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Resolving species diversity in the red algal genus *Callophyllis* (Kallymeniaceae, Gigartinales) in Canada using molecular assisted alpha taxonomy

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The COI-5P DNA barcode (the 5′ region of the mitochondrial cytochrome c oxidase I gene) has proven an effective tool for quickly screening large numbers of biological specimens and assigning them to genetic species groups for subsequent examination in an approach known as molecular-assisted alpha taxonomy (MAAT). In the present study, we applied MAAT to Canadian species of the red algal genus *Callophyllis* in order to resolve confusion surrounding this taxonomically perplexing group. A total of 563 *Callophyllis* specimens were examined (either molecularly, morphologically or both) – 504 from British Columbia along the Pacific coast of Canada and 59 from the U.S.A., Australia and Chile. In total, 16 genetic species groups were resolved including 12 in Canada although only nine species are reported in the flora. Here we report range extensions of *C. beringensis* and *C. radula* from the NW Pacific, *C. dissecta* from California, U.S.A. and the Chilean species *C. variegata* (the type species) into the Canadian flora. Our results also uncovered cryptic diversity in the genus necessitating the description of *C. schneideri* Clarkston & G.W. Saunders *sp. nov.* Subsequent analyses of a subset of specimens using the internal transcribed spacer of the ribosomal cistron (ITS) resolved the same groups as COI-5P while the universal plastid amplicon (UPA, domain V of the plastid 23S rRNA gene) had poor resolution at the species level and in one case underestimated species diversity. Finally, large-subunit ribosomal DNA (LSU), nuclear elongation factor 2 (EF2), COI-5P and a combined LSU, EF2 and COI-5P alignment were subjected to phylogenetic analyses in order to investigate relationships among Canadian species of *Callophyllis*. A significant outcome was the resolution of two well-supported lineages for the monocarpogonial versus polycarpogonial species of this genus, as had been posited in the literature.

Key words: biodiversity; biogeography; *Callophyllis*; COI-5P; DNA barcode; Florideophyceae; Gigartinales; ITS; UPA; LSU; EF2; Kallymeniaceae; Rhodophyta; systematics

Introduction

Callophyllis Kützing (1843) has been described as one of the most taxonomically perplexing genera of red algae on the Pacific coast of North America (Abbott & Norris, 1966). The genus was originally established for two species – *C. variegata* from Chile and *C. laciniata* from Europe (Norris, 1957) – but has grown into the largest genus in the family Kallymeniaceae (*ca.* 57 current species: Guiry & Guiry, 2012) with species found in most temperate seas (Abbott & Norris, 1966). Currently, there are nine species reported from British Columbia, Canada, to Southern California, U.S.A. (Gabrielson *et al.*, 2004, 2006).

There have been several important papers examining the taxonomy of *Callophyllis* species from the west coast of North America (Setchell, 1923; Doty, 1947; Dawson, 1954; Norris, 1957; Abbott & Norris, 1966). Setchell (1923) included 14 species descriptions, as well as a taxonomic key for the genus in this region. This paper was significant in that it established most of the vegetative characters (plant size, branching pattern and cystocarp placement) used to this day to distinguish species of *Callophyllis* and it "could have been a landmark in the study of this difficult genus" (Dawson, 1954). However, there were two major shortcomings. The Latin descriptions were too brief and lacked detail, with no illustrations or habitat information to accompany them, and a portion of the taxonomic key was missing due to a typographical error, rendering it unworkable. In addition, the enthusiasm Setchell displayed in designating new species has been regarded as misguided. Abbott & Norris (1966) wrote that "If one follows Setchell's (1923) interpretations, nearly every [morphological] variant seems to merit a distinct species and this is probably incorrect" and many of his

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species were subsequently synonymized (Doty, 1947; Dawson, 1954; Abbott & Norris, 1966).

Dawson (1954) emended and expanded on Setchell's work by publishing a corrected version of the key, as well as providing photographs of many of the type specimens. He also attempted to establish a more consistent suite of characters for delimiting species, such as the size of cystocarps and the number and shape of cystocarp ostioles. Unfortunately, most of the characters emphasized by Dawson have been shown to vary within a single plant (Abbott & Norris, 1966) and are therefore not useful taxonomically.

In their own examination of *Callophyllis,* Abbott & Norris (1966) proposed that the presence of one carpogonial branch per supporting cell (monocarpogony) vs. two or more (polycarpogony) can distinguish two evolutionary groups in *Callophyllis* and that there are several species from each group that may be paired (in an analogous rather than evolutionary sense) because of vegetative similarities. Regardless of whether this idea is correct, Abbott & Norris considered that most of the species within each carpogonial group could be delimited based on a combination of vegetative characters (e.g. degree of branching, branching pattern and form of ultimate branches).

Harper & Saunders (2002) were the first to include molecular data for several *Callophyllis* species in phylogenetic analyses using nuclear large subunit ribosomal DNA (LSU). Their study resolved a monophyletic *Callophyllis* clade, with the exception of species of *Pugetia* and *Euthora* that had been placed previously within *Callophyllis* (Norris, 1957; Hooper & South, 1974). Relationships among species within the genus *sensu stricto* were left largely unresolved, although polycarpogonial species grouped together with moderate to strong support (Harper & Saunders, 2002), providing consistency with this aspect of Abbott & Norris's (1966) scheme.

A modern approach to overcoming confusion in algal systematics is to use molecular-assisted alpha taxonomy (MAAT), in which specimens are screened using species-level molecular markers in order to assign them to genetic species groups (Saunders, 2008; Cianciola *et al.,* 2010). These groups are subsequently examined for additional unique and ubiquitous morphological and anatomical characters in an attempt to assign each to a known species concept (e.g. Saunders, 2008; Schneider & Lane, 2008; Le Gall & Saunders, 2010). When a group does not match a known concept, it is described as a new species. Members of *Callophyllis* found in British Columbia, Canada, are particularly good candidates for MAAT, given the confusion in the literature regarding the number of species and the lack of known reliable morphological features to delimit them.

Several molecular markers have been used in red algae for assigning specimens to species, notably the DNA barcode (COI-5P, *ca.* 664 bp from the 5′ region of the mitochondrial cytochrome c oxidase I gene: e.g. Saunders, 2005, 2008); the internal transcribed spacer of the ribosomal cistron (ITS, *ca.* 650 bp: e.g. Ross *et al.*, 2003; Clarkston & Saunders, 2012); and the universal plastid amplicon (UPA, *ca.* 400 bp, domain V of the 23S rRNA gene: e.g. Sherwood & Presting, 2007; Sherwood *et al.*, 2008; Clarkston & Saunders, 2010). Of these tools, the COI-5P is currently the standard DNA barcode marker sanctioned by the Consortium for the Barcode of Life (CBOL; http://www.barcoding.si.edu/) – an international initiative dedicated to developing short, standardized DNA sequences as species-level identification tools – and has been the primary marker used during recent and ongoing surveys of Canadian Kallymeniaceae (Clarkston & Saunders, 2010; Clarkston & Saunders, 2012).

The objectives of the current study were to (1) determine the diversity of species of *Callophyllis* in Canada, using the DNA barcode as an assessment tool; (2) assess traditional morphological and anatomical characteristics to determine which, if any, are taxonomically viable at the species level in this genus; and (3) resolve the phylogenetic affinities of Canadian *Callophyllis* species relative to one another and to other readily available members of this genus from other geographical regions, using COI-5P, LSU and EF2 (nuclear elongation factor 2 DNA) sequence data.

Materials and methods

A total of 564 *Callophyllis* specimens were examined in this study, 505 from the Pacific coast of Canada and 59 from the U.S.A., Australia and Chile. All specimens were collected either in the subtidal by SCUBA, or in the intertidal as attached individuals or drift. Supplementary Table S1 lists the genetic data generated for each specimen along with collection location; additional collection information for each specimen is available online by searching the BOLD accession number at www.barcodinglife.org. Herbarium acronyms are given according to the Index Herbariorum (Thiers, continuously updated). To provide a permanent voucher, specimens were dried either on herbarium paper or in a 20 ml scintillation vial with silica gel and a subsample of each specimen was dried in a 1.5 ml screw-cap tube with silica gel for molecular analyses.

DNA extraction and amplification

For all samples, genomic DNA was isolated following a protocol modified from Saunders (1993; see Saunders, 2008). COI-5P was amplified following the protocol of McDevit & Saunders (2009) and using primer combinations as recorded for each specimen on the Barcode of Life Data Systems website (BOLD: http://www.boldsystems.org). The most commonly used primer combinations were GWSFn (Le Gall & Saunders, 2010) and GWSRx (Clarkston & Saunders, 2012), followed by GazF1 (Saunders, 2005) and GazR1 (Saunders, 2005), and GHalF (Saunders, 2008) and GazR1

(Saunders, 2005). COI-5P failed for a few specimens (Table S1), possibly due to poor primer match or degraded DNA in the case of *Callophyllis odonthalioides*. For *C. odonthalioides,* LSU data were available from a previous study and were used for species assignment (Table S1). Nuclear (ITS) and plastid (UPA) species-level markers were used to further investigate the COI-5P results, and to assign specimens to species groups when COI-5P could not be amplified. Amplification and sequencing of ITS and UPA for selected samples (Table S1) followed the procedures of Tai *et al*. (2001) and Sherwood & Presting (2007; see Clarkston & Saunders, 2010, for a modified reverse primer), respectively.

To explore the phylogenetic affinities within *Callophyllis,* two additional nuclear markers – partial LSU and partial EF2–were amplified and sequenced for a representative of each species group (Table S1). Partial LSU was PCR amplified, cleaned and sequenced according to Harper & Saunders (2001), with the modifications of Le Gall & Saunders (2010). Partial EF2 was PCR amplified using a low-volume PCR protocol [each PCR sample contained 1.25 µL of 10× PCR buffer (Takara, Shiga, Japan), 25 mM $MgCl_2$, 8.45 µl sterile H_20, 2.5mM of each dNTP, 10 µM of each primer, 0.3 unit of Ex Taq™ (Takara, Shiga, Japan) and 1 µl DNA template] with an annealing temperature of 60°C. EF2 amplification products did not require cleaning and were sequenced following the protocol of Le Gall & Saunders (2007) All COI-5P and UPA PCR products were purified using ExoSAP-IT® (USB, Cleveland, USA) and all ITS and LSU amplification products were cleaned using a glass wool column protocol (Saunders, 1993), while EF2 amplification products did not require cleaning.

