensis Womersley	New Zealand (WES122)	HQ400601	Schmidt *et al.* (2016)

[1]Appears in GenBank as *Chrysymenia wrightii.*

Phylogenetic Analysis. An *rbc*L-based phylogenetic analysis was conducted based on 64 sequences of Rhodymeniales of which 12 were newly generated for this study (Fig. 1, Table 1). The ingroup consisted of 61 sequences of Rhodymeniaceae belonging to the genera *Chrysymenia*, *Botryocladia*, *Irvinea*, *Halopeltis*, *Rhodymenia, Coelarthrum* and *Sparlingia*. An analysis containing additional genera of the Rhodymeniaceae preserved the overall

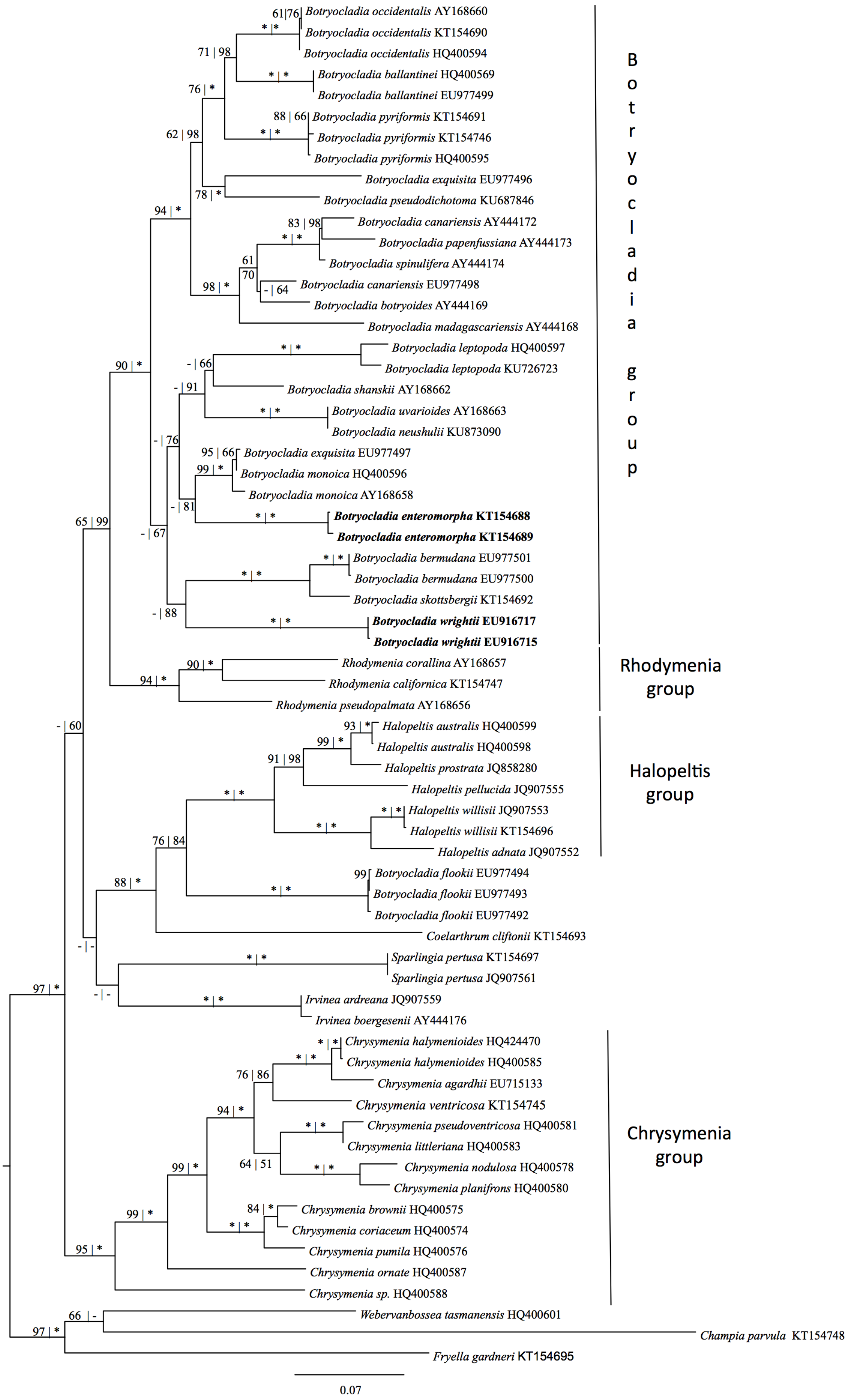


FIGURE 1. Phylogram inferred from RaxML analysis of 64 *rbc*L sequences of Rhodymeniales. The ingroup consists of 61 sequences of Rhodymeniaceae, the outgroup one representative of 3 families in the Rhodymeniales. The position of *Chrysymenia enteromorpha* and *C. wrightii* is nested inside the *Botrycladia* clade that includes the generitype, *B. botryoides*, separate from the *Chrysymenia* clade that includes the generitype, *C. ventricosa*. Numbers indicate bootstrap values at right, and BP values at left (1 represented by 100); *denotes full support. Scale bar indicates number of substitutions per site. Newly generated sequences are shown in bold.

topology of Fig. 1 (data not shown). The outgroup consisted of representatives of three families in the Rhodymeniales, namely, *Champia "parvula"* (Champiaceae), *Fryeella gardneri* (Fryeellaceae), and *Webervanbossea tasmanensis* (Faucheaceae). The alignment was analyzed in Partitionfinder (Lanfear *et al*. 2012) to determine the best fitting model of evolution and data partition. The analysis resulted in the selection of the General Time Reversible model plus gamma and a proportion of invariable sites applied separately to each codon position on the basis of the two information criteria, *i.e*. Akaike information criterion corrected (AICc) and Akaike information criterion (AIC). The alignment of the *rbc*L dataset was analyzed by Maximum likelihood (ML) as implemented by RAXML v 2.4.4 (Stamatakis 2006) with the above models and partition scheme with 1000 restarts to find the tree with the lowest likelihood score and 1000 Bootstrap (BS) replications.

