

A comparison of two DNA barcode markers for species discrimination in the red algal family Kallymeniaceae (Gigartinales, Florideophyceae), with a description of *Euthora timburtonii* sp. nov.

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Abstract: Accurate identification of many red algae to the species level using only morphological characters can be difficult. The emerging field of “molecular-assisted alpha taxonomy” can greatly alleviate this issue. In this approach, a large number of specimens are sequenced for a standard DNA marker as a first step to genetic species assignment, followed by detailed morphological observations. Regions of both the mitochondrial cytochrome *c* oxidase I gene (COI-5P) and the plastid 23S rRNA gene (UPA) have been proposed as DNA barcode markers to accomplish this task. We compared the utility of each marker as a species identification tool using members of the marine red algal family Kallymeniaceae from British Columbia, Canada. Our results indicate that COI-5P is a more sensitive marker for delimiting species, but that it can be difficult to acquire clean amplification products for many isolates of Kallymeniaceae, owing to biological contamination. This problem can be overcome by using specific primers. UPA, on the other hand, has universal primers that work in diverse lineages (e.g., red, brown, and green algae), but lower interspecific sequence variation, which has the potential to underestimate species diversity, although this was not observed in our study. During our survey, we uncovered a new species of the Kallymeniaceae, *Euthora timburtonii* Clarkston et G.W. Saunders sp. nov., which we describe here.

Key words: DNA barcode, biodiversity, COI-5P, Florideophyceae, Kallymeniaceae, UPA.

Résumé : L'identification précise de plusieurs algues rouges à l'espèce en n'utilisant que les seuls caractères morphologiques s'avère souvent difficile. L'avènement de la taxonomie alpha à l'aide des données moléculaires peut grandement améliorer cette situation. Selon cette approche, on peut séquencer un grand nombre de spécimens pour obtenir des marqueurs ADN comme première étape pour l'attribution génétique aux espèces, tout en conduisant des observations morphologiques détaillées. On a proposé les régions du gène I du cytochrome *c* oxydase mitochondrial (COI-5P) et le gène plastidique 23S rARN (UPA), comme marqueurs code-barre ADN pour réaliser ce travail. Les auteurs ont comparé l'utilité de chacun des marqueurs comme outil d'identification des espèces en utilisant des membres de la famille Kallymeniaceae d'algues rouges, en Colombie-Canadienne. Le COI-5P s'avère comme un marqueur plus sensible pour délimiter les espèces, mais il est difficile d'obtenir des produits d'amplifications précis pour plusieurs isolats de Kallymeniaceae, suite à la contamination biologique. On peut surmonter ce problème en utilisant des amorces spécifiques. D'autre part, l'UPA comporte des amorces universelles qui fonctionnent avec diverses lignées (par exemple, les algues rouges, brunes et vertes) mais diminuent la variation des séquences interspécifiques, lesquelles ont la capacité de sous-estimer la diversité des espèces, bien que les auteurs n'aient pas observé ce phénomène. Au cours de leur travail, les auteurs ont découvert une nouvelle espèce de Kallymeniaceae, l'*Euthora timburtonii* Clarkston et G. W. Saunders sp. nov., ici décrite.

Mots-clés : code-barre ADN, biodiversité, COI-5P, Florideophyceae, Kallymeniaceae, UPA.

[Traduit par la Rédaction]

Introduction

Red marine macroalgae are notoriously difficult to identify to species using traditional morphological characters. This impediment is due, in part, to the fairly simple and convergent morphologies of many red algae, which can make the accurate identification of a specimen in the field

problematic for even the experienced algal systematist (Saunders 2008). Adding to the confusion is the phenotypic variation caused by varying environmental conditions that can result in a confounding range of morphologies within a single species, and life histories with alternation of heteromorphic generations where only one life history phase is known (Saunders 2005). The use of reproductive characters for identification is problematic, owing to the cryptic and (or) ephemeral nature of these structures leading to equivocal identifications of vegetative material collected outside of the reproductive season. The end result of basing red algal descriptions solely on morphological characters is that the taxonomy of many complexes remains confused and unresolved (e.g., Saunders 2008; Walker et al. 2009).

An increasingly common practice in algal systematics is

Received 29 May 2009. Published on the NRC Research Press Web site at botany.nrc.ca on 25 February 2010.

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to include multiple types of characters (e.g., morphological, anatomical, biogeographical, ultrastructural, molecular, ecological) when describing species or completing monographs (e.g., Schneider and Lane 2008; Yamada et al. 2008). Molecular tools are increasingly guiding the assignment of collections to genetic species groups from which the previous attributes can be assessed, and for assigning cryptic specimens to known species (e.g., Fox and Swanson 2007; Guillemain et al. 2008). To date, however, there has been a lack of agreement on a standard molecular marker for quick and accurate identification of unknown algal specimens (Saunders 2008) that would enable the comparison of unknown specimens to a diverse group of known species (Ratnasingham and Hebert 2007).

Two molecular markers have been proposed as standard markers for species identification in macroalgae: COI-5P, the 5' region of the mitochondrial cytochrome *c* oxidase I gene (Saunders 2005, 2008); and UPA, the universal plastid amplicon, domain V of the 23S rRNA gene (Presting 2006). The COI-5P marker (ca. 664 bp) has been shown to resolve closely related species in many diverse lineages including animals (e.g., Hebert et al. 2003), brown algae (Kucera and Saunders 2008; McDevit and Saunders 2009), and red algae (Saunders 2005, 2008). COI-5P is a protein-coding gene, and indels (insertions/deletions) are rare, facilitating comparisons of sequences between diverse taxonomic groups (Hebert et al. 2003). The drawback of COI-5P is the need for specific amplification primers for most lineages (e.g., Kucera and Saunders 2008; Saunders 2008; McDevit and Saunders 2009). In contrast, UPA (ca. 400 bp) has been amplified from multiple species from a diverse number of algal lineages using a single set of primers (Sherwood and Presting 2007), but, excepting a recent study on a group of taxonomically complex freshwater red algae (Sherwood et al. 2008), the within versus between species divergence of UPA has yet to be explored.

The Consortium for the Barcode of Life (CBOL; www.barcoding.si.edu) is an international initiative dedicated to developing DNA barcoding — a technique that uses a short, standardized DNA sequence from a designated region of the genome as a diagnostic tool for species-level identification. CBOL has accepted COI-5P as a standard barcode region, owing to its success in most animal lineages; however, the effectiveness of COI-5P in other eukaryote lineages is still being assessed. In groups where COI-5P cannot resolve species-level differences or is otherwise ineffective, an alternative barcode region will be identified and ultimately accepted by CBOL. For red algae, COI-5P has been well explored as a species discriminating tool (Saunders 2005; Robba et al. 2006, Saunders 2008; Le Gall and Saunders 2010), while UPA has only begun to be explored (Sherwood and Presting 2007; Sherwood et al. 2008), and is not currently accepted by CBOL as a barcode region.

The Kallymeniaceae is a red algal family of ca. 20 genera united by specific aspects of female reproductive anatomy (Hansen and Lindstrom 1984). Since its elevation to familial status (Kylin 1928), the Kallymeniaceae has been the subject of several studies regarding the morphology, anatomy, distribution, and classification of its component species (e.g., Norris 1957; Abbott and Norris 1965; Hooper and South 1974). Despite these detailed examinations, disagree-

ment continues regarding the taxonomic status of many genera and species within this family. One problematic genus is *Euthora* J. Agardh, which was erected in 1847 and eventually included three species: *Euthora cristata* (C. Agardh) J. Agardh (type species), *Euthora fruticulosa* (Ruprecht) J. Agardh, and *Euthora tristanensis* Baardseth (Harper and Saunders 2002). Hooper and South (1974) subsumed *Euthora* into *Callophyllis* on the basis of morphological similarities between the two genera. They also synonymized *E. fruticulosa* with *E. cristata*. However, a recent phylogenetic examination of six kallymeniacean genera using large subunit ribosomal DNA (LSU) resolved *Euthora* as distinct from *Callophyllis* (Harper and Saunders 2002).

In this study, COI-5P and UPA were assessed for species discrimination in marine red algae using Canadian Kallymeniaceae as a test system. During our survey a new species of *Euthora*, *Euthora timburtonii* sp. nov., was uncovered and subsequently characterized anatomically and subjected to phylogenetic analyses using the LSU.

Materials and methods

All specimens were collected by SCUBA in the subtidal and as drift or attached individuals in the intertidal (Table 1). Specimens were dried on herbarium paper with a subsample dried in a vial with silica gel for subsequent molecular analyses.