Sequencing of all PCR products was carried out using the PE Applied Biosystems Big Dye (version 3.1) kit following the manufacturer's instructions except that only 1 µl of Big Dye was used per sample (ABI, Foster City, USA). Forward and reverse sequence reads (excluding the PCR primer regions) were edited using Sequencher™ 4.10 (Gene Codes Corporation, Ann Arbor, USA).

Sequencing alignments

Several samples of *Callophyllis* from Australia and Chile were fortuitously available and so were also included in our molecular analyses when possible. The COI-5P alignment (664 bp) contained 544 *Callophyllis* sequences, representing 97% of the *Callophyllis* specimens examined in this study, as well as four from other members of the Kallymeniaceae (Table S1), all of which were generated for the current study except the *Pugetia* spp. sequences (GWS001737 and GWS002184: see Clarkston & Saunders, 2012). The ITS alignment (683 bp) contained 61 sequences and the UPA alignment (371 bp) contained 82 sequences, all of these data having been generated in the current study (Table S1). The COI-5P, UPA and ITS sequences were uploaded to the Barcode of Life Data Systems database (BOLD; Ratnasingham & Hebert, 2007). For the LSU, a global alignment was constructed (2840 bp), comprising 18 *Callophyllis* species, 32 other kallymeniacean taxa and 9 outgroup taxa from the closely related families Dumontiaceae and Rhizophyllidaceae (Tai *et al*., 2001); this alignment included 34 newly determined kallymeniacean sequences (Table S1), 18 previously published kallymeniacean sequences (see Clarkston & Saunders, 2012) and nine previously published sequences [*Kraftia dichotoma* (GU176296), *Farlowia mollis* (GU176299), *Dudresnaya verticillata* (GU176301), *Gibsmithia dotyi* (GU176298), *Gibsmithia hawaiiensis* (GU176297), *Weeksia reticulata* (JF928824), *Neodilsea natashae* (JF928825), *Dilsea carnosa* (EF033609) and *Portieria hornemannii* (FJ848973)]. To reduce long branch attraction problems with the global LSU alignment, a nested (local) alignment was generated with a reduced taxon sampling (alignment provided in Supplementary information), retaining the 20 *Callophyllis* sequences and only the closest related genera (*Austrophyllis* and *Thamnophyllis*): more distant taxa were removed based on analysis of the global alignment. The EF2 alignment (1174 bp; 15 *Callophyllis* species, *Thamnophyllis lacerata* and *Pugetia fragilissima*) contained only 15 of the 18 *Callophyllis* species included in the LSU alignment because *C. lambertii*, *C. linearis* and *C. odonthalioides* failed to amplify for EF2. A reduced COI-5P alignment was also generated for phylogenetic analyses and contained one representative of each *Callophyllis* species plus two outgroup taxa (16 *Callophyllis* species, *T. lacerata* and *Austrophyllis harveyana*). Finally, a combined alignment for LSU, EF2, and COI-5P was generated and contained 16 taxa (14 *Callophyllis* species, *T. lacerata* and *P. fragilissima*) and 5016 characters. All alignments were generated manually with the assistance of MacClade version 4.08 (Maddison & Maddison, 2003). All newly determined sequences were submitted to GenBank.

Molecular analyses

For each of the COI-5P, UPA and ITS datasets, genetic species groups were visualized by distance analyses using the neighbour-joining algorithm in BOLD. For the COI-5P and UPA data the intra- and interspecific sequence divergence values for each species group were determined in BOLD using the Kimura 2-parameter distance model, whereas for the ITS data, divergence values were determined in PAUP* (Swofford, 2002) using the Kimura 2-parameter distance model.

Phylogenetic analyses were performed on the global LSU alignment, local LSU alignment, EF2 alignment, reduced COI-5P alignment and LSU + EF2 + COI-5P combined alignment to assess interspecific relationships within *Callophyllis*. For all of the alignments, maximum likelihood (ML) analyses were performed using PhyML 3.0 (Guindon & Gascuel, 2003) with a general time-reversible substitution model (selected using jModeltest version 0.1.1; Posada, 2008) and a PhyML-estimated proportion of invariable sites and gamma shape parameters. In each case, the starting tree was determined using BIONJ, Nearest Neighbour Interchanges (NNIs) branch-swapping was in effect, and tree topology and branch lengths were optimized. Branch support was estimated using both nonparametric bootstrap resampling (1000 replicates) and the Shimodaira–Hasegawa-like approximate likelihood ratio test (SH-like aLRT). The unrooted tree was imported into Geneious 5.1.4 (Drummond *et al.*, 2009) and rooted with reference to the outgroup. MrBayes 3.1 (Huelsenbeck, 2001) was used to complete Bayesian analyses under a general time reversible model on the LSU + EF2 + COI-5P combined alignment. The data

were partitioned by gene, as well as by codon for EF2 and COI-5P, for a total of seven partitions. Each analysis was run for five million generations and sampling was performed every 1000 generations and the run was replicated once. An appropriate burn-in was estimated by plotting the overall likelihood against generations prior to estimating the posterior probability distribution. The final tree topology and posterior probability values for each analysis were based on the combined results from the stationary phase of the two independent runs.

Morphological and anatomical analyses

Tissue for anatomical work was excised from herbarium specimens, rehydrated in a 4% formaldehyde and 1% Tween® 20 detergent solution for 5–30 min as needed, and sectioned using a freezing microtome (CM 1850, Leica, Heidelberg, Germany). A 1% aniline blue stain in 6% 5N hydrochloric acid was used for highlighting vegetative features and structures of the female reproductive system in all sections. Samples were 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. All images were imported into Adobe® PhotoShop® CS (Adobe Systems Inc., San Jose, USA) for plate assembly.

Unless otherwise stated, gross morphological characteristics (thallus size, order of branches, margin characteristics) were measured for at least 10 specimens (when possible) that were haphazardly selected. For other morphological characteristics (branch widths, thallus thickness, reproduction), 3–5 specimens were selected haphazardly for each species group and three measurements made per specimen.

Results

Molecular observations

A neighbour-joining clustering analysis of the COI-5P alignment resolved 16 *Callophyllis* species groups (Fig. 1), although only nine species are currently recognized (Gabrielson *et al.*, 2006). Twelve of the species groups included collections from Canada. Specimens from Australia, Chile and the U.S.A. either matched the Canadian species groups (Table S1) or resolved as distinct species (Fig. 1). Two specimens from Chile, field identified as *Callophyllis pinnata,* resolved as a distinct group, separate from *C. pinnata* specimens collected from British Columbia and California (the type locality); subsequent anatomical observations linked these specimens with a newly described Chilean species, *C. concepcionensis*. (Arakaki *et al.,* 2011). Specimens field identified as *C. violacea* resolved as two distinct groups (here referred to as *C. violacea* and *C. schneideri* Clarkston & G.W. Saunders, *sp. nov.*). In addition, specimens of the related genera *Austrophyllis, Pugetia* and *Thamnophyllis* were included for comparative purposes. The ITS and UPA markers each resolved the same species groups as COI-5P (not shown), with two exceptions for UPA discussed below.

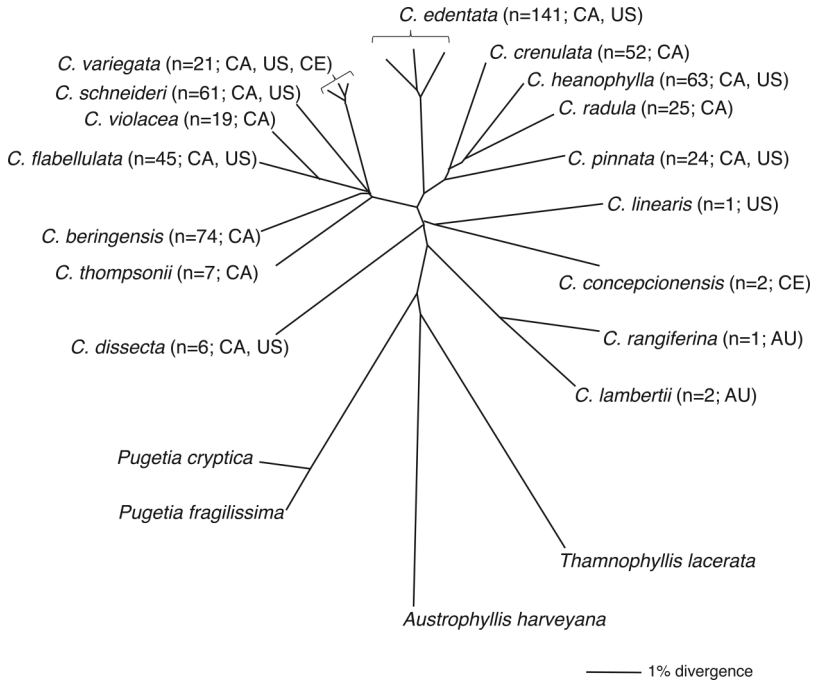


Fig. 1. Cryptic and overlooked diversity within the genus *Callophyllis* revealed using the DNA barcode (COI-5P; n = 544 *Callophyllis* specimens). Genetic species groups are represented by a single DNA barcode sequence and displayed as an unrooted phylogram (neighbour joining). Individual sequences from closely-related genera are also included for comparison. Abbreviations indicate the countries where *Callophyllis* species were collected (CA = Canada; US = United States; CE = Chile; AU = Australia).

The level of intra- and interspecific sequence divergence within *Callophyllis* varied for each marker, with COI-5P being the most variable overall (except for *C. flabellulata*, *C. thompsonii* and *C. variegata* where ITS was slightly more variable) and UPA the most conserved. Overall, UPA had poor resolution due to low interspecific divergence, with several species only 0.3% (1 bp) divergent from their nearest neighbour, including *C. edentata,* which also had higher intraspecific variation (0.5%; 2 bp). In addition, UPA failed to resolve two species delimited by both COI-5P and ITS (*C. violacea* and *C. schneideri*), indicating that this marker can underestimate species diversity as has been previously suggested (Clarkston & Saunders, 2010).

For COI-5P and ITS, the intraspecific and interspecific variation were consistent with previous reports (e.g. Saunders, 2005; Robba *et al.*, 2006; Clarkston & Saunders, 2010; Le Gall & Saunders, 2010). Intraspecific variation (excluding *C. variegata* and *C. edentata*) was 0–0.72% for COI-5P, with interspecific variation within the genus at 2.6–10.45%, and 0–0.32% for ITS intraspecific variation, with interspecific variation of 1.15–12.8%.