A Bayesian MCMC (Markov Chain Monte Carlo) was applied to the aligned dataset using MrBayes v. 3.2.5 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003). The Bayesian analysis consisted of two independent runs of 5 million generations with sampling every 1000 generations for a total of 10,002 trees. Convergence was visualized using Tracer v1.6 (Rambaut *et al*. 2014) and the first 10 percent of the trees of each run was discarded as burnin. The resulting Posterior Probabilities from Bayesian consensus trees were mapped on the ML tree.

Twenty-three SSU sequences were used to assess phylogenetic relationships within *Botryocladia* and *Chrysymenia*. The optimal model as determined by jModeltest 2.1.3 (Darriba *et al*. 2012), and as input for NJ and ML analysis calculated by the Akaike information criterion (AIC) was the HKY evolutionary model (Hasegawa, Kishino and Yano + Invariable sites) (Hasegawa *et al*. 1985). The assumed nucleotide frequencies were: A = 0.2473, C = 0.2056, G = 0.2881, T = 0.2590. The proportion of sites assumed to be invariable were 0.9160. The phylogenetic reconstruction using SSU data was performed using the maximum parsimony (MP), and neighbor-joining (NJ) algorithms as implemented in PAUP* (v 4.1b10) (Swofford 2002). RaxMLGUI was used to infer maximum likelihood optimality criterion from SSU DNA sequences (Silvestro and Michalak 2012). The robustness of the data was determined by bootstrapping the data set (Felsenstein 1985) 1000 times for ML and 2,000 times for MP and NJ Bayesian analysis of SSU data was conducted using MrBayes v3.2 by running 500,000 generations using the HKY evolutionary model (Huelsenbeck and Ronquist 2001). Trees were sampled every 100 generations, and log-likelihood scores stabilized (X-Y scatter plot) at approximately 2,000 trees. The first 2,000 trees of a possible 5,000 trees were discarded as burn- in.

Results

According to both the *rbc*L- and SSU-based phylogenies, the genus *Chrysymenia* is a monophyletic group if it excludes the species *C. enteromorpha* and *C. wrightii* (Figs 1 and 2). The Rhodymeniaceae is a well-supported clade (Bootstrap values: BS=97, Bayesian Posterior Probabilities values: BP=1) in the *rbc*L phylogeny. The true *Chrysymenia* clade was determined using the clade comprising the generitype, *Chrysymenia ventricosa* (J.V. Lamouroux) J. Agardh, from Mediterranean France. This *Chrysymenia* clade is well supported (BS=95, BP=1) and also contains taxa from the Gulf of Mexico (*C. halymenioides* Harvey*, C. pseudoventricosa* W.E. Schmidt, Gurgel & Fredericq), the Caribbean Sea (*C. nodulosa* J.N. Norris & D.L. Ballantine, *C. littleriana* J.N. Norris & D.L. Ballantine, *C. planifrons* (Melvill) J. Agardh*, C.* sp.), the south Pacific Ocean (*C. brownii* (Harvey) De Toni), the southwestern Pacific (*C. ornata* Kylin), and eastern Indian ocean (*Chrysymenia pumila* (J. Agardh) Weber-van Bosse and *C. coriacea* (Harvey) W.E. Schmidt, Gurgel & Fredericq and *Chrysymenia pumila*, *nom. illeg*.). We note the intended basionym of the latter, "*Gloiosaccion brownii* var. *coriaceum* Harvey" of Schmidt *et al*. (2016:65) is invalid, as Harvey (1859a: pl. 83) did not mention or refer to the variety name "*coriacea.*" We propose: *Chrysymenia coriacea* (Harvey) W.E. Schmidt, Fredericq et J.N. Norris, *comb. nov.*; basionym: *Gloiosaccion* var. *coriacea* Harvey (1859b:322); type: Fremantle, Western Australia (Harvey, 1857, Alg. Aust. Exsiccata no. 420).

Chrysymenia is sister with no/low support to a major clade comprising two subclades: one consisting (BS=65, BP=0.99) of the subclade *Rhodymenia sensu stricto* (BS=94, BP=1) and *Botryocladia sensu stricto* (BS=90, BP=1); and one comprising *Halopeltis*, *Irvinea, Botryocladia flookii, Sparlingia* and *Coelarthrum* with no support. The interspecific *rbc*L sequence divergence of species within the *Chrysymenia* clade ranges from 1.30% to 30.2%. All taxa within the *Chrysymenia* clade except for *C. ornata* contain filiform internal filaments (data not shown).

The genus *Botryocladia* clade that contains the generitype *B. botryoides* in the *rbc*L tree (Fig. 1) resolves as a single monophyletic clade with high support but also includes *Chrysymenia enteromorpha* from the Gulf of Mexico and *C. wrightii* from Korea (northwest Pacific) including an invasive sample from Spain (eastern Atlantic Ocean), and a suite of species of *Botryocladia* from the Gulf of Mexico, Caribbean Sea, the western and eastern Atlantic, the Mediterranean

Sea, and the western and eastern Pacific Ocean. *Botryocladia flookii* C.W.Schneider & C.E.Lane represents a sister taxon to the genus *Halopeltis* and thus does not belong in *Botrycladia*. The closest sister taxon to *C. enteromorpha* is tentatively identified as *B. monoica* from Caribbean Panama, the NW Gulf of Mexico, and Bermuda [the latter sequence in GenBank referred to incorrectly as *B. exquisita* C.W.Schneider & C.E.Lane*,* EU977497 (representing the holotype); and *B. exquisita* EU977496 in Fig. 1 results in a different clade, sister to *B. pseudodichotoma*]. The interspecific sequence divergence within *Botryocladia* ranges from 3.02% to 28.8%. The genus *Irvinea* is genetically distinct from *Botrycladia* and *Chrysymenia* with 33.3% and 30.5% sequence divergence, respectively.

The polyphyletic nature of the genus *Chrysymenia* is also evident in the 18S phylogram (Fig. 2), which includes six *Chrysymenia* species. Two clades were obtained by SSU data: one clade including *C. enteromorpha* Harvey and *C. wrightii* (Harvey) Yamada, and a second comprised of *C. nodulosa* J.N. Norris *&* D.L. Ballantine, *C. agardhii* Harvey, *C. ornata* (J. Agardh) Kylin and *C. brownii* (Harvey) DeToni. According to the 18S sequence phylogram, *C. enteromorpha* and *C. wrightii* represent sister taxa forming a moderately supported clade (Fig. 2), as result of the limited number of SSU sequences available. Although a limited number of species were included (due to sequence availability), SSU sequence analyses suggest and confirm the close relationship between *Chrysymenia enteromorpha* and *C. wrightii* within the genus *Botryocladia*.