Genomic DNA was isolated following a protocol modified from Saunders (Saunders 1993, 2008). For the majority of samples, the COI-5P region was amplified using one of the primer combinations GazF1 and GazR1 (Saunders 2005), GHalF (Saunders 2008) and GazR1, GazF1 and GazF4 (Saunders 2008), or GHalF and GHalR 5'-CTTCWGGATGRCCAAAAAATCA-3' (reverse). For several samples in which there was either poor amplification using the above primers or contamination problems due to epiphytic/endophytic biota, the following primer combinations were used: GazF2 (Lane et al. 2007) and GazR1, GWSFn (Le Gall and Saunders 2010) and GWSR3 (Saunders 2009) and GWSF5 (Saunders 2009) and GazR1. The PCR amplification profile followed Hebert et al. (2003), but used an annealing temperature of 50 °C. The UPA was PCR amplified following the protocol outlined in Sherwood and Presting (2007), but used the modified reverse primer P23SnewR 5'-TCAGCCTGTTATCCCTAGA-3', which improved sequence quality. All PCR products were purified using ExoSAP-IT® (USB, Cleveland, Ohio, USA).

Sequencing for all COI-5P and UPA PCR products was carried out using the PE Applied Biosystems Big Dye (version 3.1) kit (following the manufacturer's instructions except only 1 µL of Big Dye was used per sample; ABI, Foster City, California, USA). Forward and reverse sequence reads from the respective PCR primers were edited using Sequencher™ 4.8 (Gene Codes Corporation, Ann Arbor, Michigan, USA), and a multiple sequence alignment was constructed for each genic region using MacClade 4 (version 4.06) for OSX (Maddison and Maddison 2003).

The COI-5P alignment had no indels and included 102 specimens: 78 with bidirectional sequence reads (ca. 664 bp), and 24 with reverse reads only (ca. 575 bp). The UPA alignment had no indels and included 39 specimens (all with bidirectional reads) with 371 bp. Sequence comparisons

were conducted in PAUP* 4.0b10 (Swofford 2002). For each dataset, two distance matrices were calculated: the first comparing absolute bp differences between sequences, and the other comparing uncorrected *p* values.

To determine the relationship of *Euthora timburtonii* relative to other members of the Kallymeniaceae, a phylogeny was constructed using LSU sequence data (ca. 2800 bp). The data set included a newly determined sequence for *E. timburtonii* (GWS001340) and *Kallymenia reniformis* (GWS001453), and 14 previously published kallymeniacean sequences downloaded from GenBank (Table 1; see Harper and Saunders 2002). Eleven species of the closely related families Dumontiaceae and Rhizophyllidaceae (Tai et al. 2001) were used to root the tree (five sequences downloaded from GenBank (Le Gall and Saunders 2007), and six newly determined sequences; Table 1). Sequences were aligned using ClustalX (Higgins et al. 1996). A maximum likelihood analysis was performed using PhyML 3.0 (Guindon and Gascuel 2003) with a GTR substitution model (selected using Modeltest version 3.06; Posada and Crandall 1998) and a PhyML-estimated proportion of invariable sites and gamma shape parameters. The starting tree was determined using BIONJ, Nearest Neighbor Interchanges (NNIs) branch-swapping was in effect, and tree topology and branch lengths were optimized. Branch support was estimated using nonparametric bootstrap resampling (500 replicates) and the Shimodaira–Hasegawa-like approximate likelihood ratio test (aLRT). The unrooted tree was imported into PAUP* and rooted with reference to the outgroups Dumontiaceae and Rhizophyllidaceae.

Tissue for anatomical work was excised from herbarium specimens and rehydrated in a 4% formaldehyde, 1% Tween® 20 detergent solution for 30 min and sectioned using a freezing microtome (CM 1850, Leica, Heidelberg, Germany). Sections were stained with 1% aniline blue in either 7% acetic acid or 6% 5N hydrochloric acid, then rinsed and permanently mounted in 50% corn syrup (with 4% formaldehyde to prevent microbial growth). Photomicrographs were recorded on a Leica DFC480 digital camera mounted on a Leica DM5000B light microscope (Leica, Heidelberg, Germany). All images were imported into Adobe® PhotoShop® 5.5 (Adobe Systems Inc., San José, Calif.) for plate assembly.

Results

Molecular results

The COI-5P and UPA markers each resolved four species groups that were identical in composition. Sequence divergence levels within and between groups were much lower for UPA than for COI-5P (Fig. 1). For COI-5P, within species divergence ranged from 0%–0.9% while between species divergence was greater than 4.5%. For UPA, within species divergence ranged from 0%–0.27% while between species divergence ranged from 0.81% (the two *Euthora* species) to 6.2%. Despite lower divergence values between groups for UPA, all specimens were unequivocally assigned to the same species group as with COI-5P, i.e., for the taxa used in this study the two markers were consistent.

For each of the four COI-5P–UPA species groups, we determined the LSU sequence for one specimen. Three of the

four groups matched unequivocally to known species while the fourth had a novel sequence that grouped strongly with *Euthora cristata* in our phylogeny (Fig. 2). This group contained subtidal specimens from British Columbia that had been tentatively field identified as *E. cristata*, and are here recognized as the new species *E. timburtonii*.

Taxonomic results

Subsequent examination of specimens revealed substantial and consistent morphological, anatomical, and biogeographical differences between the two *Euthora* species groups. Based on this molecular and morphological evidence, a new species of *Euthora* is proposed.

Euthora timburtonii Clarkston et G.W. Saunders sp. nov.
(Figs. 3–9)

Plantae solum sublittorales, epizoophytiae vulgo super tubis polychaetarum. Thalli ramosissimi; rami imbricati conati saepe. Rami constantes penitus in latitudine, 1.1–2.8 mm lati ad basim; rami penultimi 0.5–2.4 mm in latitudine. Apices ramorum obtusi. Thalli 170–205 µm in crassitudine. Medulla cellularum magnarum impigmentiferarum, ovalium ad isodiametris 55–95 µm in diametro. Cellulae parvae impigmentiferae inter cellulas magnas sed typice celatae, abundantes maximam partem in regionibus basalibus.

Plants exclusively subtidal, commonly epizoic on polychaete worm tubes. Thalli highly branched; overlapping branches frequently fuse together. Branches are a consistent width throughout individuals, 1.1–2.8 mm wide near base, penultimate branches 0.5–2.4 mm wide. Branch apices blunt. Thalli 170–205 µm thick. Medulla of large, unpigmented, oval to isodiametric cells (55–95 µm). Small unpigmented cells between large cells present, but typically cryptic, most abundant in basal regions.

HOLOTYPE: B.E. Clarkston & S. Toews, 3 June 2008 (UNB, GWS010572) (Figs. 3 and 6–9).

TYPE LOCALITY: Wizard Islets (Lat: 48.8583°N, Long: 125.1588°W), subtidal at 40 ft. on worm tube, Bamfield, British Columbia, Canada.

DISTRIBUTION: Vancouver Island and the Sunshine Coast, British Columbia.

ETYMOLOGY: Named for Tim Burton, because the alga and its common substrate (polychaete worm tube) together resemble a macabre flower in the style of T. Burton's artistic contributions.

HOLOTYPE DNA BARCODE (COI-5P): GU140167

REPRESENTATIVE COLLECTIONS: GWS001340, GWS001415, GWS001416, GWS001417, GWS001418, GWS002272, GWS002729, GWS003262, GWS003263, GWS003264, GWS003265, GWS008493, GWS008738, GWS008740 (Fig. 4), GWS008742, GWS009084, GWS009085, GWS009020, GWS009499, GWS009504, GWS010101, GWS010298, GWS010318 (Fig. 5), GWS010476, GWS010571, GWS010576, GWS010578 (see Table 1 for collection details).

Observations

This new species of *Euthora* is described based on 28 collections from nine subtidal locations around Vancouver Island and the adjacent Sunshine Coast in British Columbia,

Table 1. Samples used in molecular analyses.