The two exceptions were *C. variegata* and *C. edentata. Callophyllis variegata* had high intraspecific divergence for both COI-5P (0.9%) and ITS (1.3%). COI-5P (n = 21) resolved two North American clusters (both containing specimens from British Columbia and California) and a South American cluster, all 0.9% divergent from each other. Interestingly, ITS (n = 5) resolved a similar pattern to the COI-5P. This variation could be due to a number of processes including incipient speciation, incomplete lineage sorting and/or introgression. However, we do not have sufficient data for samples across the geographical range of this species to speculate further on this interesting pattern of divergence. Morphological and anatomical examinations (see "*Morphological observations and taxonomic changes*" section) revealed no obvious distinctions between these genetic clusters and for the moment we retain *C. variegata* as a single species, pending further study.

Callophyllis edentata also had higher than expected maximum intraspecific divergence for COI-5P (2.86%; n = 141), but in this case displayed more typical ITS variation (maximum intraspecific divergence = 0.32%; n = 6). COI-5P resolved three clusters: the "main" cluster (n = 127), the "mixed" cluster (n = 11) from British Columbia and California, and the "California only" cluster (n = 3) unique to California. Divergence within each cluster was lower (0–1.04%) than between clusters (1.55–2.86%), suggesting some genetic structure. The ITS results were inconclusive in terms of resolving the same "main" and "mixed" COI-5P clusters (no ITS sequences were acquired for the "California only" cluster), but do suggest lower divergence values than COI-5P. For ITS, the "main" cluster (n = 3) had identical sequences and three fixed differences (0.16% divergence) compared to the "mixed" cluster; however, within the "mixed" cluster (n = 3) there was 0.16% divergence due to unique variation in one sample, GWS013330. The discrepancy between COI-5P and ITS variation could be due to incomplete lineage sorting of mitochondrial haplotypes but, as with *C. variegata*, more sampling and molecular work (in particular, more ITS data) are necessary to address this issue. A morphological and anatomical examination (see "*Morphological observations and taxonomic changes*" section) revealed no obvious distinctions between individuals assigned to these genetic clusters and so we also retain *C. edentata* as a single species, again pending further study.

Five alignments were analysed to assess phylogenetic relationships among the included *Callophyllis* spp.: global LSU, local LSU, EF2, reduced COI-5P and a combined alignment of LSU + EF2 + COI-5P. Presented here is the phylogram inferred from Bayesian analyses of the LSU + EF2 + COI-5P combined alignment (−LnL = 14797) (Fig. 2) with posterior probabilities, as well as SH-like aLRT and bootstrap support values from the ML analyses appended.

In the global LSU analysis, *Callophyllis* species resolved as a monophyletic lineage (including the generitype *C. variegata*) with full support. *Austrophyllis* resolved with full support as sister to *Callophyllis*, with *Thamnophyllis* and *Pugetia* the next most closely related taxa, respectively, and these taxa were therefore chosen as the outgroup for analyses of the remaining alignments. For the combined alignment, two main lineages of *Callophyllis* species from the Northeast Pacific were recovered with moderate to full support. The first contained *C. beringensis*, *C. flabellulata, C. schneideri, C. thompsonii, C. variegata* and *C. violacea,* while the second included *C. crenulata, C. edentata, C. heanophylla, C. pinnata* and *C. radula* (Fig. 2). Within the first lineage (the "*C. thompsonii*" lineage), *C. violacea* and *C. flabellulata* were closely allied with full support; other relationships within the lineage were recovered with full support in Bayesian analyses but weak to full support in ML analyses. In addition, species in this lineage were monocarpogonial (except that for *C. schneideri* there are no data). Within the second lineage (the "*C. edentata*" lineage), *C. crenulata* and *C. pinnata* were closely allied with full support, *C. heanophylla* and *C. radula* were closely allied with moderate to full support, and *C. edentata* was recovered with full support as sister to the two previous species pairs. In addition, species in this lineage were polycarpogonial (there are no data for *C. radula*). *Callophyllis rangiferina* resolved as an early divergence within *Callophyllis* with moderate support in Bayesian analyses; in ML analyses was either

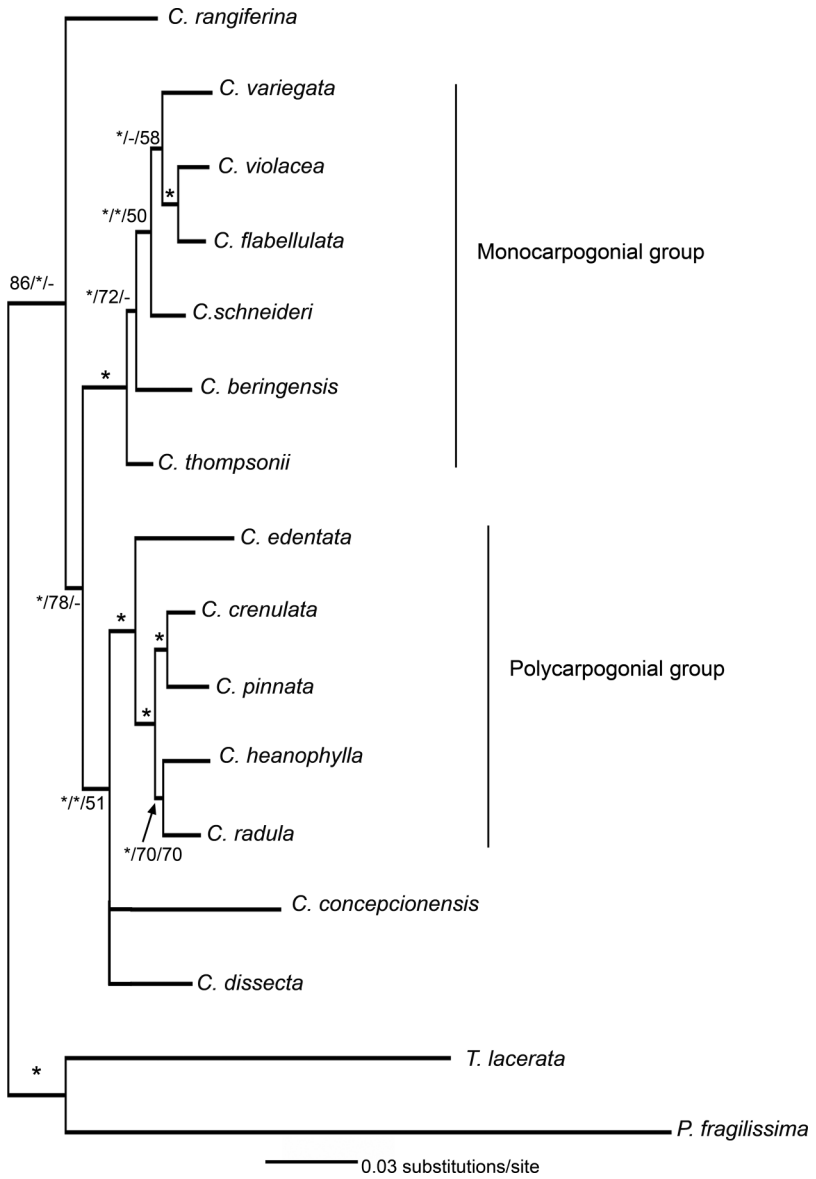


Fig. 2. Phylogenetic relationships within the genus *Callophyllis* inferred from Bayesian analyses of the combined LSU, EF2 and COI-5P alignment. Support values are listed as Bayesian posterior probabilities and SH-like aLRT and bootstrap values for maximum likelihood analyses. Asterisks denote nodes that are strongly supported (posterior probability, SH-like aLRT values and bootstraps ≥ 95%) in all analyses.

recovered as an early divergence with moderate support or was weakly allied with the *C. thompsonii* clade, the latter consistent with monocarpogony in *C. rangiferina* (Womersley, 1994). *Callophyllis dissecta* and *Callophyllis* sp. Chile resolved within *Callophyllis* with full support in all analyses. However, their positions within the genus were unresolved because, depending on the analysis, they grouped with either the monocarpogonial or polycarpogonial group. Unfortunately, none of the specimens of either *C. dissecta* or *Callophyllis* sp. Chile were female gametophytes, so we could not determine the number of carpogonial branches per supporting cell.

The local LSU alignment contained four additional species (*C. lambertii, C. cervicornis*, *C. odonthalioides* and *C. linearis*) for which COI-5P and/or EF2 sequences could not be acquired. *Callophyllis lambertii* (monocarpogonial: Womersley, 2004) allied strongly with *C. rangiferina* in all analyses. *Callophyllis cervicornis* (also monocarpogonial: Womersley, 1994) was moderately supported as sister to *C. lambertii* and *C. rangiferina* in ML analyses, but its position within the genus was unresolved in Bayesian analyses. *Callophyllis odonthalioides* grouped with *C. variegata* with moderate support in all analyses. *Callophyllis linearis* grouped with the other *Callophyllis* species with moderate support, but the position of this species relative to other members of the genus was unresolved. In addition, the local LSU alignment contained two sequences each from *C. variegata* and *C. edentata* representing individuals with the highest COI-5P intraspecific

divergence values for each species. For both species the conspecific sequences grouped together with full support and were less divergent than congeneric sequences.

Morphological observations and taxonomic changes

Callophyllis Kützing 1843, p. 400

Revised description: Plant flat or canaliculate, branched, with margins smooth, crenulate or proliferous. Holdfast small (~ 1 mm) with either no stipe or a short (1–2 mm) stipe. One to several blades expand abruptly from the holdfast, which can make the plant looked clumped or bushy. Medulla composed of multiple layers of relatively large, round to oval, unpigmented cells interspersed with filaments of smaller pigmented cells. Cortex 2–3 layers. Carpogonial branches situated just below the cortex, extending into the medulla. Monocarpogonial or polycarpogonial with one to three single-celled subsidiary filaments on the supporting cell. Supporting cell, subsidiary cells, and first cell of carpogonial branch elongate and lobed. Postfertilization development procarpic. Mature cystocarps with or without ostioles, umbonate or round, protruding from the thallus, scattered over the surface of the blade or predominantly localized along branch margins.

Lectotype species: *Callophyllis variegata* (Bory de Saint-Vincent) Kützing

Callophyllis variegata (Bory de Saint-Vincent) Kützing 1843, p. 401

(Figs 3, 4)

Basionym: *Halymenia variegata* Bory de St. Vincent 1828.

Type locality: Valparaiso, Chile.

Representative specimen: GWS000515 (Fig. 3). Collected from Los Chonos, Chile (Table S1).

Representative DNA barcode: JX034431.