Since *C. enteromorpha* and *C. wrightii* fall with high support in the *Botryocladia* clade, new combinations are provided for both species. Morphological characters of the species are illustrated and shown to fall within the generic concept of *B. occidentalis,* a common tropical and subtropical species of *Botryocladia*.

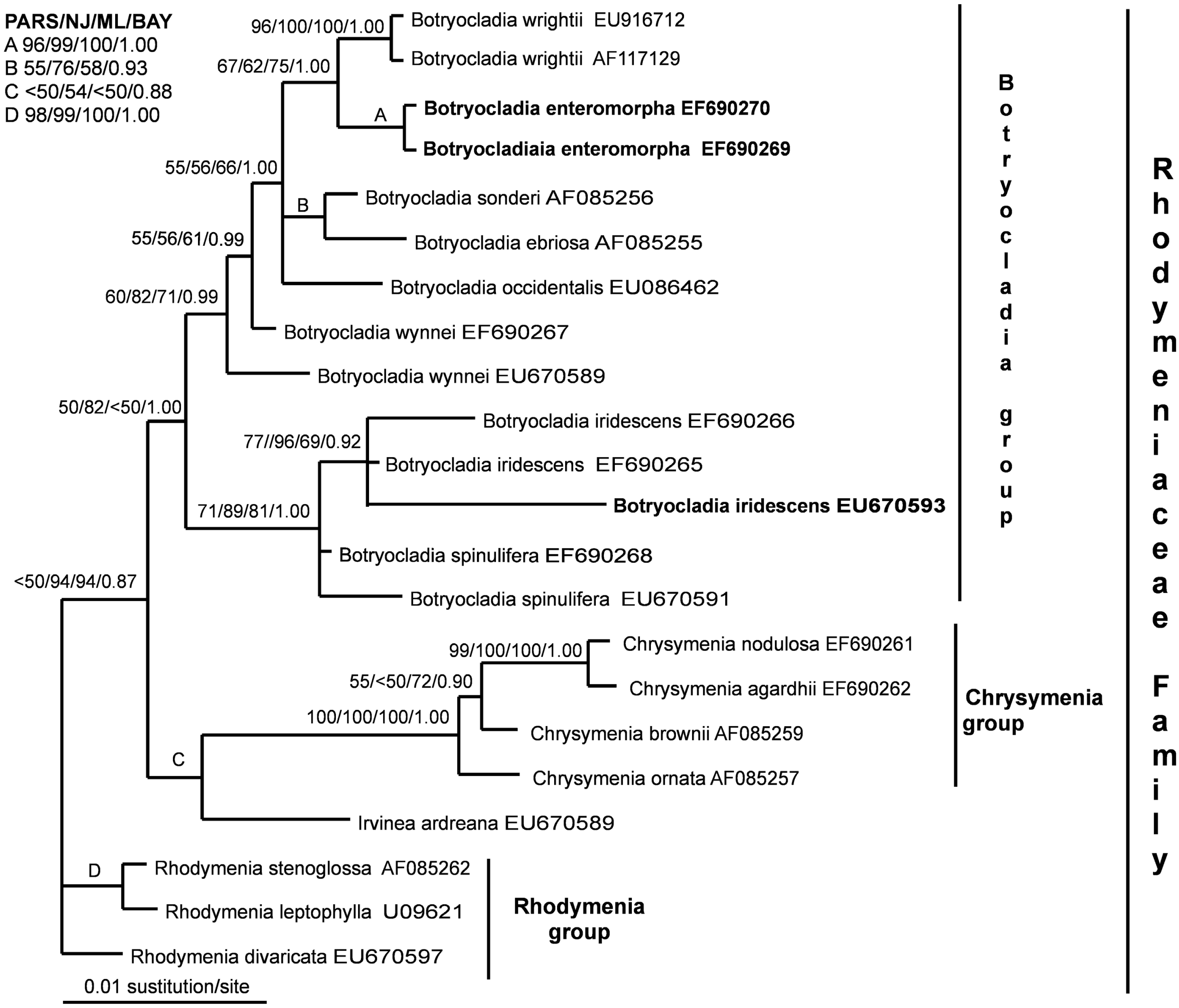


FIGURE 2. Phylogram showing *Botryocladia* and *Chrysymenia* species inferred from Bayesian analysis of 22 SSU sequences, using *Rhodymenia* species as the outgroup taxa. Support values reflect all bootstrap values (parsimony, neighbor joining, maximum likelihood, except for Bayesian Posterior Probabilities).

Taxonomy

Botryocladia enteromorpha (Harvey) W.E. Schmidt, Lozada-Troche, D.L.Ballantine et Fredericq, comb. nov.

Figures 3–14

BASIONYM: Chrysymenia enteromorpha Harvey 1853, Smithsonian Contributions to Knowledge 5(5): pp. 187–188.

TYPE LOCALITY: Key West, Florida, USA.

DISTRIBUTION: All references below as "*Chrysymenia enteromorpha*": NE Atlantic: Canary Islands (Price *et al.* 1986; Haroun *et al*. 2002). W Atlantic: Bermuda, North Carolina, South Carolina, Georgia, Florida (Schneider and Searles 1991), Caribbean (Taylor 1960, Littler and Littler 2000, Ballantine and Aponte 2002, Wynne 2011, Guiry and Guiry 2017), NW Gulf of Mexico: Flower Garden Banks National Marine Sanctuary, Outer Shelf Banks (Rezak *et al*. 1985, Gavio and Fredericq 2005; Fredericq *et al*. 2009). SE Atlantic: Ghana (Price *et al.* 1986, Lawson and John 1987). Indian Ocean: Bangladesh, Seychelles (Silva *et al*. 1996). This species has not been collected again from offshore Louisiana in the NW Gulf of Mexico following the 2010 Deepwater Horizon oil Spill (Fredericq *et al*. 2014, Felder *et al*. 2014).