| Species & voucher | Collection details* | GenBank accession No. | | |
|--|---|-----------------------|----------|------------------|
| | | COI-5P | UPA | LSU [†] |
| Family Kallymeniaceae | | | | |
| <i>Erythrophyllum delesserioides</i> J. Agardh | | | | |
| GWS000072 | Piedras Blancas, California, USA: G.W.S. July 1996 | GU140104 | GU140205 | AF419123 |
| GWS001645 | Seppings Is., Bamfield, B.C., Canada: G.W.S. May 2003 | GU140109 | N.D. | N.D. |
| GWS001664 | Cape Beale, Bamfield, B.C., Canada: G.W.S. May 2003 | GU140100 | GU140203 | N.D. |
| GWS001665 | Cape Beale, Bamfield, B.C., Canada: G.W.S. May 2003 | GU140103 | N.D. | N.D. |
| GWS001746 | Cape Beale, Bamfield, B.C., Canada: G.W.S. June 2003 | GU140099 | GU140202 | N.D. |
| GWS001747 | Cape Beale, Bamfield, B.C., Canada: G.W.S. June 2003 | GU140113 | GU140213 | N.D. |
| GWS001752 | Cape Beale, Bamfield, B.C., Canada: G.W.S. June 2003 | GU140110 | GU140210 | N.D. |
| GWS002910 | Blowhole at Bradys Beach, Bamfield, B.C., Canada: G.W.S. June 2005 | GU140107 | GU140208 | N.D. |
| GWS002911 | Blowhole at Bradys Beach, Bamfield, B.C., Canada: G.W.S. June 2005 | GU140105 | GU140206 | N.D. |
| GWS002945 | Seppings Is., Bamfield, B.C., Canada: G.W.S. June 2005 | GU140106 | GU140207 | N.D. |
| GWS003275 | Seppings Is., Bamfield, B.C., Canada: G.W.S. September 2005 | GU140101 | N.D. | N.D. |
| GWS003295 | Cape Beale, Bamfield, B.C., Canada: G.W.S. & R. Withall September 2005 | GU140102 | GU140204 | N.D. |
| GWS004405 | Botanical Beach, Port Renfrew, B.C., Canada: B.E.C., G.W.S. & D. McDevit June 2006 | GU140112 | GU140212 | N.D. |
| GWS004688 | Palmerston Recreation Reserve, Vancouver Is., B.C., Canada: B.E.C. & D. McDevit July 2006 | GU140111 | GU140211 | N.D. |
| GWS006868 | “Lands End”, Pachena Bay, Bamfield, B.C., Canada: B.E.C., D. McDevit, K. Roy, & H. Kucera June 2007 | GU140108 | GU140209 | N.D. |
| <i>Euthora cristata</i> (C. Agardh) J. Agardh | | | | |
| GWS000026 | Maces Bay, N.B., Canada: G.W.S. April 1996 | GU140142 | N.D. | AF419124 |
| GWS001120 | Partridge Pt, Widby Is., WA, USA: G.W.S. May 2001 | GU140130 | GU140215 | N.D. |
| GWS002314 | Green Is., Cape Breton, N.S., Canada: G.W.S. August 2004 | GU140128 | GU140214 | N.D. |
| GWS002325 | Louisbourg, Cape Breton, N.S., Canada: G.W.S. August 2004 | GU140141 | N.D. | N.D. |
| GWS002332 | Mira Bay, Cape Breton, N.S., Canada: G.W.S. September 2004 | GU140124 | N.D. | N.D. |
| GWS002677 | Cape Neddick, Maine, USA: G.W.S. April 2005 | GU140125 | N.D. | N.D. |
| GWS002691 | Owls Head Lighthouse, Rockland, Maine, USA: G.W.S. April 2005 | GU140127 | N.D. | N.D. |
| GWS003605 | Cape Neddick, Maine, USA: G.W.S. April 2006 | GU140155 | GU140221 | N.D. |
| GWS003606 | Cape Neddick, Maine, USA: G.W.S. April 2006 | GU140126 | N.D. | N.D. |
| GWS003721 | Pettes Cove, Grand Manan, N.B., Canada: B.E.C., G.W.S. & D. McDevit May 2006 | GU140134 | N.D. | N.D. |
| GWS003877 | Lepreau, N.B., Canada: L. Le Gall May 2005 | GU140131 | N.D. | N.D. |
| GWS003879 | Lepreau, N.B., Canada: L. Le Gall May 2005 | GU140136 | N.D. | N.D. |
| GWS005193 | Meadow Cove, N.B., Canada: B.E.C. & G.W.S. August 2006 | GU140144 | N.D. | N.D. |
| GWS005196 | Meadow Cove, N.B., Canada: B.E.C. & G.W.S. August 2006 | GU140139 | GU140218 | N.D. |
| GWS006973 | Cape Ray, N.L., Canada: L. Le Gall & J. Utge July 2006 | GU140129 | N.D. | N.D. |
| GWS007016 | Norris Point, Bonne Bay, N.L., Canada: L. Le Gall & J. Utge July 2006 | GU140138 | GU140217 | N.D. |
| GWS007091 | Bonne Bay, N.L., Canada: L. Le Gall & J. Utge, July 2006 | GU140133 | GU140216 | N.D. |
| GWS007112 | Bonne Bay, N.L., Canada: L. Le Gall, B. Hooper, & J. Utge, July 2006 | GU140132 | N.D. | N.D. |
| GWS007114 | Bonne Bay, N.L., Canada: L. Le Gall, B. Hooper, & J. Utge, July 2006 | GU140135 | N.D. | N.D. |
| GWS007127 | Bonne Bay, N.L., Canada: L. Le Gall, B. Hooper, & J. Utge, July 2006 | GU140154 | N.D. | N.D. |
| GWS007239 | Bonne Bay, N.L., Canada: L. Le Gall, B. Hooper, & J. Utge, July 2006 | GU140153 | N.D. | N.D. |
| GWS007397 | Bottle Cove, N.L., Canada: D. McDevit, J. Utge, & L. Le Gall July 2006 | GU140137 | N.D. | N.D. |

Table 1 (continued).

| Species & voucher | Collection details* | GenBank accession No. | | |
|---|---|-----------------------|----------|----------|
| | | COI-5P | UPA | LSU† |
| GWS007484 | English Harbour, N.L., Canada: D. McDevit, J. Utge, & L. Le Gall July 2006 | GU140147 | N.D. | N.D. |
| GWS007488 | English Harbour, N.L., Canada: D. McDevit, J. Utge, & L. Le Gall July 2006 | GU140149 | N.D. | N.D. |
| GWS007854 | Cap St. Mir, Digby, N.S., Canada: L. Le Gall & J. Utge August 2006 | GU140145 | N.D. | N.D. |
| GWS007914 | Cape St. Marys, N.S., Canada: L. Le Gall & J. Utge July 2006 | GU140140 | GU140219 | N.D. |
| GWS008785 | Cable Head, P.E.I., Canada: B.E.C., K. Dixon & K. Roy August 2007 | GU140151 | N.D. | N.D. |
| GWS008790 | Cable Head, P.E.I., Canada: B.E.C., K. Dixon & K. Roy August 2007 | GU140152 | GU140220 | N.D. |
| GWS008792 | Cable Head, P.E.I., Canada: B.E.C., K. Dixon & K. Roy August 2007 | GU140150 | N.D. | N.D. |
| GWS008805 | Gadd Point, Bonne Bay, N.L., Canada: B. Hooper & S. Clayden September 2007 | GU140143 | N.D. | N.D. |
| GWS008807 | Gadd Point, Bonne Bay, N.L., Canada: B. Hooper & S. Clayden September 2007 | GU140148 | N.D. | N.D. |
| GWS008811 | Gadd Point, Bonne Bay, N.L., Canada: B. Hooper & S. Clayden September 2007 | GU140146 | N.D. | N.D. |
| GWS011845 | Paddys Head, N.S., Canada: G.W.S. & D. McDevit May 2009 | GU140120 | N.D. | N.D. |
| GWS011846 | Paddys Head, N.S., Canada: G.W.S. & D. McDevit May 2009 | GU140121 | N.D. | N.D. |
| GWS011849 | Paddys Head, N.S., Canada: G.W.S. & D. McDevit May 2009 | GU140122 | N.D. | N.D. |
| GWS011858 | Paddys Head, N.S., Canada: G.W.S. & D. McDevit May 2009 | GU140123 | N.D. | N.D. |
| GWS012622 | Mazarredo Is., Haida Gwaii, B.C., Canada: G.W.S. & D. McDevit June 2009 | GU140119 | N.D. | N.D. |
| GWS012623 | Mazarredo Is., Haida Gwaii, B.C., Canada: G.W.S. & D. McDevit June 2009 | GU140118 | N.D. | N.D. |
| GWS012624 | Mazarredo Is., Haida Gwaii, B.C., Canada: G.W.S. & D. McDevit June 2009 | GU140117 | N.D. | N.D. |
| GWS012637 | Mazarredo Is., Haida Gwaii, B.C., Canada: G.W.S. & D. McDevit June 2009 | GU140116 | N.D. | N.D. |
| GWS012638 | Mazarredo Is., Haida Gwaii, B.C., Canada: G.W.S. & D. McDevit June 2009 | GU140115 | N.D. | N.D. |
| GWS012641 | Mazarredo Is., Haida Gwaii, B.C., Canada: G.W.S. & D. McDevit June 2009 | GU140114 | N.D. | N.D. |
| <i>Euthora timburtonii</i> Clarkston et G.W. Saunders | | | | |
| GWS001340 | Wizard Islet, Bamfield, B.C., Canada: G.W.S. May 2002 | GU140180 | GU140229 | GU176302 |
| GWS001415 | Wizard Islet, Bamfield, B.C., Canada: G.W.S. October 2002 | GU140179 | N.D. | N.D. |
| GWS001416 | Wizard Islet, Bamfield, B.C., Canada: G.W.S. October 2002 | GU140182 | N.D. | N.D. |
| GWS001417 | Wizard Islet, Bamfield, B.C., Canada: G.W.S. October 2002 | GU140181 | N.D. | N.D. |
| GWS001418 | Wizard Islet, Bamfield, B.C., Canada: G.W.S. October 2002 | GU140173 | N.D. | N.D. |
| GWS002272 | Wizard Islet, Bamfield, B.C., Canada: G.W.S. June 2004 | GU140176 | GU140228 | N.D. |
| GWS002729 | Pachena Beach, Bamfield, B.C., Canada: G.W.S. June 2005 | GU140172 | GU140226 | N.D. |
| GWS003262 | Wizard Islet, Bamfield, B.C., Canada: J. Provencher September 2005 | GU140168 | N.D. | N.D. |
| GWS003263 | Wizard Islet, Bamfield, B.C., Canada: J. Provencher September 2005 | GU140174 | GU140227 | N.D. |
| GWS003264 | Wizard Islet, Bamfield, B.C., Canada: J. Provencher September 2005 | GU140169 | GU140223 | N.D. |
| GWS003265 | Wizard Islet, Bamfield, B.C., Canada: J. Provencher September 2005 | GU140183 | GU140230 | N.D. |
| GWS008493 | Tuwanek, Sechelt, B.C., Canada: B.E.C., G.W.S., D. McDevit, & K. Roy June 2007 | GU140171 | GU140225 | N.D. |
| GWS008738 | Wizard Islet, Bamfield, B.C., Canada: B.E.C., G.W.S, D. McDevit, & K. Roy June 2007 | GU140157 | N.D. | N.D. |
| GWS008740 | Wizard Islet, Bamfield, B.C., Canada: B.E.C., G.W.S, D. McDevit, & K. Roy June 2007 | GU140156 | GU140222 | N.D. |
| GWS008742 | Wizard Islet, Bamfield, B.C., Canada: B.E.C., G.W.S, D. McDevit, & K. Roy June 2007 | GU140170 | GU140224 | N.D. |
| GWS009020 | Gilbert Is., Broken Group Islands, B.C., Canada: B.E.C. & G.W.S. September 2007 | GU140178 | N.D. | N.D. |
| GWS009084 | Wizard Islet, Bamfield, B.C., Canada: B.E.C. & G.W.S September 2007 | GU140175 | N.D. | N.D. |
| GWS009085 | Wizard Islet, Bamfield, B.C., Canada: B.E.C. & G.W.S September 2007 | GU140177 | N.D. | N.D. |
| GWS009499 | Pipers Point, Sechelt, B.C., Canada: B.E.C., G.W.S., D. McDevit, & K. Hind May 2008 | GU140159 | N.D. | N.D. |
| GWS009504 | Pipers Point, Sechelt, B.C., Canada: B.E.C., G.W.S., D. McDevit, & K. Hind May 2008 | GU140160 | N.D. | N.D. |