Confirmed distribution: Thus far only reported with certainty from Chile, California, U.S.A. and British Columbia (Vancouver Island and Haida Gwaii), Canada (Table S1). Also reported (not confirmed genetically) throughout South America (e.g. Kylin & Skottsberg, 1919) and from Alaska, U.S.A. (Lindstrom, 1977), Africa (Silva *et al.*, 1996), Antarctica (e.g. Kylin & Skottsberg, 1919) and the Indo-Pacific (e.g. Adams, 1994, Silva *et al.*, 1996).

Habit and vegetative anatomy: Of the 23 *C. variegata* specimens examined during this study, most were collected in the subtidal (5–15 m depth) or as drift, with a single specimen collected from the low intertidal in California. Specimens were found growing on rock or occasionally on red algae. Plants were often found subtidally growing as single blades and intertidally growing in bushy clumps. Plants were 3–9 cm in height, with 5–12 orders of branching and smooth margins (Fig. 3, 4). Branch width generally decreased from basal branches (2–10 mm) towards the apices (1–4 mm), with acute or irregularly rounded terminal branches (0.25–1 mm wide × 0.25–1 mm long). Blades were 170–240 μm thick near the apex.

Reproductive anatomy: Female gametophytes and tetrasporophytes were not observed during this study. Spermatangia were produced from spermatangial mother cells in the outer cortex throughout younger regions of blades.

Additional observations: A morphological and anatomical examination of *C. variegata* specimens revealed no definitive differences among the COI-5P genetic clusters (Fig. 1) or for specimens collected from the Northern versus the Southern hemisphere. *Callophyllis variegata* appears to be a widely distributed species along the Eastern Pacific coast, however, the occurrence of this species in other oceans has yet to be confirmed genetically.

As part of a separate study we have generated COI-5P sequence data for 26 Chilean (type region) isolates field-identified as *C. variegata* and, not surprisingly, uncovered overlooked diversity in the form of three genetically–divergent clusters (Buschman & Saunders, unpublished data). However, only one of these genetic species ($n = 6$) actually resolves with the genus *Callophyllis* in phylogenetic analyses, the other two groups forming an independent species pair that may represent a new genus (possibly including *Callophyllis macrostiolata*, which was only remotely allied to *bona fide Callophyllis* spp. in the phylogeny of Arakaki *et al.* 2011). Anatomical observations link our concept of *C. variegata* with that of Arakaki *et al.* (2011), although our plants are shorter and thicker. Unfortunately, attempts to borrow the type material to confirm the identifications here and of Arakaki *et al.* (2011) were unsuccessful, as the type could not be located (L. Le Gall, personal communication). Norris (1957) did examine an isotype in UC, but provided no details of the vegetative anatomy. However, the gross morphology presented (Norris, 1957, plate 35), as well as the original description (Bory, 1828) are a strong match to our collections. For now, we are content to consider our British Columbian collections as representative of *C. variegata* and accept the possibility that a name change for Canadian collections may be necessitated following a detailed molecular and morphological assessment of the Chilean flora.

Monocarpogonial group

Callophyllis schneideri* Clarkston & G.W. Saunders, *sp. nov.

(Figs 5–8)

Description: Plants usually subtidal. Thalli erect, flat and flabellately to irregularly branched, 2–6 cm in height with 5–11 orders of branching. Often coated with invertebrates (e.g. bryozoans).

Diagnosis: Distinguished from other species of *Callophyllis* by COI-5P and ITS sequence data.

Holotype: G.W. Saunders and K. Dixon, June 7, 2010 (UNB GWS020918; vegetative) (Fig. 5; Table S1).

Type locality: Indian Head (Lat. 53.24805, Long. –131.98369), Skidegate, Haida Gwaii, B.C., Canada, subtidal (10 m), on rock.

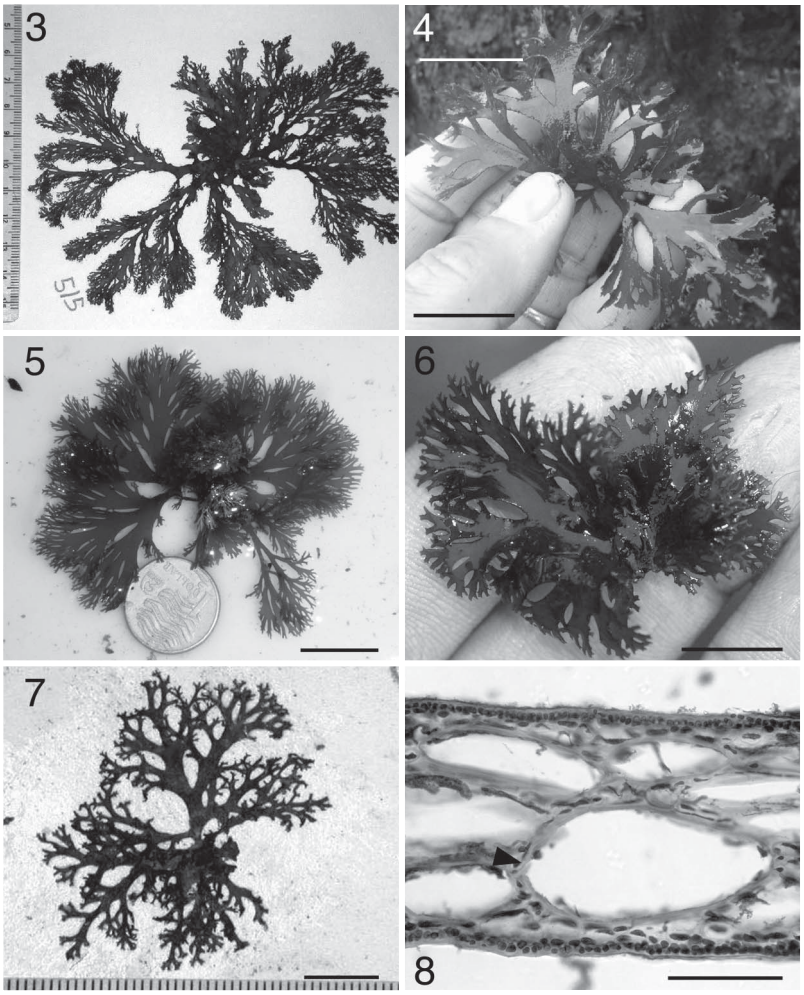
Holotype DNA Barcode: HQ545179.

Representative DNA Barcodes: See Table S1.

Etymology: Named for Dr. Craig W. Schneider in honour of his numerous contributions to red algal taxonomy.

Distribution: Thus far only reported with certainty from along the coast of British Columbia, Canada and from Pacific Grove, California, U.S.A. (See Table S1).

Habit and vegetative anatomy: Of the 63 specimens collected during this study, the majority were collected in the subtidal (3–17 m depth) or as drift, with a single specimen (GWS005117) collected from the low intertidal in northern British Columbia (Table S1). Specimens were found growing on rock, algae (often *Ahnfeltia*) or invertebrates (e.g. sponges, polychaete worm tubes).



Figs 3–8. Gross morphology of the *Callophyllis* generitype, *C. variegata* (Figs 3, 4) and morphology and anatomy of *C. schneideri sp. nov.* (Figs 5–8). **3.** Representative specimen of C. *variegata* (GWS000515). **4.** *Callophyllis variegata* specimen with fewer orders of branching (GWS013172). **5.** *Callophyllis schneideri* holotype (GWS020918; vegetative). **6, 7.** Fresh specimen of GWS005113 and pressed voucher for GWS006428, respectively, showing variation in blade morphology. **8.** Vegetative cross-section near apex of plant showing medulla with large, unpigmented cells and filaments of small, pigmented cells (arrowhead) (GWS006428; stained with aniline blue). Scale bars = centimetre ruler (Fig. 3), 2 cm (Figs 4, 5), 1 cm (Figs 6, 7) and 100 üm (Fig. 8).

Plants were found growing either as single blades or, more frequently, as several blades together in a small clump (Figs 5–7). Plants were 2–6 cm in height with 5–11 orders of branching and had smooth or occasionally dentate margins. Branch widths generally decreased from basal branches (1–4 mm) toward the apices (0.25–1 mm). The terminal branches were acute (0.5–1 mm wide × 0.5 mm or long). Blades were 205–230 μm thick near the apex.

The internal anatomy of *C. schneideri* was typical for the genus, with a medulla composed of one to several irregular cell layers, these cells variable in size, round to oval, and unpigmented (Fig. 8). Filaments of small, round to slightly elongate, pigmented cells were interspersed among the larger medullary cells (Fig. 8) and were common throughout the thallus. The cortex was composed of 2–3 layers – an inner layer of round to periclinal cells and 1–2 (mostly 1) outer layers of smaller, round to slightly anticlinal, pigmented cells (Fig. 8).

Reproductive anatomy: None of the *C. schneideri* collections were reproductive.

Additional observations: *Callophyllis schneideri* is generally found in the subtidal and morphologically resembles *C. flabellulata*. It is likely that *C. schneideri* has been overlooked in the past because it is a predominantly subtidal species or because it was mistaken for *C. flabellulata*.

Callophyllis beringensis **Perestenko (1996), pp. 98, 201, pl. 26, fig. 6**

(Fig. 9)

Holotype: J.E. Pterov, July 27, 1962 (herbarium not listed).

Type locality: Cape Chaplino, Russia.

Representative specimen: GWS004471 (Fig. 9). Collected from Bamfield, B.C., Canada (Table S1).

Representative DNA barcode: JX034138.

Confirmed distribution: Thus far only reported with certainty from British Columbia, Canada (Table S1) and the type locality in Russia.

Habit and vegetative anatomy: Of the 74 *C. beringensis* specimens collected during this study, the majority (65) were collected subtidally (5–12 m) from sites around Vancouver Island and Prince Rupert, with the rest (nine) collected intertidally at two locations in northern British Columbia (Prince Rupert and Haida Gwaii). Specimens were found growing on rock or occasionally on kelp stipes or invertebrates.

Plants were typically found with several blades growing together in a small clump or occasionally as single blades and were often coated with a large amount of epiphytic invertebrates (e.g. bryozoans) as compared to other species of *Callophyllis.* Plants were 1.5–5.5 cm in height, with 5–8 orders of branching and smooth margins. Branch widths generally decreased from the basal branches (1–10 mm) towards the apices (0.25–1.5 mm). Several specimens had branches that narrowed abruptly and gave the plant a distinctly stunted appearance (Fig. 9), while others narrowed gradually. The terminal branches were small (0.25–1 mm wide × 0.25–1 mm long). Blades were 120–280 μm thick near the apex, with cystocarpic and male plants thinner (120–170 μm) than tetrasporic plants (200–280. μm).