MATERIAL EXAMINED: Campeche Banks, Mexico: SW Gulf of Mexico, *LAF-NSF-II-17-6 (WES33)*, 22˚15'150" N, 90˚43'290" W, 7.vi.2005, 52–53 m depth; *LAF-NSF-II-83-5 (WES24)*, 21˚36'540" N, 91˚04'600" W, 15.vi.2005, 29–20 m depth; *LAF-NSF-II-96-21*, 22˚08'004" N, 91˚23'670" W, 17.vi.2005, 52–53 m depth. Caribbean Sea: Puerto Rico, 1.5 Km seaward of Media Luna Reef, La Parguera, 17m, 22.xii.2003, coll. DLB *(CLT-143)*; and Margarita Reef, La Parguera, Puerto Rico, 24m, 7.vii.2006, coll. DLB and H.Ruiz *(CLT-201)*.

HABIT, VEGETATIVE AND REPRODUCTIVE STRUCTURE: Thalli are elongated, sparsely branched vesicles (Figs 3–4) comprising a main axis that is slightly wider than the secondary and tertiary vesicular branches. Young vesicles, as lateral branches, are strongly constricted at their bases (Fig. 5).

The medullary layer is monostromatic, composed of large hexagonal cells (Fig. 6). The larger medullary cells at their margin are subtended by 1–2 layers of small, pigmented surface cortical cells (Figs 7–8). Mature specimens have nearly complete surface cortication (Fig. 7), while in younger specimens the surface cortication is incomplete except for medullary glandiferous cells (Figs 7–8). Medullary cells that cut off gland cells (Fig. 9) are often corticated as opposed to medullary cells that do not bear gland cells (Figs. 7–8). Gland cells are pyriform (Figs 8–9). The middle portion of the stipe is of solid construction, lacking internal filaments (Figs 10–11). Tetrasporangia are cruciately divided (Fig. 12) and scattered over the thallus surface. A centrally located ostiole in the pericarp (Fig. 13) surrounds a carposporophyte of which all gonimoblast cells convert into carposporangia (Fig. 14).

REPRESENTATIVE SEQUENCES: KT154688, KT154689 (*rbc*L); EF690270, EF690269 (SSU rDNA).

Botryocladia wrightii (Harvey) W.E. Schmidt, D.L.Ballantine et Fredericq, comb. nov.

Figures 15–22.

BASIONYM: *Halosaccion wrightii* Harvey 1860: 332, *Proceedings of the American Academy of Arts and Sciences* 4: 332.

HOMOTYPIC SYNONYMS: *Chylocladia wrightii* (Harvey) Okamura 1902: 45

Chrysymenia wrightii (Harvey) Yamada 1932: 118

TYPE LOCALITY: Hakodate, Hokkaido, Japan

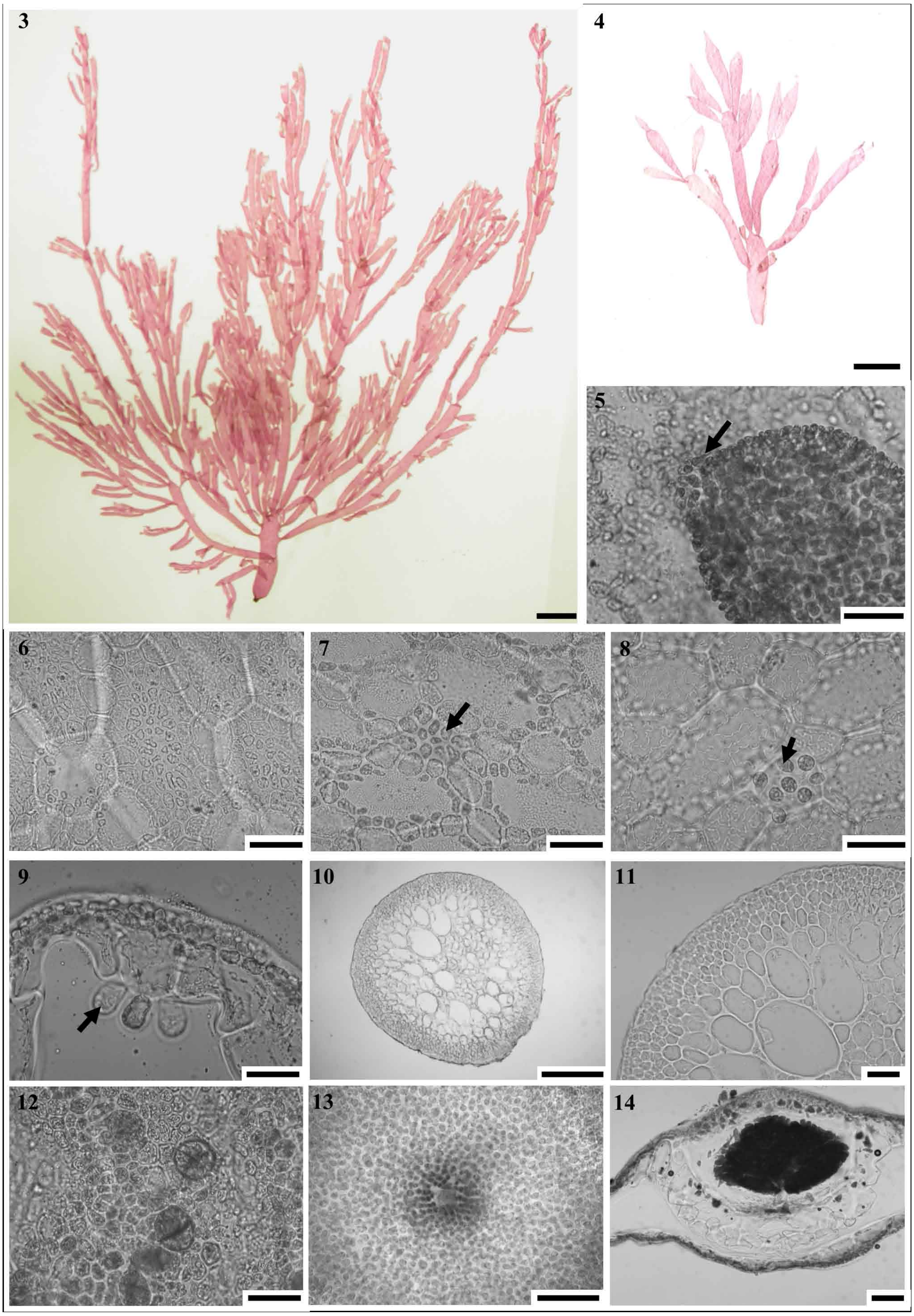
DISTRIBUTION: Northwest Pacific, i.e. Japan, China and Korea (Guiry and Guiry 2017). An invasive species in the Eastern Atlantic, Spain and France (Ben Maïz *et al*. 1987, Bárbara *et al*. 2008).