Table 1 (continued).

| Species & voucher | Collection details* | GenBank accession No. | | |
|--|---|-----------------------|----------|-----------------|
| | | COI-5P | UPA | LSU† |
| GWS010101 | McKay Passage, Tahsis, B.C., Canada: B.E.C. & G.W.S. May 2008 | GU140161 | N.D. | N.D. |
| GWS010298 | Bodega Is., Tahsis, B.C., Canada: B.E.C. & G.W.S. May 2008 | GU140158 | N.D. | N.D. |
| GWS010318 | Mozino Pt., Tahsis, B.C., Canada: B.E.C. & G.W.S. May 2008 | GU140162 | N.D. | N.D. |
| GWS010476 | Execution Rock, Bamfield, B.C., Canada: B.E.C. & S. Toews June 2008 | GU140165 | N.D. | N.D. |
| GWS010571 | Wizard Islet, Bamfield, B.C., Canada: B.E.C. & S. Toews June 2008 | GU140164 | N.D. | N.D. |
| GWS010572 | Wizard Islet, Bamfield, B.C., Canada: B.E.C. & S. Toews June 2008 | GU140167 | N.D. | N.D. |
| GWS010576 | Wizard Islet, Bamfield, B.C., Canada: B.E.C. & S. Toews June 2008 | GU140166 | N.D. | N.D. |
| GWS010578 | Wizard Islet, Bamfield, B.C., Canada: B.E.C. & S. Toews June 2008 | GU140163 | N.D. | N.D. |
| <i>Kallymenia reniformis</i> (Turner) J. Agardh | | | | |
| GWS001453 | Doaghbeg, Fanad Head, Ireland: C.A. Maggs September 2002 | N.D. | N.D. | GU176295 |
| <i>Kallymeniopsis oblongifruca</i> (Setchell) G.I. Hansen | | | | |
| GWS001164 | Seapool Rock, Bamfield, B.C., Canada: G.W.S. May 2001 | GU140196 | GU140238 | AY171613 |
| GWS003039 | Seapool Rock, Bamfield, B.C., Canada: G.W.S. & J. Mortimer June 2005 | GU140201 | N.D. | N.D. |
| GWS003040 | Seapool Rock, Bamfield, B.C., Canada: G.W.S. & J. Mortimer June 2005 | GU140186 | GU140232 | N.D. |
| GWS003044 | Seapool Rock, Bamfield, B.C., Canada: G.W.S. & J. Mortimer June 2005 | GU140184 | N.D. | N.D. |
| GWS003055 | Seapool Rock, Bamfield, B.C., Canada: G.W.S. & J. Mortimer June 2005 | GU140200 | N.D. | N.D. |
| GWS003058 | Seapool Rock, Bamfield, B.C., Canada: G.W.S. & J. Mortimer June 2005 | GU140187 | N.D. | N.D. |
| GWS003066 | Seapool Rock, Bamfield, B.C., Canada: G.W.S. & J. Mortimer June 2005 | GU140185 | GU140231 | N.D. |
| GWS003143 | Seapool Rock, Bamfield, B.C., Canada: R. Withall September 2005 | GU140197 | N.D. | N.D. |
| GWS004496 | Seapool Rock, Bamfield, B.C., Canada: B.E.C., G.W.S., & D. McDevit June 2006 | GU140199 | GU140240 | N.D. |
| GWS004530 | Seapool Rock, Bamfield, B.C., Canada: B.E.C., G.W.S., & D. McDevit June 2006 | GU140194 | GU140236 | N.D. |
| GWS004531 | Seapool Rock, Bamfield, B.C., Canada: B.E.C., G.W.S., & D. McDevit June 2006 | GU140195 | GU140237 | N.D. |
| GWS004532 | Seapool Rock, Bamfield, B.C., Canada: B.E.C., G.W.S., & D. McDevit June 2006 | GU140192 | N.D. | N.D. |
| GWS004533 | Seapool Rock, Bamfield, B.C., Canada: B.E.C., G.W.S., & D. McDevit June 2006 | GU140198 | GU140239 | N.D. |
| GWS004534 | Seapool Rock, Bamfield, B.C., Canada: B.E.C., G.W.S., & D. McDevit June 2006 | GU140191 | N.D. | N.D. |
| GWS004535 | Seapool Rock, Bamfield, B.C., Canada: B.E.C., G.W.S., & D. McDevit June 2006 | GU140190 | GU140235 | N.D. |
| GWS004909 | Stenhouse Reef, Prince Rupert, B.C., Canada: B.E.C. & G.W.S. July 2006 | GU140189 | GU140234 | N.D. |
| GWS004939 | Stenhouse Reef, Prince Rupert, B.C., Canada: B.E.C. & G.W.S. July 2006 | GU140188 | GU140233 | N.D. |
| GWS008676 | Seapool Rock, Bamfield, B.C., Canada: B.E.C., G.W.S., D. McDevit & K. Roy June 2007 | GU140193 | N.D. | N.D. |
| Family Dumontiaceae | | | | |
| <i>Dudresnaya australis</i> J. Agardh ex Setchell | | | | |
| GWS000980 | Flinders Jetty, Victoria, Australia: G.T. Kraft January 2001 | N.D. | N.D. | GU176300 |
| <i>Dudresnaya verticillata</i> (Withering) Le Jolis | | | | |
| GWS001090 | Co. Galway, Ireland: C. Maggs November 2000 | N.D. | N.D. | GU176301 |
| <i>Farlowia mollis</i> (Harvey et Bailey) Farlow et Setchell | | | | |
| GWS000845 | Seppings Is., Bamfield, B.C., Canada: G.W.S. May 2000 | N.D. | N.D. | GU176299 |
| <i>Gibsmithia dotyi</i> Kraft et R.W. Ricker | | | | |
| GWS002048 | Islands off Balls Pyramid, Lord Howe Island, Australia: G.W.S. February 2004 | N.D. | N.D. | GU176298 |
| <i>Gibsmithia hawaiiensis</i> Doty | | | | |
| GWS001343 | Maunalua Bay, Oahu, Hawaii, U. S.A.: J. Huisman April 2002 | N.D. | N.D. | GU176297 |
| <i>Kraftia dichotoma</i> Shepley et Womersley | | | | |

Table 1 (concluded).

| Species & voucher | Collection details* | GenBank accession No. | | |
|---|--|-----------------------|------|-----------------|
| | | COI-5P | UPA | LSU† |
| GW5000924 | Queenscliff Jetty, Port Phillip Heads, Victoria, Australia: G.W.S. December 2000 | N.D. | N.D. | GU176296 |
| Family Rhizophyllidaceae | | | | |
| <i>Portieria hornemannii</i> (Lyngbye) P.C. Silva | Culture isolate: J. West September 1994 | N.D. | N.D. | FJ848973 |
| G0232 | | | | |

Note: N.D., not determined.