Reproductive anatomy: Carpogonial branches were monocarpogonial. Mature cystocarps were umbonate with a single ostiole, which protruded prominently from the thallus, and were found mostly along the branch margins, but occasionally in the middle of a branch. Tetrasporophytes and male gametophytes were not observed in this study.

Additional observations: Collecting topotype material from Russia was beyond the scope of this study and the type specimen could not be located. However, our collections are morphologically and anatomically similar to *C. beringensis* as described and illustrated in the type description by Perestenko (1996), in particular with respect to branching order and branch size. We therefore consider our British Columbia collections as representative of *C. beringensis*. The possibility remains, however, that a name change may be necessary for our Canadian collections, following a detailed molecular assessment of the Russian Pacific flora similar to that completed here for our waters.

Callophyllis flabellulata **Harvey (1862), p. 171**

(Figs 10, 11)

Lectotype: D. Lyall, February 1860 (TCD 0011792; cystocarpic). We designate this specimen to be the lectotype of *C. flabellulata*.

Type locality: Esquimalt, B.C., Canada, specimens dredged from 8 fathoms and “cast ashore”.

Representative specimen: GWS004575 (Fig. 10). Collected from Bamfield, B.C., Canada (Table S1).

Representative DNA barcode: JX034285.

Confirmed distribution: Thus far only reported with certainty from British Columbia, Canada, and the Monterey Peninsula, California, U.S.A. (Table S1). Also reported (but not confirmed genetically) from Washington State, U.S.A. (Scagel *et al.*, 1989), Oregon, U.S.A. (Hansen, 1997) and Mexico (Dawson, 1954).

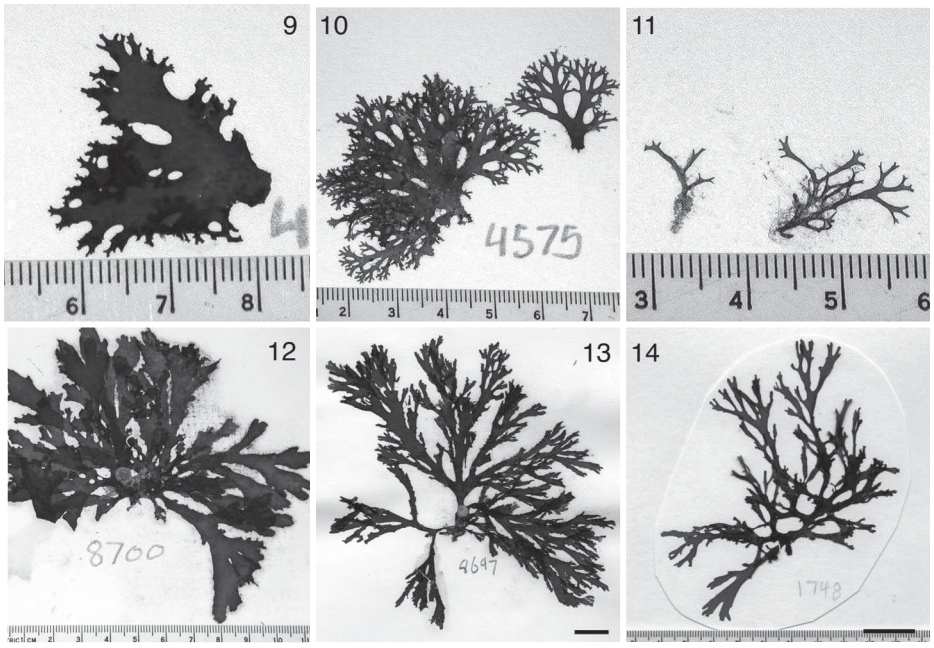
Habit and vegetative anatomy: The 45 *C. flabellulata* specimens collected during this study were collected from the subtidal (5–14 m) from numerous sites around Vancouver Island and one site in California (Table S1). The majority of specimens were found growing on polychaete worm tubes, other invertebrates or algae, with the remainder on rock.

Plants were typically found with several blades growing together in a small bushy clump or rarely as single blades and, as with *C. flabellulata,* plants were often coated with a large amount of epiphytic invertebrates (e.g. bryozoans), compared to other species of *Callophyllis.* Plants were 1–4.5 cm in height, with 6–12 orders of branching and smooth margins (Figs 10, 11). In general, branch widths decreased from basal branches (1–6.5 mm) toward the apices (0.5–1 mm). Several specimens had narrow branches throughout and few orders of branching (Fig. 11). The terminal branches were acute (0.5–1 mm wide × 0.5–1 mm long). Overlapping branches occasionally fused together by extension of the outer cortical cells. Blades were 240–400 μm thick near the apex.

Reproductive anatomy: Carpogonial branches were monocarpogonial. Mature cystocarps were umbonate, with a single ostiole, which protruded prominently from the thallus, and were found mostly along the margins and occasionally in the middle of branches throughout the upper half of the plant. Tetrasporophytes and male gametophytes were not observed in this study.

Additional observations: The type locality of *C. flabellulata* is given only as "Esquimalt", which is a busy harbour municipality in British Columbia that has undergone considerable development since 1862. We did attempt to collect *C. flabellulata* specimens from this area but were unsuccessful. In lieu of topotype material we have chosen a representative specimen that is from another site on Vancouver Island and closely matches the morphological description of this species (see below).

Some confusion exists regarding the type collection of *C. flabellulata* because in Harvey's original description (1862) he did not specify a holotype, but instead discussed several specimens and did not list any collection numbers. Dawson (1954) reported that twelve syntypes of *C. flabellulata* are housed in the Trinity College Herbarium, Dublin (TCD). However, a request to TCD for images of the syntypes resulted in photos of fourteen specimens (TCD 0011790–0011795 and eight unnumbered specimens). All fourteen were collected by David Lyall from the type locality between 1858–1860 and were apparently examined by Harvey when he described *C. flabellulata*. In the description, Harvey specifically mentioned characteristics of the cystocarp and therefore a cystocarpic specimen should be chosen from the syntypes in order to designate a lectotype for this species. Two of the fourteen specimens (TCD 0011791, 0011792) are cystocarpic and, from these two, we chose the one



Figs 9–14. Gross morphology of *Callophyllis* species from the NE Pacific belonging to the monocarpogonial lineage. **9.** Representative specimen of *C. beringensis* with 'stunted' morphology (GWS004471). **10.** Representative specimen of *C. flabellulata* with overall round, fan-shaped appearance (GWS004575). **11.** *Callophyllis flabellulata* specimen showing narrow, relatively unbranched morphology (GWS006536). **12.** Representative specimen of *C. thompsonii*, showing distinctly rounded terminal branches (GWS008700). **13.** Representative specimen of *C. violacea*, showing wide, highly branched morphology (GWS008697). **14.** *Callophyllis violacea* specimen showing narrow, less branched morphology (GWS001748). Scale bars = centimetre ruler (Figs 9–12) and 2 cm (Figs 13, 14).

(TCD 0011792) that best matched Harvey's description of a small, fan-shaped, deep red, subdichtomous or digitate, much divided plant with cystocarps on the surface of the blade or near the margins. We designate this specimen the lectotype of *C. flabellulata*.

Callophyllis thompsonii Setchell (1923), p. 399

(Fig. 12)

Holotype: N.F. Thompson, July 1917 (UC 367784; cystocarpic and tetrasporic).

Type locality: Canoe Island, Washington State, U.S.A., dredged.

Representative specimen: GWS008700 (Fig. 12). Collected from Bamfield, B.C., Canada (Table S1).

Representative DNA barcode: JX034426.

Confirmed distribution: Thus far only reported with certainty from British Columbia, Canada (Table S1), and the type locality in Washington State. Also reported (but not confirmed genetically) from California, U.S.A. (Abbott & Norris, 1966).

Habit and vegetative anatomy: All but one of the eight *C. thompsonii* specimens collected during this study were from one site (Seapool Rock) in Bamfield, British Columbia, with the remaining specimen from northern British Columbia (Table S1). All samples were collected subtidally (8–15 m) and were found growing on rock.

Plants typically consisted of one or two blades and overall lacked a bushy or clumped appearance. Plants were 3–7 cm in height, with 3–6 orders of branching and smooth or dentate margins. In general, branch widths decreased from basal branches (3.5–10 mm) toward the apices (1–6 mm). The terminal branches (1–4 mm wide × 2–5 mm long) often had a distinctive rounded shape (Fig. 12). Blades were 280–320 μm thick near the apex.

Reproductive anatomy: Carpogonial branches were monocarpogonial. Mature cystocarps were umbonate, with a single ostiole, which protruded prominently from the thallus, and were found mostly along the margins and occasionally in the middle of branches throughout the upper half of the plant. Tetrasporophytes and male gametophytes were not observed in this study.

Additional observations: The type locality of *C. thompsonii* is given as Canoe Island, Washington, and collecting in this part of the United States was beyond the scope of this study. Canoe Island is located off the southern end of Vancouver Island, British Columbia, where we collected extensively, but did not find specimens that were either a genetic or morphological match to *C. thompsonii.* Our specimens are from a location on Vancouver Island approximately 225 km from the type locality and are morphologically and anatomically similar to *C. thompsonii* as described by Setchell (as translated from Latin by Abbott & Norris, 1966) and pictured by Dawson (1954, which contains a photograph of the holotype) in terms of branching and the distinctly rounded terminal branches. Thus, we consider our specimens and Setchell's to be the same species.

Our collections were from only two sites, despite extensive sampling throughout British Columbia. Before our study *C. thompsonii* was known only from a few collections around the type locality in Washington State (Abbott & Norris, 1966; as well as one unconfirmed collection from California). These findings suggest that *C. thompsonii* has a narrow distribution and/or habitat range.

All of the *C. thompsonii* specimens stained herbarium paper brown. To the best of our knowledge, this brown discolouration has not been reported previously in the literature for any *Callophyllis* species and may be taxonomically useful.

Callophyllis violacea J. Agardh (1885), p. 34

(Figs 13, 14)

Holotype: Dr. Dimmick (undated) (Agardh Herbarium 25032; cystocarpic).

Type locality: Santa Barbara, California, U.S.A.

Representative specimen: GWS008697 (Fig. 13). Collected from Bamfield, B.C., Canada (Table S1).

Representative DNA barcode: JX034442.

confirmed distribution: Thus far reported with certainty only from the type locality in California, U.S.A. and British Columbia, Canada (Table S1). Also reported (but not genetically confirmed) from Washington State, U.S.A. (Scagel *et al.*, 1989), Oregon, U.S.A. (Hansen, 1997), Florida, U.S.A. (D'Archino *et al.*, 2010) and Mexico (Dawson, 1954).