SPECIMENS EXAMINED: Henashi (Fukaura), Aomori Prefecture (Aomori-ken) Japan, June 10, 1966, coll. M. Yoshizaki, *MY-18-054*.

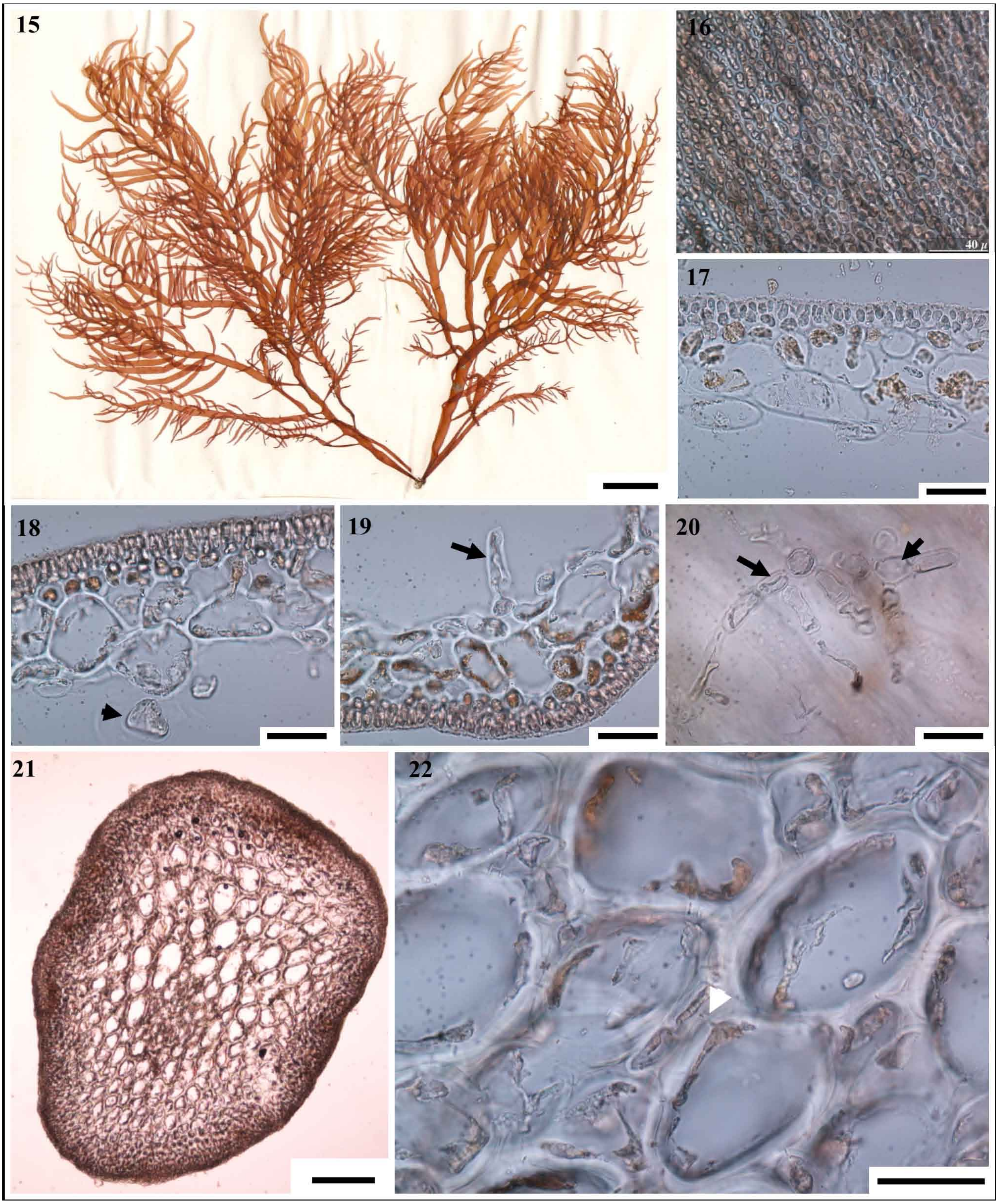
RESULTS: Thalli (Fig. 15) are completely corticated (Fig. 16), composed of one to two cortical cell layers and two to three medullary layers of cells increasing in size toward the interior (Fig. 17). Gland cells are borne on unmodified medullary cells that extend further into the medulla (Figs 18–19), and may appear flattened (Fig. 19). Internal filaments also found throughout the thallus (Fig. 20) [Fig. 19 is poor], developing from smaller cells cut off by larger medullary cells (Fig. 19). The middle portion of holdfast (Figs 21–22) is solid.

REMARKS. The presence of gland-bearing medullary cells extending further into the medulla was also observed by Bárbara *et al.* (2008).