*Collectors' initials B.E.C. and G.W.S. for B.E. Clarkston and G.W. Saunders, respectively.

†Accession numbers in bold type indicate sequences were acquired from GenBank

Canada (Table 1). The majority of specimens were collected from around Wizard Islets, Bamfield, B.C. and were epizoic on polychaete worm tubes protruding from the sand. The majority of Wizard Islets collections are coated, excepting the newest branch tips, in epiphytic animals including bryozoans, crustaceans, and hydroids (Fig. 4). Specimens from outside the Bamfield area were found on more varied substrates (e.g., rocks, cup coral) and have far less epiphytic biota (Fig. 5).

Thalli are erect, to 69 mm, highly branched, branching distichous. There are several specimens potentially greater than 69 mm, but the bushy and clumped (Fig. 3) habit of the plants make individuals difficult to measure. Overlapping branches frequently fuse together (Fig. 7), a character unreported for *E. cristata*. The branches are generally a consistent width from base to apex and the branch apices are relatively blunt (Fig. 6). In comparison, branches of *E. cristata* (Figs. 10–19) are typically narrow acropetally, and branch apices are acute (Fig. 13). While it remains undetermined whether thallus construction is uniaxial or multi-axial in *Euthora*, our *E. cristata* specimens have an obvious single apical cell on most terminal branchlets (Fig. 14), whereas no apical cell is apparent on branches of *E. timburtonii*.

In the vegetative section, thalli are flattened in cross section (Fig. 8) compared with the more terete *E. cristata* (Fig. 16). The medulla is tightly packed with large, unpigmented cells (55–95 µm wide) ranging from oval to isodiametric in outline (Fig. 8). Small, unpigmented cells between the large cells, typical of some Kallymeniaceae, are conspicuous only in basal sections of *E. timburtonii*, while in *E. cristata* they are clearly visible throughout most of the thallus (Fig. 16). The cortex is composed of two layers: the outer layer of densely packed, ovoid to periclinal, pigmented cells (3–7 µm wide), and the inner layer of larger, sparsely packed, oval, pigmented cells (Fig. 9) (pigmentation of the cortex was observed prior to staining with aniline blue.) Despite intensive searching, no reproductive structures were observed for *E. timburtonii*.

There is another *Euthora* species reported from the North Pacific, *Euthora fruticulosa*, which Ruprecht erected based on terete and filiform specimens from Kamchatka (Hooper and South 1974). Hooper and South (1974) synonymized this species with *E. cristata*, arguing that the *E. fruticulosa* morphology was a phenotypic extreme of *E. cristata*, which we have observed in the range of morphologies assigned to *E. cristata* based on our genetic work here. *Euthora timburtonii* differs from *E. fruticulosa* because thalli are flattened, wide, and not terete or filiform. We therefore did not resurrect the name *fruticulosa* for this species.

Euthora cristata (C. Agardh) J. Agardh (Figs. 10–19)

TYPE LOCALITY: Arctic and North Atlantic Oceans.

REPRESENTATIVE DNA BARCODE (COI-5P): GU140144 (GWS005193; Fig. 11; see Table 1 for collection details).

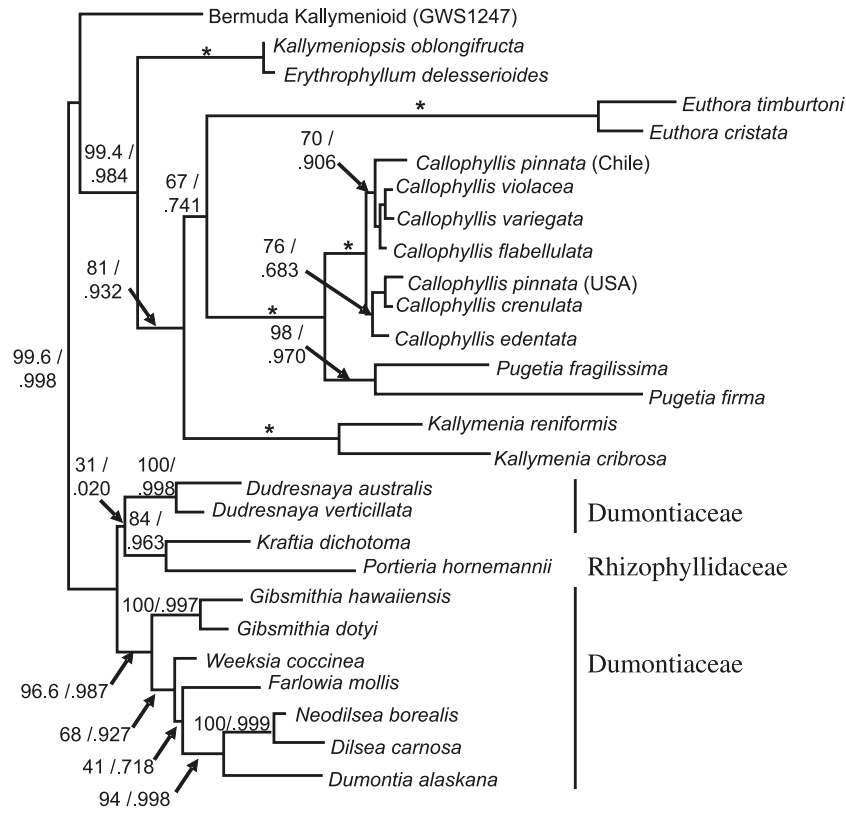
REPRESENTATIVE COLLECTIONS: See Table 1.

OBSERVATIONS: The majority of *Euthora cristata* specimens were found in the subtidal zone (4.5–30 m). Intertidal specimens were only collected from the Bay of Fundy in New Brunswick and Nova Scotia. Specimens were epilithic ex-

Fig. 1. COI-5P and UPA distance matrices for members of *Euthora*, *Kallymeniopsis*, and *Erythrophyllum* showing within and between species sequence divergences. Numbers above are absolute nucleotide differences while percentages in parentheses are sequence divergences (uncorrected *p* values).

| | | <i>E. cristata</i> | <i>E. timburtonii</i> | <i>K. oblong.</i> | <i>E. del.</i> |
|--------------------------------------|---------------------------|------------------------|------------------------|----------------------|-------------------|
| <i>Euthora cristata</i> | COI-5P (<i>n</i> =41) | 0–6 (0%–0.90%) | | | |
| | UPA (<i>n</i> =8) | 0 | | | |
| <i>Euthora timburtonii</i> | COI-5P (<i>n</i> =28) | 29–31 (4.5%–5.2%) | 0–1 (0%–0.15%) | | |
| | UPA (<i>n</i> =9) | 3 (0.81%) | 0 | | |
| <i>Kallymeniopsis oblongifructa</i> | COI-5P (<i>n</i> =18) | 61–74 (10.5%–11.3%) | 56–65 (9.8%–10.3%) | 0–5 (0%–0.75%) | |
| | UPA (<i>n</i> =10) | 18 (4.8%) | 19 (5.1%) | 0 | |
| <i>Erythrophyllum delesserioides</i> | COI-5P (<i>n</i> =15) | 66–76 (11.0%–11.7%) | 58–68 (10.1%–10.7%) | 57–60 (8.6%–9.0%) | 0–5 (0%–0.75%) |
| | UPA (<i>n</i> =12) | 21–22 (5.7%–5.9%) | 22–23 (5.9%–6.2%) | 9–10 (2.4%–2.7%) | 0–1 (0%–0.27%) |