Habit and vegetative anatomy: Of the 20 *C. violacea* specimens collected during this study, most (14) were collected subtidally (6–12 m) from sites in Bamfield, British Columbia, and the rest (six) were collected intertidally from Bamfield and two other sites on Vancouver Island (Table S1). Specimens were found growing on rock or occasionally on coralline algae.

Plants typically consisted of one or two blades and lacked a bushy or clumped appearance. Plants were 4.5–13 cm in height, with 5–11 orders of branching and smooth or occasionally dentate margins. In general, branch widths decreased from basal branches (2–11 mm) towards the apices (1–5.5 mm) (Figs 13, 14). Several specimens had relatively wide branches overall and a higher order of branching (Fig. 13) compared to the other collections (Fig. 14). The terminal branches (0.25–0.5 mm wide × 0.25–0.5 mm long)

were irregularly rounded to acute and occasionally incised. Blades were 280–330 μm thick near the apex.

Reproductive anatomy: Carpogonial branches were monocarpogonial. Mature cystocarps were umbonate, with one or two ostioles, which protruded prominently from the thallus, and were found mostly along the margins and occasionally in the middle of branches throughout the upper half of the plant. Tetrasporophytes and male gametophytes were not observed in this study.

Additional observations: Collecting topotype material from Santa Barbara, California was beyond the scope of this study. However, we did attempt to collect *C. violacea* from further north in California but were unable to find it in the Monterey and Santa Cruz areas.

Both Dawson (1954) and Abbott & Norris (1966) considered *C. violacea* to be a morphologically variable species and consequently synonymized several other members of the genus with it. Our results indicate that *C. violacea* does display phenotypic variation, particularly with regard to branching characteristics, as several of the specimens are large, with wide branches overall (Fig. 13), while others are smaller in height and have narrower branches (Fig. 14). The narrow specimens strongly resemble a specimen that Dawson (1954) described "as very much like the Agardh type" and we consider this genetic group to be *C. violacea*.

Polycarpogonial group

***Callophyllis crenulata* Setchell (1923), p. 400**

(Figs 15, 16)

Holotype: N.L. Gardner (N.L. Gardner 145, undated) (UC 92775; cystocarpic).

Type locality: Whidbey Island, Washington State, U.S.A.

Representative specimen: GWS004475 (Fig. 15). Collected from Bamfield, B.C., Canada (Table S1).

Representative DNA barcode: JX034178.

Confirmed distribution: Thus far reported with certainty only from Washington State, U.S.A. and British Columbia, Canada (Table S1). Also reported (but not genetically confirmed) from Alaska, U.S.A. (Scagel *et al.*, 1989) and Oregon, U.S.A. (Hansen, 1997).

Habit and vegetative anatomy: Of the 53 *C. crenulata* specimens collected during this study, 23 were collected subtidally (2–15 m) from sites around Vancouver Island and Prince Rupert and Haida Gwaii, in northern British Columbia, 27 were collected intertidally from several locations along the same three coastlines, and three were drift (Table S1). Specimens were found growing on rock or occasionally on other algae.

Plants were often found subtidally growing as single blades and intertidally growing in clumps. Plants were 3–14 cm in height, with 3–7 orders of branching (Fig. 15). Most but not all plants had crenulate margins (Fig. 16). Branch widths were relatively consistent throughout a given plant, from 2–8 mm basally to 1.5–13 mm near the apices. The terminal branches were blunt (1–4 mm wide × 1–7 mm long). Blades were 240–370 μm thick near the apex.

Reproductive anatomy: Carpogonial branches were polycarpogonial. Mature cystocarps were umbonate and protruded slightly to prominently from the thallus, with one ostiole or occasionally no ostiole, and were found scattered throughout the upper half of the plant. Tetrasporophytes and male gametophytes were not observed in this study.

Additional observations: The type locality of *C. crenulata* is given as Whidbey Island, Washington, and collecting in this part of the United States was beyond the scope of this study. However, we collected several *C. crenulata* specimens from the southern end of Vancouver Island, British Columbia, which is near Whidbey Island. These were genetically identical to our chosen representative specimen, GWS004475.

Setchell's original description lists the holotype collection as containing both cystocarpic and tetrasporic plants, while Dawson (1954) mentions that "data on the [holotype] sheet indicates that the specimen chosen as the technical type is a cystocarpic one". Abbott & Norris (1966) considered the "canaliculate nature of the blade segments and the crisp margins" to be diagnostic of the species, but several of the genetically-identified *C. crenulata* specimens had neither character. In addition, several genetically-identified collections of *C. edentata* and *C. dissecta* have crenulate margins, though this character is less common in these species than in *C. crenulata*. We consider this genetic group to be *C. crenulata* based on the common occurrence of canaliculate thalli and crenulate margins, as well as proliferous marginal branches in several specimens (e.g. GWS004415), which is a character present in the holotype of *C. crenulata*.

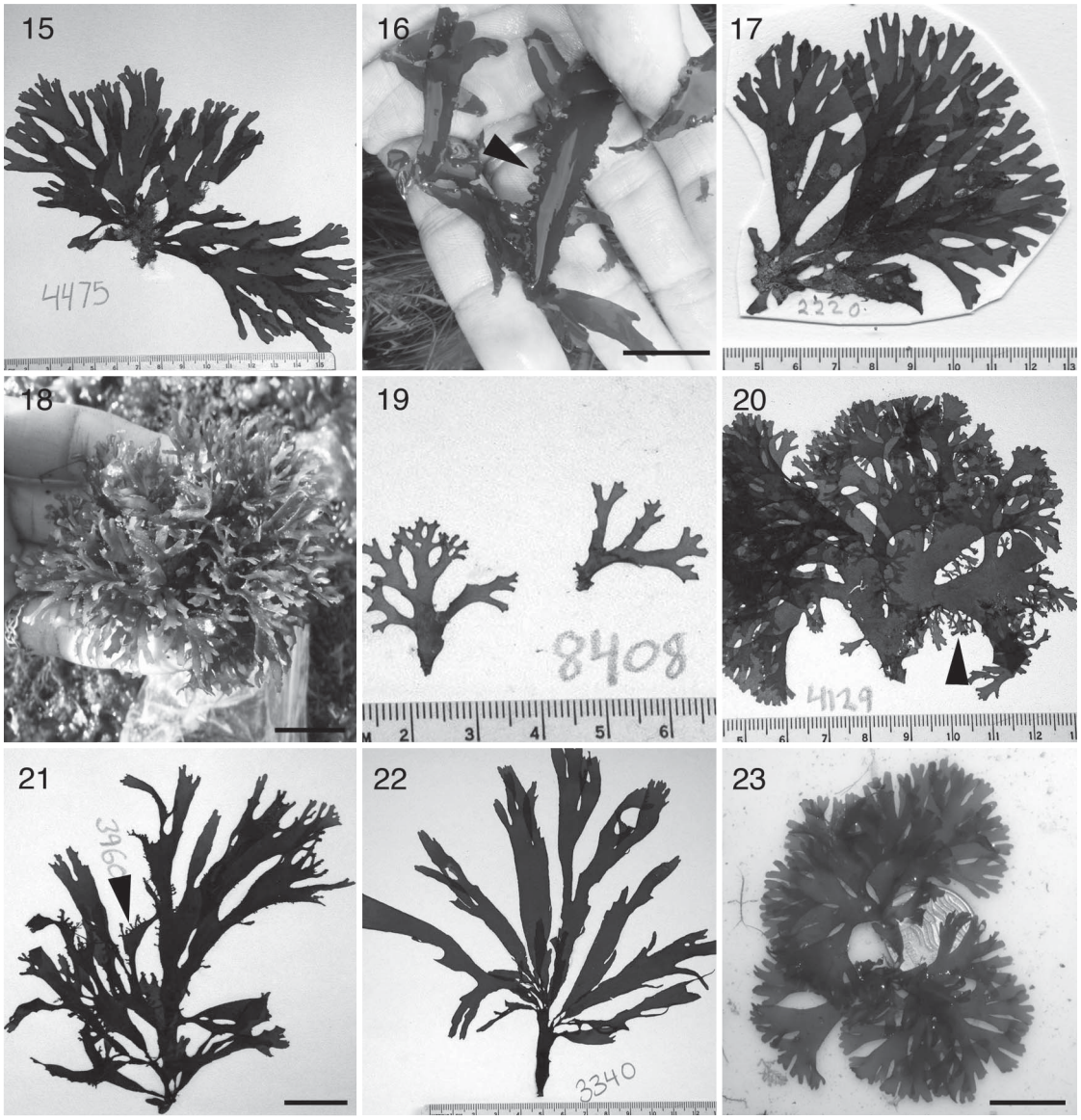
***Callophyllis edentata* Kylin (1925), p. 34**

(Figs 17, 18).

Holotype: Not designated. An isotype in the UC Herbarium is dated July 22, 1924 (Dawson, 1954).

Type locality: Turn Island, Washington State, U.S.A., dredged from 5–10 fathoms.

Representative specimen: GWS002220 (Fig. 17). Collected from Port Hardy, B.C., Canada (Table S1).



Figs 15–23. Gross morphology of *Callophyllis* species from the NE Pacific belonging to the polycarpogonial lineage. **15.** Representative specimen of *C. crenulata* (GWS004475). **16.** *Callophyllis crenulata* specimen showing crenulate margin (arrowhead) (GWS020052). **17.** Representative specimen of *C. edentata* Kylin showing large, flat morphology (GWS002220). **18.** *Callophyllis edentata* specimen showing short, turf-like morphology (GWS020432). **19.** Representative specimen of *C. heanophylla* showing narrow morphology (GWS008408). **20.** *Callophyllis heanophylla* specimen showing wide morphology and proliferous marginal branches (arrowhead) (GWS004129). **21.** Representative specimen of *C. pinnata* with proliferous marginal branches (arrowhead) (GWS003960). **22.** *Callophyllis pinnata* specimen with long ultimate and penultimate branches (GWS003340). **23.** Representative specimen of *C. radula* showing overall flabellate morphology (GWS0020798). Scale bars = centimetre ruler (Figs 15, 17, 19, 20, 22) and 2 cm (Figs 18, 21, 23).

Representative DNA barcode: JX034252.

Confirmed distribution: Thus far only reported with certainty from Washington State and California, U.S.A., and British Columbia, Canada (Table S1). Also reported (but not genetically confirmed) from Alaska, U.S.A. (Lindstrom, 1977), Oregon, U.S.A. (Hansen, 1997) and Mexico (Dawson, 1954).