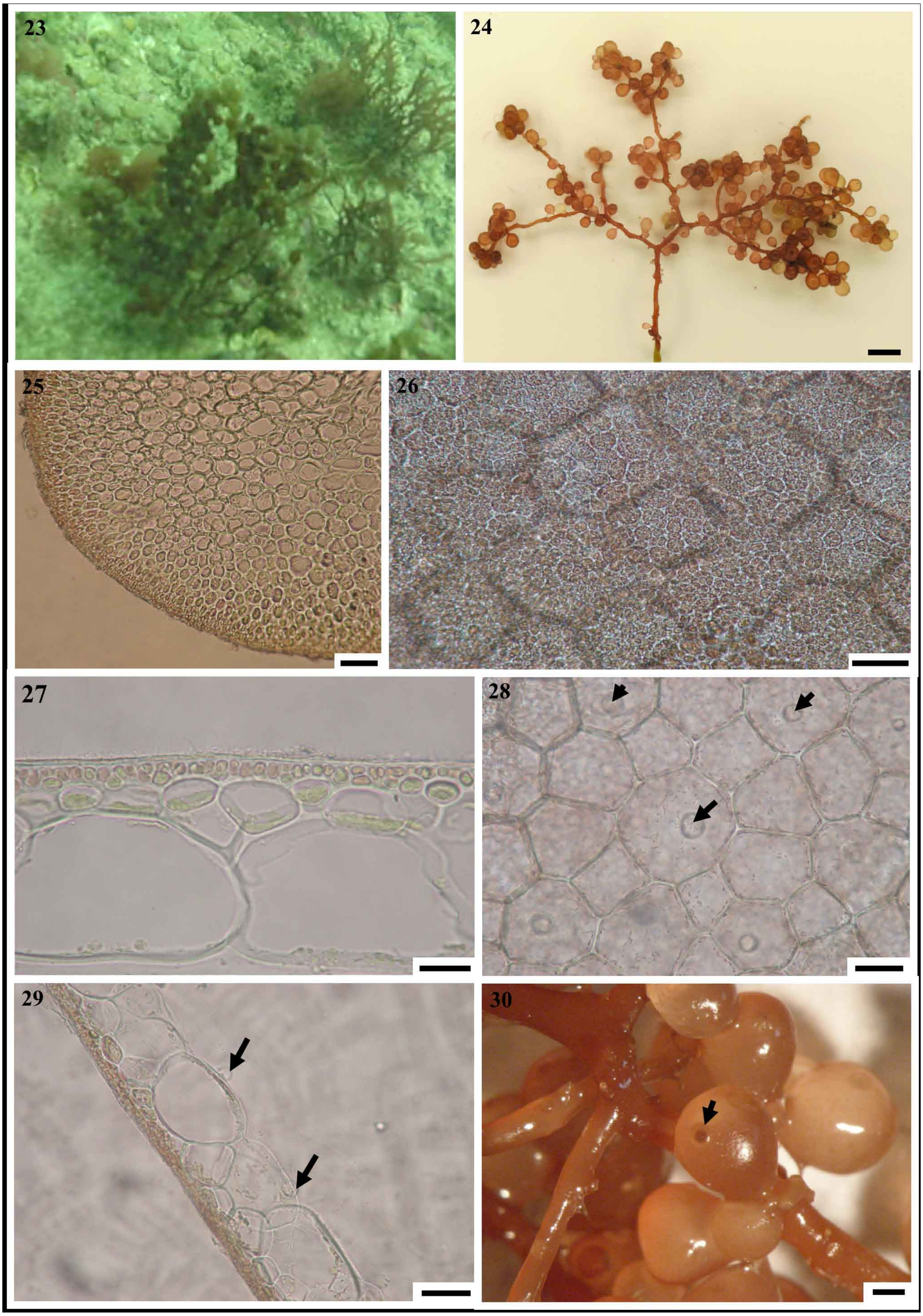
REPRESENTATIVE SEQUENCES: EU977501, EU916717 (*rbc*L); EU916712, AF117129 (SSU rDNA).



FIGURES 3–14. *Botryocladia enteromorpha* (Harvey) W.E. Schmidt, C. Lozada-Troche, D.L. Ballantine et Fredericq, comb. nov. Vicinity of the Florida Middle Grounds: Figs. 2, 12–13.LAF-NSF-III-7-9-06-9-2, Fig. 3. LAF-NSF-III-7-5-06-3-7. Campeche Banks: Figs. 4–10, LAF-NSF-II-78-3, Fig. 11. LAF-NSF-II-96-21. 3) Habit of female gametophyte with cystocarps. Scale bar 2 cm. 4) Habit of non-reproductive thallus. Scale bar 1 cm. 5) Surface view of young branch on main axis with constricted base (arrow). Scale bar 40 µm. 6) Surface view of mature specimen with nearly complete cortication. Scale bar 40 µm. 7) Surface view of younger specimen with incomplete cortication except for glandiferous cells bearing gland cells (arrow). Scale bar 40 µm. 8) Surface view of medullary, glandiferous, and gland cells (arrow). Scale bar 40 µm. 9) Cross section through vesicle wall with glandiferous cell bearing three gland cells (arrow). Scale bar 40 µm. 10) Cross section through holdfast. Scale bar 0.5 mm. 11) Organization of cortical and medullary cells in holdfast cross section. Scale bar 100 µm. 12) Surface view of cortex with cruciately divided tetrasporangia (arrowhead). Scale bar 40 µm. 13) Surface view of pericarp ostiole topping carposporophyte. Scale bar 100 µm. 14) Longitudinal section through carposporophyte. Scale bar 100 µm.



FIGURES 15–22. *Botryocladia wrightii* (Harvey) W.E. Schmidt, D.L. Ballantine et Fredericq, comb. nov., from Henashi, Aomori-ken, Japan, MY 18-054. 15) Habit of non-reproductive plant. 16) Surface view of thallus showing complete cortication. Scale bar 40 µm. 17) Cross section through mid thallus region showing pigmented cortical cells and hyaline medullary cells. Scale bar 40 µm. 18) Cross section through middle portion of thallus region, showing irregular gland cell (arrowhead) on modified glandiferous medullary cell. Scale bar 40 µm. 19) Cross section through mid thallus region showing cortical cells, and medullary cell giving rise to internal filament (arrow). Scale bar 40 µm. 20) Medullary cell and internal filament (arrows). Scale bar 40 µm. 21) Cross section through middle part of holdfast showing cortical cells and medullary cells with dense aggregations of internal filaments. Scale bar 0.25 mm. 22) Internal medullary filaments in mid holdfast region, with pit-connection (arrowhead) linking one cell to a large medullary cell. Scale bar 40 µm.



FIGURES 23–30. *Botryocladia occidentalis* from Bocas del Toro, Panama, LAF-8-17-09-1-10. 23). Habit of specimens *in situ*. Scale bar 2 cm. 24) Habit of female gametophyte with cystocarps. Scale bar 1 cm. 25). Cross section through axis. Scale bar 50 µm. 26) Surface view of vesicle with complete cortication. Scale bar 50 µm. 27) Cross section through vesicle showing cortical and medullary cell layers. Scale bar 25 µm. 28) Surface view of medullary cells bearing gland cells (arrows). Scale bar 50 µm. 29) Cross section through vesicle showing gland cells (arrows) borne on unmodified medullary glandiferous cells. Scale bar 50 µm. 30) Raised carposporophyte (arrow) on female gametophyte. Scale bar 1 mm.