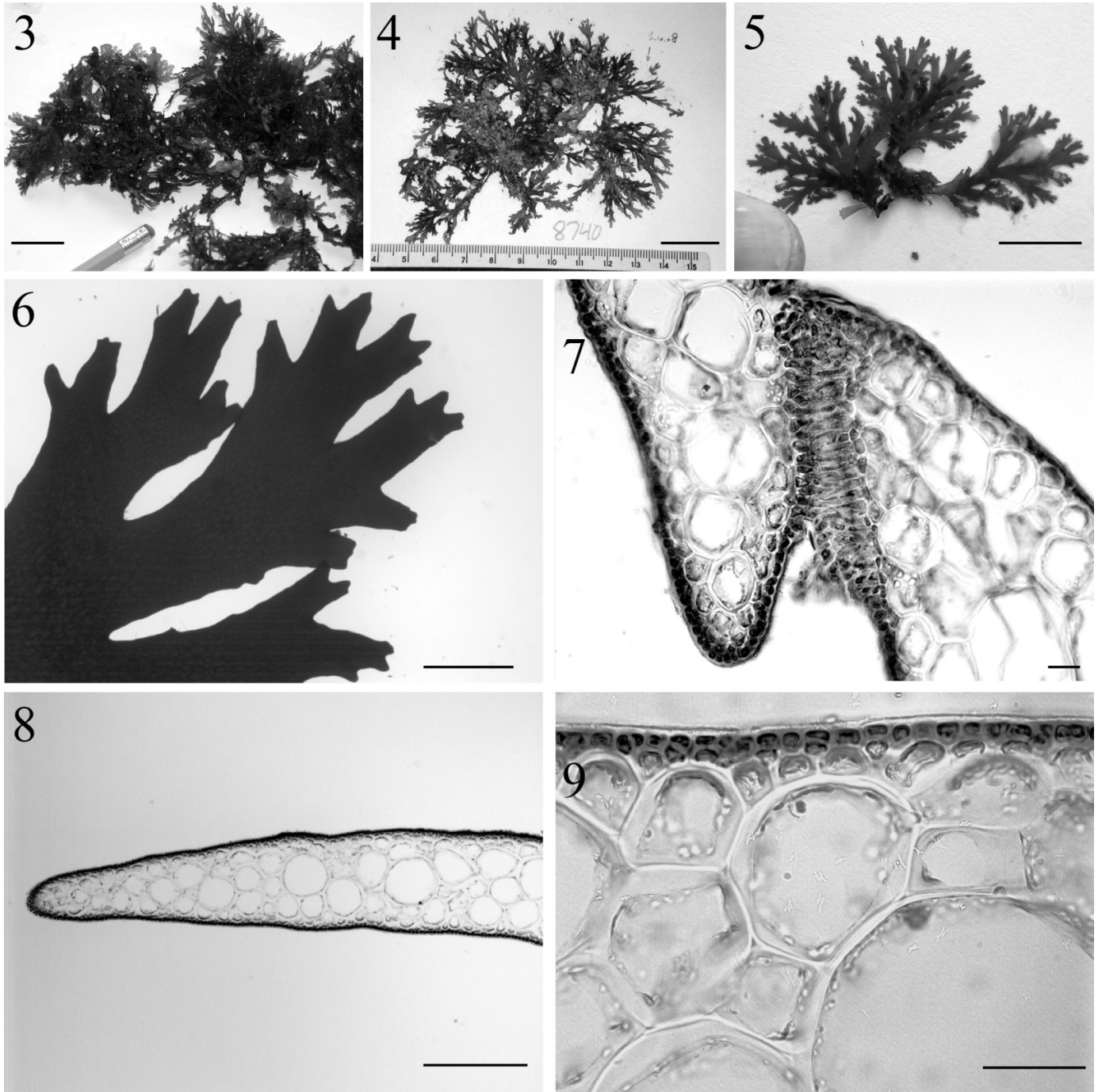
Fig. 2. Phylogenetic tree for representative members of the Kallymeniaceae based on maximum likelihood analysis of LSU sequence data. Values indicate bootstrap (percentage of 500 replicates) and aLRT support, respectively. An asterisk (*) denotes full support for a branch (100% for bootstrap and 1.00 for aLRT). Scale bar = 0.01 substitutions/site.



cept for those collected in Newfoundland (6 of 11 collections were epiphytic on other red algae, one was epizoic on bryozoans), and Prince Edward Island (3 collections, all drift, and epiphytic on other red algae).

Our specimens range in thallus height from 27–90 mm. In general, each small discoid holdfast gives rise to a single blade. Blades are highly branched (>7 orders), branching is distichous (Figs. 10–12). Basal branches are wider (0.7–

Figs. 3–9. Morphology and anatomy of *Euthora timburtonii* sp. nov. Figs. 3–5. Pressed vouchers for GWS010572 (holotype), GWS008740, and GWS010318, respectively. Scale bars = 2 cm. Fig. 6. Terminal branches (GWS010572) with blunt apices and broad penultimate branches. Scale bar = 1 mm. Fig. 7. Fusing branches (GWS010572). Scale bar = 20 μm . Fig. 8. Flat habit of thallus (GWS010572). Scale bar = 200 μm . Fig. 9. Close-up of cortex (GWS010572). Scale bar = 20 μm .



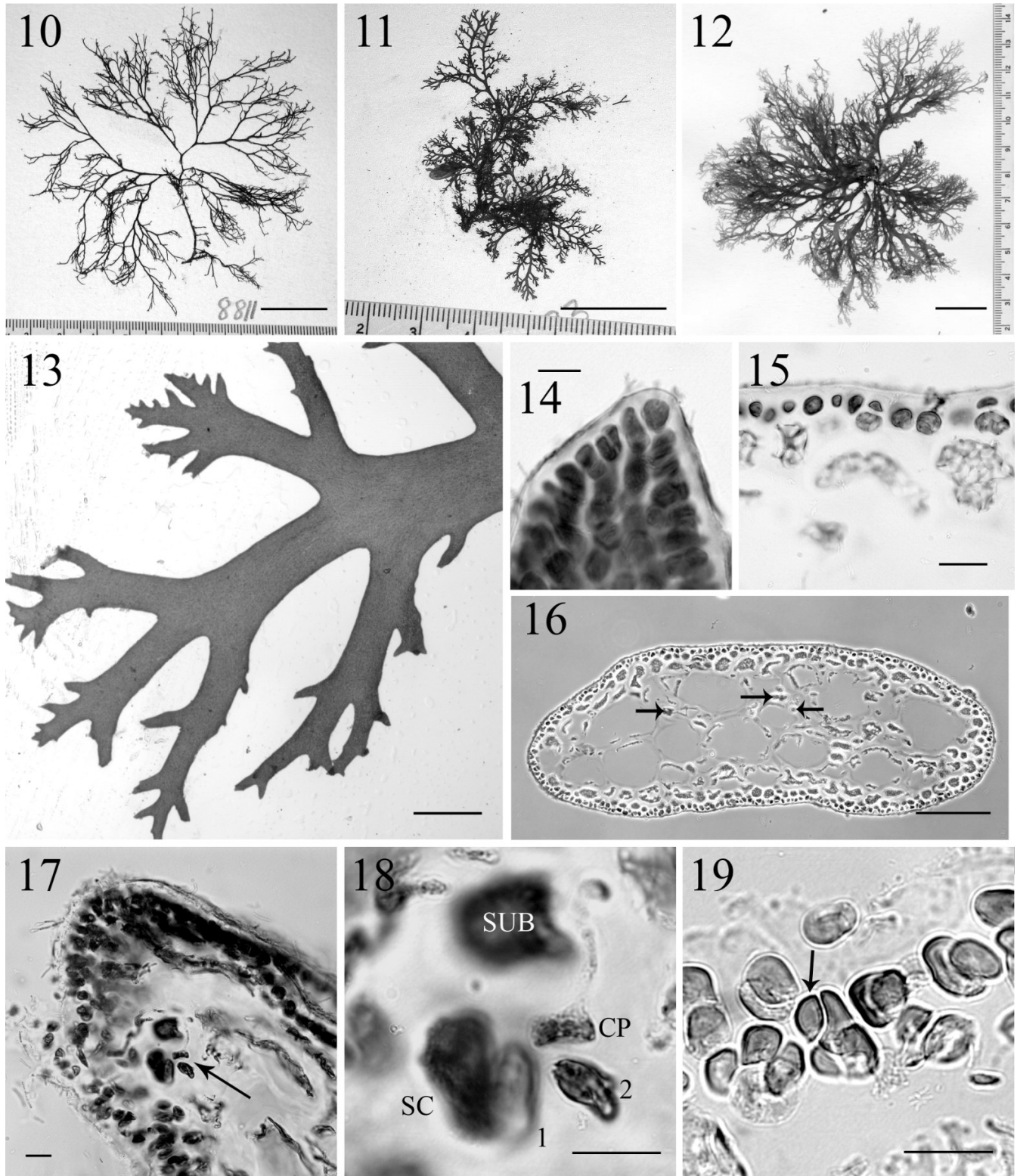
4.1 mm) than upper branches (140–580 μm) (Fig. 13). Branch tips are acute, and a single apical cell (Fig. 14) is obvious on most young branches. Some specimens have narrow, marginal proliferations, especially near the branch tips.