Habit and vegetative anatomy: Of the 141 *C. edentata* specimens collected during this study, most were collected from the low intertidal, and the rest were subtidal (3–14 m depth) or drift (Table S1). Specimens were found growing on rock or occasionally other algae.

Plants were often found subtidally growing as single blades (Fig. 17) and intertidally growing in clumps (Fig. 18). Plants were 1.5–17.5 cm in height with 5–10 orders of branching and smooth or occasionally crenulate margins. Branch widths were relatively consistent throughout a given plant, from 2.5–13 mm basally to 2.5–9 mm near the apices. The terminal branches ranged from narrow to blunt (1–4 mm

wide × 1–4 mm long). Blades were 320–450 μm thick near the apex.

Reproductive anatomy: Carpogonial branches were polycarpogonial. Mature cystocarps were umbonate with a single ostiole, which protruded prominently from the thallus, and were found scattered throughout the upper half of the plant. Tetrasporophytes and male gametophytes were not observed in this study.

Additional observations: As with *C. crenulata,* collecting from the type locality of *C. edentata* was beyond the scope of this study. However, we collected specimens from nearby on the southern end of Vancouver Island, British Columbia. Many of our collections closely resemble the type specimen of *C. edentata* (Kylin, 1925), as well as Kylin's description of this species as having "small, roundish to obtuse [ultimate lobes]" and "smooth, sometimes finely crisped [margins]", and we consider our collections and Kylin's to be the same species. In addition, we observed that *C. edentata* is highly phenotypically variable and can resemble *C. crenulata* or *C. violacea,* as has been previously reported (Abbott & Norris, 1966).

Callophyllis heanophylla Setchell (1923), p. 401

(Figs 19, 20)

Holotype: N.L. Gardner, July 1910 (UC 651624).

Type locality: Shaw Island, Washington State, U.S.A., dredged from 5–30 fathoms.

Representative specimen: GWS008408 (Fig. 19). Collected from Beaver Island, B.C., Canada (Table S1).

Representative DNA barcode: JX034321.

Confirmed distribution: Thus far only reported with certainty from Washington State and California, U.S.A. and British Columbia, Canada (Table S1). Also reported (but not genetically confirmed) from Alaska, U.S.A. (Lindstrom, 1977) and Oregon, U.S.A. (Hansen, 1997).

Habit and vegetative anatomy: Of the 64 *C. heanophylla* specimens collected during this study, the majority were collected subtidally (5–15 m) from numerous sites around Vancouver Island and the Sunshine Coast, two sites around Haida Gwaii in northern British Columbia and one site in California (Table S1). Two specimens were collected intertidally from two sites in British Columbia and two were drift. Specimens were often found growing on invertebrates (e.g. polychaete worm tubes, tunicates), coralline algae, kelp stipes or rock.

Plants were typically found with several blades growing together in a small clump or occasionally as single blades. Plants were 2–7.5 cm in height, with 3–6 orders of branching and a distinct soft and silky texture (very much like, and at times confused with, local species of *Gloeocladia,* Faucheaceae and Rhodymeniales). Branch margins were either smooth (Fig. 19) or proliferous (Fig. 20). Branch widths were relatively consistent throughout; however, some plants had an overall narrower morphology (~ 1 mm branch widths) (Fig. 19) and others a wider morphology (up to 8 mm branch width) (Fig. 20). The terminal branches were broad (1–2 mm wide × 1–2 mm long). Blades were 100–140 μm near the apex.

Reproductive anatomy: We did not observe carpogonial branches but this species is likely polycarpogonial, as it groups in our phylogenetic analyses with species known to be polycarpogonial (Abbott & Norris, 1966). Mature cystocarps were round, protruded prominently from the thallus, lacked ostioles and were found along the branch margins, in the middle of branches and on the marginal proliferations. Tetrasporophytes were not observed in this study and male gametophytes have not been reported for this species.

Additional observations: Although collecting from the U.S. type locality of *C. heanophylla* was beyond the scope of this study, we did collect a specimen from Galiano Island, a Canadian Southern Gulf island that is part of the same larger archipelago as the American San Juan Islands and close to Shaw Island.

The holotype of *C. heanophylla* consists of two plants, both lacking cystocarps, and Setchell (1923) makes no mention of marginal proliferations in his brief description. Abbott & Norris (1966) described *C. heanophylla* as having a "slippery and soft" texture relative to other *Callophyllis* species. We consider this species group to be *C. heanophylla* based on the distinctive thallus texture, broad, round terminal branches and thallus thickness, which is reported to be thin for *C. heanophylla* compared to other members of the genus (Norris, 1957).

Callophyllis pinnata Setchell & Swezy in Setchell (1923), p. 400

(Figs 21, 22).

Holotype: W.A. Setchell, April 6–7, 1896 (UC 92762; cystocarpic).

Type locality: Duxbury Reef, Bolinas, California, U.S.A., low intertidal on stipe of *Laminaria sinclairii*.

Representative specimen: GWS003960 (Fig. 21). Collected from Bamfield, B.C., Canada (Table S1).

Representative DNA barcode: JX034369.

Confirmed distribution: Thus far only reported with certainty from British Columbia, Canada, and California, U.S.A. (Table S1). Also reported (but not genetically confirmed) from Alaska, U.S.A. (Lindstrom, 1977), Washington, U.S.A. (Scagel

et al., 1989), Oregon, U.S.A. (Hansen, 1997), Mexico (Dawson, 1954) and Chile (Ramírez & Santelices, 1991); this last record, from Chile, is particularly uncertain, given the results here (Fig. 1).

Habit and vegetative anatomy: Of the 25 *C. pinnata* specimens collected during this study, the majority (18) were collected from the lower intertidal from several exposed sites around Vancouver Island and California, with the rest (seven) collected subtidally (6–12 m) from three highly exposed sites on Vancouver Island and Haida Gwaii (Table S1). Specimens were found growing on rock or kelp stipes.

Plants typically consisted of one or two blades and overall lacked a bushy or clumped appearance. Plants were 7.5–24 cm in height, with 3–10 orders of branching and smooth or occasionally proliferous margins (Fig. 21). In general, branch width increased from the basal branches (1.5–10 mm) toward the apices (5–23 mm) (Fig. 22). The terminal branches (1.5–12 mm wide × 2.5–39 mm long) were long, irregularly rounded and often dentate. Blades were 250–270 μm thick near the apex.

Reproductive anatomy: Carpogonial branches were polycarpogonial. Mature cystocarps were round, protruded slightly from the thallus, had a single ostiole and were found scattered throughout the upper half of the plant. Tetrasporophytes and male gametophytes were not observed in this study.

Additional observations: Although we could not collect topotype material of *C. pinnata* from Bolinas, California, we did collect specimens from nearby, in Santa Cruz, California. The holotype of *Callophyllis pinnata* is extremely proliferous (see Dawson, 1954), as were several of our collections; we chose one of these as a representative specimen rather than a California specimen because of the morphological resemblance to the holotype. We assign this genetic group to *C. pinnata* based on the large, wide terminal branches, the polycarpogonial condition, and the occurrence of proliferous branches. *Callophyllis obtusifolia* is reported to be morphologically similar to *C. pinnata,* but is monocarpogonial (Abbott & Norris, 1966); we did not encounter this species in any of our Canadian samplings.

Callophyllis radula **Perestenko (1996), pp. 100, 201, pl. 36, figs 2–4**

(Fig. 23)

Holotype: A. Popov, 1931 (herbarium not listed).

Type locality: Kamchatka, Russia.

Representative specimen: GWS020798 (Fig. 23). Collected from Haida Gwaii, B.C., Canada (Table S1).

Representative DNA barcode: HQ545092.

Confirmed distribution: Thus far only reported with certainty from British Columbia, Canada (Table S1), and the type locality in Russia.

Habit and vegetative anatomy: The 25 *C. radula* specimens were collected subtidally (5–12 m depth) from five sites on Vancouver Island and two sites in Haida Gwaii and were found growing on rock, invertebrates or red algae (Table S1).

Plants were flat with smooth margins, 3–4.5 cm in height, with 3–4 orders of branching. Plants were often found with several blades growing together in a small clump or rarely as single blades. Branch widths were relatively consistent throughout, from 2.5–7mm basally to 2.4–5 mm toward the apices (Fig. 23). Terminal branches were blunt or occasionally acute. Blades were *ca.* 100 μm thick near the apex, however, only one specimen was measured as members of this species rehydrated poorly, which made intact sections difficult to obtain.

Reproductive anatomy: Carpogonial branches were polycarpogonial. Mature cystocarps were round, protruded prominently from the thallus, and were found scattered throughout the middle to lower half of the plant. Tetrasporangia were cruciately divided and scattered throughout the cortex.

Additional observations: As with *C. beringensis,* collecting topotype material from Russia was beyond the scope of this study and the type specimen could not be located. Perestenko (1996) described *C. radula* as 3–4 cm tall, 145–315 μm thick and resembling *C. heanophylla* in morphology. Our collections match hers in thallus size and general resemblance to *C. heanophylla,* though our collections are slightly smaller in blade thickness. Based on the above features we assign this species group to *C. radula.* However, as with *C. beringensis* (see above), the possibility remains that a name change may be necessary for our Canadian collections, following a detailed assessment of the Russian Pacific flora.

Species unresolved in phylogenetic analyses

Callophyllis dissecta **Setchell & Swezy in Setchell (1923), p. 401**

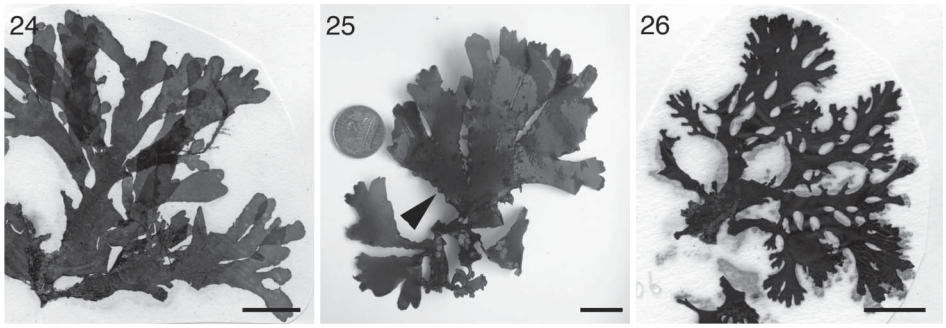
(Figs 24, 25).

Holotype: H.D. Johnston, August, 1898 (UC 92776; cystocarpic).

Type locality: San Pedro, California, U.S.A.

Representative specimen: GWS001743 (Fig. 24). Collected from Haida Gwaii, B.C., Canada (Table S1).