***Botryocladia occidentalis* (Børgesen) Kylin 1931**, p. 18

Figures 23–30.

BASIONYM**:** *Chrysymenia uvaria* var. *occidentalis* Børgesen 1920: 403, fig. 388 (as *Chrysymenia uvaria* (L.) J. Ag.).

TYPE LOCALITY: Between St. John and St. Thomas, US Virgin Islands, Lesser Antilles, Caribbean Sea,Western Atlantic.

DISTRIBUTION: From the Carolinas and Bermuda to Brazil, including the Gulf of Mexico and Caribbean Sea (Ballantine and Aponte 2002, Gavio and Fredericq 2003, Dawes and Mathieson 2008).

SPECIMENS EXAMINED: Caribbean Panama: STRI Beach, Bocas del Toro, *LAF-8-31-09-1-18*, 9°21'7.02"N, 82°15'19.85"W, in the drift, 31.viii.2009 (Figs 22–29). SW Gulf of Mexico: Campeche Banks, *LAF-NSF-II-30-4 (WES227)*, 21°06'60", N 92°08'66" W, 9.vi.2005, 51–54 m depth; *LAF-NSF-II-16-18* (WES38), 22°16' 020" N, 90°43'230" W, 7.vi.2005, 51–52 m depth. NE Gulf of Mexico: Florida Middle Grounds, FL, *LAF-NSF-III-7-4-06-12-3 (WES 90)*, 28°10' 280" N, 84°01'950" W, 4.vii.2006, 41–42 m depth, and *LAF-NSF-III-7-4-06-3-10* (WES67), 28°37'260" N, 84°24'380" W, 4.vii.2006, 45 m depth.

RESULTS: Thalli are rose red to golden-pink, erect, up to 11cm in length, with irregular pseudodichotomous branching (Figs 23–24) of the stipe. The main axes and branches are terete, 0.9 to 2.5 mm diam., of solid construction, composed of small pigmented round to oval, cortical cells (5 µm in diameter) gradually increasing in size inward; medullary cells are large, hyaline, ovoid, up to 50 µm wide (Fig. 25). The axes and branches bear pyriform vesicles (Fig. 24), 3–7 mm long by 3–4.5 mm in diameter. The vesicle walls are 2–(3)–4 cells wide, 66–100–135 µm thick (Fig. 26). The cortices of vesicles are complete (Fig. 27), uni-to bistratose, pigmented, composed of round to ovoid cells 3–6 µm in diameter, and distal to the larger medullary cells. The medulla is composed of 1–(2) layers of hyaline, polygonal cells, 77–(100–132)–180 µm wide; the outer medulla of intermediate-sized cells. Gland cells are spherical, borne singly, rarely in pairs on unmodified medullary gland-bearing cells (Figs. 28–29). Carposporophytes are borne on the vesicles, visible as slightly raised protrusions extending both inwardly and outwardly (Fig. 30).

REPRESENTATIVE SEQUENCES: AY168660, KT154746, HQ400594 (*rbc*L); EU086462 (SSU rDNA).

Discussion

Four genera in the Rhodymeniaceae have members that contain saccate vesicles that lack diaphragms, i.e. *Chrysymenia* (including *Gloiosaccion)*, *Irvinea*, *Cresia* and *Botryocladia* (Wynne 2005, Schneider and Lane 2008, Lozada-Troche *et al*. 2010, Schmidt *et al*. 2016). These genera, however, are not well defined by other morphological characteristics (Afonso-Carrillo *et al*. 2006). Earlier research by Le Gall *et al*. (2008) and Saunders *et al*. (1999) using nuclear markers for the Rhodymeniales revealed that most of these genera appear to be polyphyletic. This study and that by Schmidt *et al.* (2016) have partially supported this finding on the basis of plastid-encoded gene sequence analysis and increased taxon sampling of *Chrysymenia* and *Botryocladia* species.

Previous studies of *Chrysymenia* by Le Gall *et al*. (2008) and Saunders *et al*. (1999) were limited to *Chrysymenia ornata* and *C. wrightii*, which led to an informal proposal of the resurrection of the genus *Cryptarachne* (Harvey) Kylin due to the lack of internal rhizoids in *C. ornata* and their presence in *C. wrightii.* However, with the increased taxon sampling presented here, the members of the *Chrysymenia* clade contain species that both possess and lack internal rhizoids. This finding corroborates the opinion of Okamura (1936) and later that of Abbott and Littler (1969) that the division of *Chrysymenia* and *Cryptarachne* is unfounded.

A taxon previously referred to *Chrysymenia*, *C. enteromorpha,* resolves strongly within *Botryocladia*. In earlier morphological studies, Norris and Ballantine (1995) suggested that the presence of filaments in the stipe versus a solid stipe may be a character to separate *Botryocladia* and *Chrysymenia*, and transferred *Chrysymenia bullosa* Levring on the basis of a solid stipe into *Botryocladia*. However, the results of this study with the finding of filiform filaments present in *Chrysymenia wrightii* holdfasts do not support the absolute state of this character for generic discrimination. Previously Saunders *et al*. (1999) found *C. wrightii* to be closely related to *Botryocladia*, a taxonomic opinion confirmed here.

Afonso and Sabrino's (2003) original suggestion that determinate vesicle size is restricted to *Botryocladia* was later abandoned by Afonso *et al*. (2006). On the basis of new information on *Botryocladia enteromorpha* it was also not supported by this study. Our results suggest that the *Chrysymenia* clade contains only specimens that produce their gland cells directly on unmodified medullary cells as opposed to other genera, such as *Botryocladia* and *Cresia*, that may not. Previous research of Saunders *et al*. (1999) found a sister relationship between *Gloiosaccion brownii* and

Chrysymenia ornata, but with the increased taxon sampling used, *Gloiosaccion* was found to resolve unambiguously within *Chrysymenia* (Schmidt *et al*. 2016).