The blades range in thickness from 190 to 480 μm (Fig. 16). The medulla is tightly packed with large, unpigmented cells (20–128 μm) ranging from oval to isodiametric in outline. Small, round, unpigmented cells are visible between the large cells throughout the plant, although they are

most abundant near the base (Fig. 16). The cortex is composed of two layers: an outer layer of closely packed, small, ovoid, pigmented (pre-staining) cells (3–8 μm), and a sparse inner layer of oval, pigmented cells (Figs. 15 and 16).

The gametophytes (Fig. 11) and tetrasporophytes (Fig. 12) are isomorphic. On female gametophytes, the carpogonial branches are dispersed along the margins of younger branches, and are found just below the cortex (Fig. 17). The carpogonial branch consists of three cells on a supporting

Figs. 10–19. Morphology and anatomy of *Euthora cristata*. Figs. 10–12. Pressed vouchers for GWS008811, GWS005193, and GWS003606, respectively. Scale bars = 2 cm. Fig. 13. Terminal branches with acute apices and narrow penultimate branches (GWS005193). Scale bar = 1 mm. Fig. 14. Close-up of apical cell (GWS003606). Scale bar = 10 μ m. Fig. 15. Close-up of cortex (GWS003606). Scale bar = 10 μ m. Fig. 16. Vegetative section showing terete habit of thallus (GWS007488). Arrows indicate small cells between large medullar cells. Scale bar = 100 μ m. Fig. 17. Position of carpogonial branch (arrow) just below the cortex near the margin (GWS002677). Scale bar = 10 μ m. Fig. 18. Close-up of single, three-celled carpogonial branch attached to a supporting cell with a single subsidiary cell (CP, carpogonium; 1, 2 indicate branch cells progressively distal to the supporting cell; SC, supporting cell; SUB, subsidiary cell) (GWS002677). Scale bar = 10 μ m. Fig. 19. Outer cortical cell bearing a spermatium (arrow) (B2–9). Scale bar = 10 μ m.



cell, with usually one subsidiary cell, and is monocarpogonial (Fig. 18). Mature cystocarps are 300–560 μm wide and are marginal, but can expand completely across narrow branches. No obvious ostiole is formed. None of the specimens collected for this study were male. A borrowed specimen from Alaska (see B2–9, Wynne and Heine (1992) for collection details) is monoecious, and revealed that spermatia are cut off from outer cortical cells, which are scattered or in small groups on younger portions of plants (Fig. 19).

Tetrasporangia are cruciate to irregular, and are scattered throughout the cortex of younger portions of sporophytes, but are most numerous along the margins.

Discussion

For many red algae, accurately identifying a specimen to the species level using traditional morphological characters can be frustrating and overwhelming (Saunders 2005). In this study, specimens of *Euthora timburtonii* lacked any reproductive features and, prior to the molecular results, could not be distinguished convincingly from some species of *Callophyllis* that can look similar in vegetative morphology. The difficulty in identifying red macroalgae using morphological characters can be greatly alleviated by an emerging method termed “molecular-assisted alpha taxonomy” (Saunders 2008). Using this approach a large number of specimens from a diverse range of geographical and ecological regions are sequenced as a first step to “species” assignment. The genetic “species” assignment is then augmented by subsequent detailed morphological and anatomical observations. The power of the molecular-assisted alpha taxonomy method is increased if a standard marker (or set of markers) is widely used because then sequences from unidentified specimens can be compared to many known species. In addition, by attaching a standard marker sequence to each described species (i.e., a “holotype barcode” sequence; Saunders 2008), we can conduct more comprehensive ecological studies, examine biogeography more closely, and gain a better understanding of red algal biodiversity.

Both COI-5P and UPA have been proposed as species markers for red algae (Saunders 2005; Sherwood and Presting 2007). COI-5P has high variability, especially in the third codon position, making it effective for discriminating between even closely related species (Hebert et al. 2003). The variability between species for the COI-5P dataset in this study was ca. 10 \times greater than within-species variation, which is consistent with other studies of red algae (Saunders 2005; Robba et al. 2006; Saunders 2008; Walker et al. 2009), brown algae (Kucera and Saunders 2008; McDevit and Saunders 2009), and some animals (Hebert et al. 2003). Within-species variation was slightly higher for *Erythrophyllum delesserioides*, *Kallymeniopsis oblongifracta* (0%–0.75% for both), and *Euthora cristata* (0.90%) than is typically reported in red algae. For *E. delesserioides*, this variation is likely due to geography, since the greatest divergence values occurred between specimens from British Columbia and a single collection from California (Table 1), while among the B.C. collections themselves divergence values (0%–0.30%) fell within the typically reported range. For *K. oblongifracta*, the highest variation occurred among several specimens collected from the same location in B.C.; how-

ever, more research is required before this divergence can be attributed to a phenomenon such as incipient speciation, introgression, etc. The variation between *E. delesserioides* and *K. oblongifracta* (8.6%–9.0%), which are closely related to each other (Figs. 1 and 2) was still >10 \times the within-species variation for either species, and all specimens were unequivocally assigned to the correct species group by COI-5P. For *E. cristata*, divergence values were highest (0%–0.90%) among specimens collected from the Haida Gwaii Islands in northern B.C., and were lower (0%–0.30%) among the other *E. cristata* specimens from both the Pacific and the Atlantic. As with *K. oblongifracta*, this is an interesting result in need of further study.

For UPA, the within and between species divergence values were lower than for COI-5P. UPA sequence divergence between *Euthora cristata* and *Euthora timburtonii* sp. nov., for example, was only 0.81% (3 bp) compared with 5.2% (29–31 bp) using COI-5P. Despite lower interspecific divergence values for UPA, all specimens studied here were assigned unequivocally to the same species group using both markers, a result consistent with the findings of Sherwood et al. (2008) for the freshwater red algal genus *Batrachospermum*. However, there remains the possibility that UPA may lack resolving power among closely related species, which could lead to an underestimation of diversity, and though this has not yet been observed, it is an attribute in need of further study for UPA as a potential marker for DNA barcoding.

Unfortunately, COI-5P is not without its problems, the major drawback being the current lack of universal amplification primers. The original red-algal primers (Saunders 2005) are successful in some groups, but show mixed results in other lineages, which may be due to heterogeneity within a species at positions near the 3' end of the primers (Saunders 2008). The four species resolved in this study using COI-5P required seven different primer combinations to successfully amplify 102 samples, and there were several samples for which a second or even third primer combination was needed to overcome weak amplification or contamination due to epi/endophytic biota. UPA, on the other hand, has universal primers that can amplify product from many diverse lineages including red, green, and brown algae (Sherwood and Presting 2007). For this study, amplification and high quality UPA sequences were never problematic using our modified primers and there was no contamination. Both markers thus have strengths and weaknesses as a DNA barcode, and further study, especially for closely related species pairs (UPA) and primer design (COI-5P), is warranted.

When describing species, a holistic approach that includes a combination of character types (e.g., morphological, molecular, ecological, etc.) should be used (Saunders 2008). However, the question remains, what constitutes a species? Traditional species descriptions in red algae are almost exclusively based on morphological characters and are therefore morphospecies. Saunders (2008) reasoned that genetic species groups are most likely consistent with biological species because genomes acquire independent mutations as populations become reproductively isolated. Using COI-5P, Saunders (2008) resolved seven species groups for the Dumontiaceae instead of the expected four, and all groups were subsequently supported using morphological and anatomical

characters. Sherwood et al. (2008), however, also resolved more species groups than expected in the genus *Batrachospermum* using both COI-5P and UPA markers, but subsequent morphological examination could not resolve unequivocally the same groups. Typically, a molecular marker contains hundreds to thousands of characters, whereas even the most thorough morphological analysis examines, at most, only a few dozen characters. In cases where multiple species-level markers (e.g., nuclear and organellar) resolve the same groups but extensive morphological examination does not, we contend that the molecular data should take precedence and the groups recognized as different species. For the current study, this was not a concern because our collections of *E. timburtonii* differed in many aspects of morphology, anatomy, ecology, and molecular data from *E. cristata*.

Acknowledgements

All of the collectors listed in Table 1 are acknowledged for their help with this project, as are Kathryn Roy, J. Todd Harper, Tanya Moore, Bethany Hermann, and Alexandra Johnson for generating some of the sequence data. C. Schneider provided advice on microscopy techniques. M. Wynne is thanked for providing a loan of *Euthora cristata* male plants and Pacific collections. The Latin diagnosis was generously provided by A. Friend. Thank you to two anonymous reviewers for comments on the manuscript. Finally, the Bamfield Marine Sciences Centre and Taitsa Dive Charters in British Columbia are thanked for hosting much of the field component of this research. This research was supported through funding to the Canadian Barcode of Life Network from Genome Canada through the Ontario Genomics Institute, Natural Sciences and Engineering Research Council of Canada and other sponsors listed at www.BOLNET.ca. Additional support was provided by the Canada Research Chair Program, as well as infrastructure support from the Canada Foundation for Innovation and the New Brunswick Innovation Foundation.