Representative DNA barcode: JX034184.



Figs 24–26. Gross morphology of *Callophyllis* species from the NE Pacific with unresolved positions in our phylogenetic analyses. **24.** Representative specimen of *C. dissecta* showing large, wide branches with bluntly rounded termini (GWS001743). **25.** *Callophyllis dissecta* specimen with margins (arrowhead) (GWS022367). **26.** Representative specimen of *C. odonthalioides* showing blackened stains left on the herbarium paper by the dried plant (GWS001706). Scale bars = 1 cm.

Confirmed distribution: Thus far reported with certainty only from the type locality as well as Pacific Grove, California, U.S.A. and Haida Gwaii, British Columbia, Canada (Table S1). Also reported (but not genetically confirmed) from Mexico (Dawson, 1954).

Habit and vegetative anatomy: Of the six *C. dissecta* specimens collected during this study, two were collected subtidally (20 m) from Haida Gwaii in British Columbia, and four were collected subtidally (5–11 m) from Pacific Grove, California (Table S1). Specimens were found growing on rock. This species appears to be rare, at least for the sites and seasons we collected.

Plants were found either with several blades growing together in a small clump or as single blades. Plants were 6–11 cm in height, with 2–6 orders of branching. Specimens from British Columbia had smooth or dentate margins (Fig. 24) while specimens from California were crenulate (Fig. 25). Branch widths were relatively consistent throughout, from 11–13 mm basally to 8–21 mm near the apices. Terminal branches were blunt (6–9 mm wide × 6–9 mm long).

Reproductive anatomy: Female gametophytes and tetrasporophytes were not observed during this study. Male gametophytes produced small, colourless spermatangia (~ 2 μm) from spermatangial mother cells in the outer cortex throughout younger regions and possibly only on one side of the blade.

Additional observations: While we could not collect topotype material of *C. dissecta* from San Pedro, California, we did collect specimens from Monterey, California. We chose our representative specimen for this species because it closely resembles the original morphological and anatomical description (Setchell, 1923), in particular with respect to the large, round terminal branches; we provisionally consider our collections and Setchell's to be conspecific.

***Callophyllis odonthalioides* Setchell (1923), p. 399**

(Fig. 26)

Holotype: C.L. Anderson (undated) UC 367783; cystocarpic.

Type locality: Santa Cruz, California, U.S.A.

Representative specimen:GWS001706 (Fig. 26). Collected from Haida Gwaii, B.C., Canada (Table S1).

Confirmed distribution: Thus far only reported with certainty from the Haida Gwaii Islands in British Columbia, Canada, and the type locality in Santa Cruz, California (Table S1). There appears to be a disjunct distribution from the type locality in California to northern British Columbia, a biogeographical pattern that we are uncovering with some regularity among seaweeds (Saunders, unpublished observations).

Habit and vegetative anatomy: The five *C. odonthalioides* specimens collected during this study were collected from the subtidal (12–20m) from two sites on Haida Gwaii in northern British Columbia (Table S1). Specimens were found growing on rock. This species appears to be rare, at least for the sites and seasons we collected.

Plants typically consisted of one or two blades and overall lacked a bushy or clumped appearance. Plants were red when collected, but unlike other *Callophyllis* species, they turned a deep red-to-black colour when dried. Plants were 4–17.5 cm in height, with 7–13 orders of branching and smooth or occasionally dentate margins. In general, branch widths decreased from the basal branches (5–11 mm) toward the apices (2–4 mm) (Fig. 26). The terminal branches were round (1–3 mm wide × 1–3 mm long) and occasionally dentate. Blades were 170–210 μm thick near the apex.

Reproductive anatomy: None of the *C. odonthalioides* collections were reproductive.

Additional observations: We collected from the type locality of Santa Cruz, California but were unable to

find specimens that matched *C. odonthalioides,* either genetically or morphologically, and other topotype material was not available for this study. The disjunct distribution (northern British Columbia and California) may be a result of insufficient sampling and needs to be further investigated.

Abbott & Norris (1966) reported that most of the collections they identified as *C. odonthalioides* turned black when dried – the only such report we could find in the literature for this genus – yet despite this distinctive feature they synonymized *C. odonthalioides* with *C. flabellulata.* In his original description of *C. odonthalioides,* Setchell (1923) did not mention a colour change, but his description is quite brief and lacking in detail. We assign our genetic species group to *C. odonthalioides* because all of these specimens turned black when dried, a feature we did not observe in any other members of the genus.

Although we were unsuccessful at amplifying either the COI-5P and EF2 markers for any of the isolates of *C. odonthalioides*, for one specimen (GWS001706) we were able to sequence the entire LSU region used in this study (~2840 bp) and for several other specimens we were able to acquire a shorter portion of the LSU, the D2/D3 region (~860 bp). Based on the LSU data, we are reasonably confident in assigning the following specimens to this species: GWS001707, GWS001732, GWS001734, GWS001735 and GWS001745 (Table S1; see Clarkston & Saunders, 2010).

Discussion

For the genus *Callophyllis,* numerous vegetative (e.g. plant size, branching pattern, branch width) and reproductive characteristics (e.g. ostiole number and shape, mono- vs. polycarpogony) and combinations of these have been proposed over the years for delimiting species (e.g. Setchell, 1923; Dawson, 1954; Abbott & Norris, 1966). Abbott & Norris (1966) reported the high morphological variability found in many *Callophyllis* species, and here we conclude that most of the morphological characters typically used for species delimitation must be used in conjunction with molecular data for reliable identification. Nonetheless, the number of carpogonial branches per supporting cell (monocarpogony vs. polycarpogony) does appear to differentiate two evolutionary lineages within the genus, as has been suggested previously (Abbott & Norris, 1966). Thus, when attempting to identify an unknown specimen, mono- vs. polycarpogony can be useful for quickly narrowing down the number of potential species matches. However, this requires the unknown specimen to be in a particular life history stage, namely a female gametophyte. For routine identification purposes, diagnostic features should be present in all life history stages and finding such features is what we attempted to accomplish in this study.

Existing species names should be assessed and utilized when appropriate for unnamed genetic species groups. However, there comes a point when the energy expended in assessing all available species names outweighs the benefits. For example, there are *ca.* 54 species of *Callophyllis* worldwide, and many more currently synonymized names that could be applied to an unnamed genetic group. In addition, most *Callophyllis* species described in the past have been based on only a few highly variable morphological features and many of the written descriptions are so brief and uninformative that they are basically useless for deciding among potential matches. Borrowing the type specimens for every potential match would represent a considerable time investment and one that would not guarantee success. The only definitive way to assign available names to genetic groups.would be to acquire sequences from type specimens; however, some researchers consider that current DNA extraction and amplification methodologies applied to archival material may be unreliable because the aged and often degraded nature of the material increases the chance of amplifying a contaminant (Saunders & McDevit, 2012). For the purposes of this study, we adopted a pragmatic solution, which was to apply a valid and demonstrably appropriate name to each of our genetic groups. In the future, we hope to sequence fresh collections from more of the type localities in order to genetically 'test' our species assignments, which, while still not definitive, might lend support to our taxonomic conclusions.

Abbott & Norris (1966) suggested that geographically small-scale studies, such as the one they conducted in the Pacific North-East, are a necessary first step towards a global assessment of *Callophyllis* and we agree. We believe that our study is such a step forward because we have provided an indication of how many species are actually present and a link between traditional taxonomic concepts and modern genetic data. This link is essential for increasing the accessibility of taxonomy to researchers around the world and is the only way to truly assess *Callophyllis,* or any taxonomic group, on a global scale.

Key to species of *Callophyllis* in Canada (*C. dissecta* is poorly known and not included)

1. Plant typically < 5 cm in height **2**

1. Plant typically > 5 cm in height **5**

2. Plants with 3–4 orders of branching; closely resembling the broad morph of *C. heanophylla* but not feeling silky; polycarpogonial; rare in British Columbia; reliably distinguished by DNA only..... ..... ***C. radula***

2. Plants with 5 or more orders of branching; monocarpogonial or polycarpogonial; common or rare in British Columbia..... **3**

3. Branches narrowing abruptly near apices, making terminal branches appear stunted; 5–8 orders of branching; monocarpogonial; common in the subtidal in British Columbia, as well as intertidal in northern British Columbia..... ..... ***C. beringensis***

3. Branches narrowing gradually; terminal branches acute; 6–12 orders of branching; monocarpogonial or polycarpogonial..... **4**

4. Overlapping branches may fuse; monocarpogonial; subtidal only; reliably distinguished from *C. schneideri* only by DNA..... ***C. flabellulata***

4. Overlapping branches not reported to fuse; likely monocarpogonial; common in subtidal throughout British Columbia, also rare in the intertidal in northern British Columbia; reliably distinguished from *C. flabellulata* by DNA only.. ..... ***C. schneideri***

5. Plant turning black when dried; subtidal; found only in northern British Columbia.… ***C. odonthalioides***

5. Plant not turning black when dried **6**

6. Herbarium paper under plant turning brown; subtidal only; rare in British Columbia ……..... ..... ***C. thompsonii***

6. Herbarium paper not stained brown..... **7**

7. Terminal branch segments > 1 cm wide; branch widths typically increasing toward apices; margins smooth or proliferous; polycarpogonial; uncommon in British Columbia..... ..... ***C. pinnata***

7. Terminal branch segments <1 cm wide; branch widths typically decreasing toward apices; margins smooth, proliferous or crenulate..... **8**

8. Plant with a distinct silky texture; margins smooth or proliferous but not crenulate; plant < 150µm thick; branch widths relatively constant from base to apex (1–8 mm) with individual plants having either a 'broad' or 'narrow' morph; common in subtidal, rare in intertidal..... ***C. heanophylla***

8. Plant with a smooth but not silky texture; > 200 µm thick; branch margins smooth or crenulate **9**

9. Branch widths 2–8 mm at base to 1.5–13 mm near apices; terminal branches blunt (1–4 mm wide × 1–7 mm long); polycarpogonial; relatively common; reliably distinguished from *C. edentata* by DNA only..... ..... ***C. crenulata***

9. Branch widths 2.5–13 mm at base to 2.5–9 mm near apices; terminal branches blunt or narrow (1–4 mm wide × 1–4 mm long); polycarpogonial; extremely common in British Columbia; reliably distinguished from *C. crenulata* and *C. violacea* by DNA only..... ***C. edentata***

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Supplementary information

The following supplementary material is accessible via the Supplementary Content tab on the article's online page at http://

Table S1. List of examined specimens and accession numbers in GenBank.

Alignment of LSU sequences.

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