Currently the genus *Botryocladia* is generally accepted to include morphologically heterogenous species (Brodie and Guiry 1988; Norris 1989; Gavio and Fredericq 2003, 2005; Schneider and Lane 2000; Wilkes *et al*. 2006; Afonso-Carrillo *et al*. 2006). Even though molecular studies have begun to reveal more natural groups within the family with the creation of the genus *Irvinea* and subsequent transfers (Saunders *et al*. 1999, Wilkes *et al*. 2006), species diversity in the Rhodymeniaceae remains under-estimated. Several sequences of *Botryocladia* species downloaded from GenBank that were found to be problematic (*i.e.* short sequences, unresolved nucleotides) were not included in the analysis. *Botryocladia exquisita* (Schneider and Lane 2010) from Bermuda (EU977497) is found to be a synonym of *B. monoica* Schnetter (1978; type locality is Caribbean Colombia) from the Gulf of Mexico (AY168658, Gavio and Fredericq 2005) and Caribbean Panama. Even though the two GenBank numbers of *Botryocladia exquisita* in Schneider and Lane (2008) show up separately in different clades in the current paper, they are adjacent in fig. 13 of Schneider and Lane, implying that one of the Genbank numbers is incorrect. In addition, downloaded sequences from GenBank designated as *B. canariensis* (EU977498) without assigned locality, and from the Canary Islands (AY444172), represent separate taxa as do the two sequences of *"B. exquisita"* from Bermuda that also encompass two species (EU977496, EU977497) as can be readily seen from a distance tree generated BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Placement of a new taxon in *Botryocladia* by Schneider and Lane (2008, as *Botryocladia flookii*) was reportedly supported both by molecular and morphological evidence. However, the samples used in Schneider and Lane's (2008) phylogenetic analysis were limited to a few species of *Botryocladia* and *Irvinea* which led to its inaccurate placement into the genus *Botryocladia*, when in fact molecular evidence presented here suggests erection of a new elongate-saccate genus within the Rhodymeniaceae. In light of the misidentifications of species and problematic assignment of species names to taxa worldwide, a comprehensive revision of the genus *Botryocladia* is needed with careful examination of type material.

Conclusion

Chrysymenia enteromorpha and *C. wrightii* do not belong in the genus *Chrysymenia* but instead are species of *Botryocladia* on the basis of morphological and molecular evidence. The habit-based taxonomic sections of *Botryocladia* that were conceived by Feldmann (1945), *i.e. Botryocladia* sect. Microphyseae and *B*. sect. Botryoideae, are not supported in this study as forming natural groups in accordance with the conclusion of Afonso-Carrillo *et al*. (2006). The genera in "*B*. sect. Microphyseae" can be further divided into forms with spherical, ovate or pyriform vesicles, and forms with elongate, cylindrical or fusiform vesicles. It is apparent that these forms have arisen multiple times throughout the evolution of *Botryocladia*.

Table 2. Species used in SSU analysis. Accession numbers in bold represent new sequences in this study.

Taxa	Collection information / source	SSU GenBank Accession No.	Reference
Botryocladia ebriosa A. Millar	GenBank	AF085255	Saunders *et al.* (1999)
Botryocladia enteromorpha (Harvey) W.E. Schmidt, Lozada-Troche, D.L.Ballantine & Fredericq *comb. nov*.	La Parguera, Puerto Rico	**EF690270**	**This study**
Botryocladia enteromorpha (Harvey) W.E. Schmidt, Lozada-Troche, D.L.Ballantine & Fredericq *comb. nov*.	La Parguera, Puerto Rico	**EF690269**	**This study**
Botryocladia iridescens D.L. Ballantine & H. Ruí	GenBank	EF690265	Lozada *et al.* (2010)
Botryocladia iridescens D.L. Ballantine & H. Ruí	GenBank	EF690266	Lozada *et al.* (2010)
Botryocladia iridescens D.L. Ballantine & H. Ruí	La Parguera, Puerto Rico	**EU670593**	**This study**
Botryocladia leptopoda (J.Agardh) Kylin	GenBank	DQ343160	Le Gall & Saunders (2007)
Botryocladia occidentalis (Børgesen) Kylin	GenBank	EU086462	Lozada *et al.* (2010)
Botryocladia sonderi P.C. Silva	GenBank	AF085256	Saunders *et al.* (1999)
Botryocladia spinulifera W.R.Taylor & I.A. Abbott	GenBank	EU690268	Lozada *et al.* (2010)

...continued on the next page

TABLE 2. (Continued)

Taxa	Collection information / source	SSU GenBank Accession No.	Reference
Botryocladia spinulifera W.R.Taylor & I.A. Abbott	GenBank	EU670591	Lozada *et al.* (2010)
Botryocladia wynnei D.L. Ballantine	GenBank	EF690267	Lozada *et al.* (2010)
Botryocladia wynnei D.L. Ballantine	GenBank	EU670589	Lozada *et al.* (2010)
Botryocladia wrightii (Harvey) W.E. Schmidt, D.L.Ballantine & Fredericq *comb. nov.* [1]	GenBank	EU916712	Barbara *et al*. (2008)
Botryocladia wrightii (Harvey) W.E. Schmidt, D.L.Ballantine & Fredericq *comb. nov.* [1]	GenBank	AF117129	Saunders *et al*. (1999)
Chrysymenia agardhii Harvey	GenBank	EF690261	Ballantine *et al*. (2010)
Chrysymenia brownii (Harvey) De Toni	GenBank	AF085259	Saunders *et al*. (1999)
Chrysymenia nodulosa J.N. Norris & D.L. Ballantine	GenBank	AF085254	Ballantine *et al*. (2010)
Chrysymenia ornata (J. Agardh) Kylin	GenBank	AF085257	Saunders *et al*. (1999)
Irvinea ardreana (J. Brodie & Guiry) Guiry	GenBank	EU670589	Saunders *et al*. (1999)
Rhodymenia divaricata E.Y. Dawson	GenBank	EU670597	Lozada & Ballantine (2010)
Rhodymenia leptophylla J. Agardh	GenBank	U09621	Saunders & Kraft (1994)
Rhodymenia stenoglossa J. Agardh	GenBank	AF085262	Saunders *et al*. (1999)

[1] Appears in GenBank as *Chrysymenia wrightii*.

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