References

- Abbott, I.A., and Norris, R.E. 1965. Studies on *Callophyllis* (Rhodophyceae) from the Pacific coast of North America. *Nova Hedwigia*, **10**: 67–84.
- Fox, C.H., and Swanson, A.K. 2007. Nested PCR detection of microscopic life-stages of laminarian macroalgae and comparison with adult forms along intertidal height gradients. *Mar. Ecol. Prog. Ser.* **332**: 1–10. doi:10.3354/meps332001.
- Guillemin, M.L., Akki, S.A., Givernaud, T., Mouradi, A., Valero, M., and Destombe, C. 2008. Molecular characterisation and development of rapid molecular methods to identify species of Gracilariaceae from the Atlantic coast of Morocco. *Aquat. Bot.* **89**(3): 324–330. doi:10.1016/j.aquabot.2008.03.008.
- Guindon, S., and Gascuel, O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol.* **52**(5): 696–704. doi:10.1080/10635150390235520. PMID:14530136.
- Hansen, G.I., and Lindstrom, S.C. 1984. A morphological study of *Hommersandia maximicarpa* gen. et. sp. nov. (Kallymeniaceae, Rhodophyta) from the North Pacific. *J. Phycol.* **20**(4): 476–488. doi:10.1111/j.0022-3646.1984.00476.x.
- Harper, J.T., and Saunders, G.W. 2002. Using molecular data to resolve taxonomic limits of the genera *Callophyllis*, *Euthora* and *Pugetia* (Kallymeniaceae, Rhodophyta). *Phycol. Res.* **50**(4): 275–281. doi:10.1111/j.1440-1835.2002.tb00160.x.
- Hebert, P.D.N., Cywinska, A., Ball, S.L., and DeWaard, J.R. 2003. Biological identifications through DNA barcodes. *Proc. R. Soc. Lond. B. Biol. Sci.* **270**(1512): 313–321. doi:10.1098/rspb.2002.2218.
- Higgins, D.G., Thompson, J.D., and Gibson, T.J. 1996. Using CLUSTAL for multiple sequence alignments. *Methods Enzymol.* **266**: 383–402. doi:10.1016/S0076-6879(96)66024-8. PMID:8743695.
- Hooper, R., and South, G.R. 1974. A taxonomic appraisal of *Callophyllis* and *Euthora* (Rhodophyta). *Br. Phycol. J.* **9**(4): 423–428. doi:10.1080/00071617400650491.
- Kucera, H., and Saunders, G. 2008. Assigning morphological variants of *Fucus* (Fucales, Phaeophyceae) in Canadian waters to recognized species using DNA barcoding. *Botany*, **86**(9): 1065–1079. doi:10.1139/B08-056.
- Kylin, H. 1928. *Entwicklungsgeschichte der Florideen*. Lunds Univ. Arsskr. N. F. Avd. **2**(21): 1–127.
- Lane, C.E., Lindstrom, S.C., and Saunders, G.W. 2007. A molecular assessment of northeast Pacific *Alaria* species (Laminariales, Phaeophyceae) with reference to the utility of DNA barcoding. *Mol. Phylogenet. Evol.* **44**(2): 634–648. doi:10.1016/j.ympev.2007.03.016. PMID:17544704.
- Le Gall, L., and Saunders, G.W. 2007. A nuclear phylogeny of the Florideophyceae (Rhodophyta) inferred from combined EF2, small subunit and large subunit ribosomal DNA: establishing the new red algal subclass Corallinophycidae. *Mol. Phylogenet. Evol.* **43**(3): 1118–1130. doi:10.1016/j.ympev.2006.11.012. PMID:17197199.
- Le Gall, L., and Saunders, G.W. 2010. DNA barcoding is a powerful tool to uncover algal diversity: a case study of the Phylloporaceae (Gigartinales, Rhodophyta) in the Canadian flora. *J. Phycol.* In press.
- Maddison, W., and Maddison, D. 2003. *MacClade*. 4.06. Sunderland, Sinauer Associates, Mass.
- McDevit, D.C., and Saunders, G.W. 2009. On the utility of DNA barcoding for species differentiation among brown macroalgae (Phaeophyceae) including a novel extraction protocol. *Phycol. Res.* **57**(2): 131–141. doi:10.1111/j.1440-1835.2009.00530.x.
- Norris, R.E. 1957. Morphological studies on the Kallymeniaceae. *Univ. Calif. Publ. Bot.* **28**: 251–334.
- Posada, D., and Crandall, K.A. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics*, **14**(9): 817–818. doi:10.1093/bioinformatics/14.9.817. PMID:9918953.
- Presting, G. 2006. Identification of conserved regions in the plastid genome: implications for DNA barcoding and biological function. *Can. J. Bot.* **84**(9): 1434–1443. doi:10.1139/B06-117.
- Ratnasingham, S., and Hebert, P.D.N. 2007. BOLD: The Barcode of Life Data System (<http://www.barcodinglife.org>). *Mol. Ecol. Notes*, **7**(3): 355–364. doi:10.1111/j.1471-8286.2007.01678.x. PMID:18784790.
- Robba, L., Russell, S., Barker, G., and Brodie, J. 2006. Assessing the use of the mitochondrial *cox1* marker for use in DNA barcoding of red algae (Rhodophyta). *Am. J. Bot.* **93**(8): 1101–1108. doi:10.3732/ajb.93.8.1101.
- Saunders, G.W. 1993. Gel purification of red algal genomic DNA: an inexpensive and rapid method for the isolation of polymerase chain reaction-friendly DNA. *J. Phycol.* **29**(2): 251–254. doi:10.1111/j.0022-3646.1993.00251.x.
- Saunders, G.W. 2005. Applying DNA barcoding to red macroalgae: a preliminary appraisal holds promise for future applications.

- Phil. Trans. R. Soc. Lond. Ser. B. Biol. Sci. **360**: 1879–1888. doi:10.1098/rstb.2005.1719.
- Saunders, G.W. 2008. A DNA barcode examination of the red algal family Dumontiaceae in Canadian waters reveals substantial cryptic species diversity. 1. The foliose *Dilsea*–*Neodilsea* complex and *Weeksia*. Botany, **86**(7): 773–789. doi:10.1139/B08-001.
- Saunders, G.W. 2009. Routine DNA barcoding of Canadian Gracilariales (Rhodophyta) reveals the invasive species *Gracilaria vermiculophylla* in British Columbia. Mol. Ecol. Resour. **9**: 140–150. doi:10.1111/j.1755-0998.2009.02639.x.
- Schneider, C., and Lane, C.E. 2008. Notes on the marine algae of the Bermudas. 9. The genus *Botryocladia* (Rhodophyta, Rhodymeniaceae), including *B. bermudana*, *B. exquisita* and *B. flookii* spp. nov. Phycologia, **47**(6): 614–629. doi:10.2216/08-44.1.
- Sherwood, A.R., and Presting, G.G. 2007. Universal primers amplify a 23S rDNA plastid marker in eukaryotic algae and cyanobacteria. J. Phycol. **43**(3): 605–608. doi:10.1111/j.1529-8817.2007.00341.x.
- Sherwood, A.R., Vis, M., Entwisle, T., Necchi, O., Jr., and Presting, G.G. 2008. Contrasting intra versus interspecies DNA sequence variation for representatives of the Batrachospermales (Rhodophyta): Insights from a DNA barcoding approach. Phycol. Res. **56**(4): 269–279. doi:10.1111/j.1440-1835.2008.00508.x.
- Swofford, D.L. 2002. PAUP: Phylogenetic Analysis Using Parsimony, version 4.08a. Computer program distributed by the Illinois Natural History Survey, Champaign, Ill.
- Tai, V., Lindstrom, S.C., and Saunders, G.W. 2001. Phylogeny of the Dumontiaceae (Gigartinales, Rhodophyta) and associated families based on SSU rDNA and internal transcribed spacer sequence data. J. Phycol. **37**(1): 184–196. doi:10.1046/j.1529-8817.2001.037001184.x.
- Walker, R.H., Brodie, J., Russell, S., Irvine, L.M., and Orfanidis, S. 2009. Biodiversity of coralline algae in the Northeast Atlantic including *Corallina caespitosa* sp. nov. (Corallinoideae, Rhodophyta). J. Phycol. **45**(1): 287–297. doi:10.1111/j.1529-8817.2008.00637.x.
- Wynne, M.J., and Heine, J.N. 1992. Collections of marine red algae from St. Matthew and St. Lawrence Islands, the Bering Sea. Nova Hedwigia, **55**: 55–97.
- Yamada, T.K., Miyaji, K., and Nozaki, H. 2008. A taxonomic study of *Eudorina unicocca* (Volvocaceae, Chlorophyceae) and related species, based on morphology and molecular phylogeny. Eur. J. Phycol. **43**(3): 317–326. doi:10.1080/09670260701763484.