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Applying DNA barcoding to red macroalgae: a preliminary appraisal holds promise for future applications

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Marine macroalgae, especially the Rhodophyta, can be notoriously difficult to identify owing to their relatively simple morphology and anatomy, convergence, rampant phenotypic plasticity, and alternation of heteromorphic generations. It is thus not surprising that algal systematists have come to rely heavily on genetic tools for molecular assisted alpha taxonomy. Unfortunately the number of suitable marker systems in the three available genomes is enormous and, although most workers have settled on one of three or four models, the lack of an accepted standard hinders the comparison of results between laboratories. The advantages of a standard system are obvious for practical purposes of species discovery and identification; as well, compliance with a universal marker, such as *cox1* being developed under the label 'DNA barcode', would allow algal systematists to benefit from the rapidly emerging technologies. Novel primers were developed for red algae to PCR amplify and sequence the 5′ *cox1* 'barcode' region and were used to assess three known species-complex questions: (i) *Mazzaella* species in the Northeast Pacific; (ii) species of the genera *Dilsea* and *Neodilsea* in the Northeast Pacific; and (iii) *Asteromenia peltata* from three oceans. These models were selected because they have all caused confusion with regards to species number, distribution, and identification in the field, and because they have all been studied with molecular tools. In all cases the DNA barcode resolved accurately and unequivocally species identities and, with the enhanced sampling here, turned up a variety of novel observations in need of further taxonomic investigation.

Keywords: *cox1*; cryptic species; DNA barcode; Florideophyceae; mitochondrial DNA; Rhodophyta

1. INTRODUCTION

From the student taking an introductory course in Phycology to the seasoned field biologist, there is a common, at times overwhelming, frustration when tasked with the identification of many macroalgal species. Even for the experienced systematist confronted with exclusively vegetative material—reproduction often betraying the ordinal and familial affinities of a collection—or even with reproductive material among species in a genus, accurate identification can remain elusive. This frustrating situation derives from a few commonalities of marine macroalgae that tend to confound attempts at identification viz., simple morphology and anatomy, rampant convergence (in part owing to the previous), remarkable degrees of phenotypic plasticity in response to environmental factors, and incompletely understood life histories with alternation of heteromorphic generations.

In light of the previous it is not unexpected that algal systematists have, for close to two decades, come to rely increasingly on molecular tools to resolve and identify species (see Harper & Saunders 2001). Examples include the internal transcribed spacer of the ribosomal cistron (ITS; Tai *et al.* 2001; Ross *et al.* 2003), the rubisco operon (*rbcL*; Hughey *et al.* 2001), and variable portions of the large subunit of the ribosomal cistron (LSU; Saunders & Lehmkuhl in press). The previous examples serve to highlight an unsatisfactory shortcoming in the current efforts among algal systematists—the lack of a universally applied marker has resulted in multiple, independent, and not easily comparable systems being used. Although there is ample justification for the development of multiple and divergent molecular markers for phylogenetics, agreement on a standard marker for the purposes of quick and accurate species identification would be a powerful tool for the practising taxonomist.

Genetic barcoding has championed the use of the mitochondrial marker cytochrome oxidase subunit I (*cox1*). In a pair of landmark publications Hebert *et al.* (2003*a*,*b*) established the utility of *cox1* as the 'core of a global bioidentification system for animals'. These authors reported that, for a wide variety of animal species at least, this gene could be used to assign unknown species to higher-level taxa, and where comprehensive *cox1*-5′ databases were established species level assignments were possible. The authors clearly articulate the power of this approach to species identification when phenotypic plasticity is a concern, morphology-based keys are only useful for particular stages in the life history or stages are unknown, or cryptic species are likely to be an issue—all of these, as noted above, considerations where macroalgae are concerned. Hebert *et al.* (2003*a*,*b*) justify the choice

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One contribution of 18 to a Theme Issue 'DNA barcoding of life'.

Table 1. List of samples for which *cox1*-5′ sequences were determined in this study.

order/family	species	voucher	collection details	Genbank
Ceramiales				
Ceramiaceae	*Ptilota serrata* Kützing	GWS002173	Vancouver I., BC, Canada. GWS	AY970640
Gigartinales				
Cystocloniaceae	*Rhodophyllis* sp.	GWS001945	Australia. GWS & R. Withall	AY970627
Dumontiaceae	'*Dilsea* Exposed'	GWS002248	Vancouver I, BC, Canada. GWS	AY970638
	Dilsea californica (J. Agardh) Kuntze	GWS001671/1687/1689	Vancouver I., BC, Canada. GWS	AY970573/572/571
	Dilsea californica	GWS002171/2238/2252	Vancouver I, BC, Canada. GWS & C. Lane	AY970636/639/637
	'*Dilsea*(?) Exposed'	GWS002283	Vancouver I, BC, Canada. GWS	AY970582
	Dilsea carnosa (Schmidel) Kuntze	GWS000746	Northern Ireland. C. Maggs	AY971151
	Dilsea carnosa	GWS001216	France. GWS	AY970635
	Dilsea integra (Kjellman) Rosenvinge	GWS001850	Nunavut, Canada. GWS	AY970633
	Dilsea integra	GWS002334	Cape Breton, NS, Canada. GWS	AY970634
	Dumontia contorta (Gmelin) Ruprecht	CSM005A-C	Cape St Marys, NS, Canada. GWS	AY971154/ 155/ 156
	Dumontia contorta	GWS001815	Ireland. GWS	AY970583
	Dumontia contorta	GWS002137-2141	Plymouth, England, UK. C. Maggs	AY971157/158/ 147/148/149
	Dumontia contorta	GWS002142-2146	Portaferry, N. Ireland, UK. C. Maggs	AY971150/159/ 160/161/152
	Dumontia contorta	PC004A-C	Peggys Cove, NS, Canada. GWS	AY970568/569/570
	Dumontia simplex Cotton	GWS000209	Alaska, USA. S. Lindstrom	AY971153
	Neodilsea borealis (Abbott) Lindstrom	GWS001681/1683/2176	Vancouver I, BC, Canada. GWS	AY970614/626/625
	'Neodilsea borealis' Exposed	GWS002232/2281/2282	Vancouver I, BC, Canada. GWS	AY970617/615/616
	Neodilsea natashae Lindstrom	G0224	Alaska, USA. S. Lindstrom	AY970624
Gigartinaceae	*Chondrus crispus* Stackhouse	PC001B	Peggys Cove, NS, Canada. GWS	AY970567
	Mazzaella affinis (Harvey) Fredericq	GWS001333/2259	Vancouver I, BC, Canada. GWS	AY970577/578
	Mazzaella affinis	GWS002256	Vancouver I, BC, Canada. C. Bates	AY970576
	Mazzaella flaccida (Setchell *et* Gardner) Fredericq	GWS002235/2245	Vancouver I, BC, Canada. GWS & C. Lane	AY970575/574
	Mazzaella laminarioides (Bory) Fredericq	GWS000131	Chacao, Chile. B. Rudolph	AY970593
	Mazzaella linearis (Setchell *et* Gardner) Fredericq	GWS000910C/1173A–D/F–J	Vancouver I, BC, Canada. GWS	AY971162/ 592–584
	Mazzaella oregona (Doty) Hughey, P.C. Silva *et* Hommersand	GWS002199/2258	Vancouver I, BC, Canada. GWS	AY970602/603
	Mazzaella parksii (Setchell *et* Gardner) Hughey *et al.*	GWS001115	Vancouver I, BC, Canada. GWS	AY970601
	Mazzaella rosea (Kylin) Fredericq	GWS001109	Vancouver I, BC, Canada. GWS	AY970600
	Mazzaella sanguinea (Setchell *et* Gardner) Hommersand	GWS001146/1165	Vancouver I, BC, Canada. GWS	AY970598/599
	Mazzaella splendens (Setchell *et* Gardner) Fredericq	GWS001128/1173E/ 1174A–J/1175A–C, E, F, H–J	Vancouver I, BC, Canada. GWS	AY970597- 594/ 613–604/ 623–618

(*Continued.*)

Table 1. (*Continued.*)

order/family	species	voucher	collection details	Genbank
Rhodymeniales				
Faucheaceae	*Leptofauchea pacifica* Dawson	GWS001708/1713/2226/JD032	Vancouver I, BC, Canada. GWS	AY970580/579/581/566
Rhodymeniaceae	*Asteromenia peltata* (Taylor) Huisman *et* Millar Bermuda	GWS001252	Bermuda, GWS	AY970560
	Asteromenia peltata Bermuda	CL033401/402	Bermuda, C. Lane & C. Schneider	AY970564/565
	Asteromenia peltata Bermuda	6188/6274/6275/6268	Puerto Rico. H. Ruiz & D. Ballantine	AY970561/562/563/628
	Asteromenia peltata LHA	GWS001062	Lord Howe I, Australia. C. O'Brien	AY970632
	Asteromenia peltata LHA	GWS001079/2072	Lord Howe I, Australia. GWS	AY970630/631
	Asteromenia peltata LHB	GWS002050	Lord Howe I, Australia. GWS	AY970629

of a protein coding mitochondrial gene because of the relatively rapid rate of divergence in animals, the haploid mode of inheritance, the ability to design 'universal' primers at constrained portions of the gene, and the low prevalence of indels, which greatly facilitates alignment across phyla. They acknowledge that there is no *a priori* reason for the selection of one mitochondrial protein gene over another, but point to two advantages of *cox1*: (i) the universality of existing primers for amplification of the 5′ end of this gene in a wide variety of animals; and (ii) the broad phylogenetic range covered by the gene (Hebert *et al.* 2003*a*).

It is admittedly uncertain how well *cox1*-5′ will function for species discrimination in the other kingdoms of life because the mode of inheritance, rate of divergence, as well as many of the other attributes discussed above for this marker, are poorly known outside of the animals and land plants. For the latter, it was established that mitochondrial genes are generally more slowly evolving than in animals (Barkman *et al.* 2000), but that the more rapidly evolving plastid genome may provide sequences that would be adequate candidates for genetic barcoding (Chase *et al.* 2005). As for the many unicellular and multicellular protists, little is known and only through exploratory research will the utility of *cox1*-5′ as a genetic marker be established for the various lineages. At the time of writing this manuscript only four red algal *cox1* genes were accessible from GenBank and these were largely associated with mitochondrial genome projects.

There are obviously many advantages to algal systematists in adopting a standard marker for the purposes of species identification. By choosing the *cox1*-5′ system, should it prove suitable, there is the added advantage of being consistent with work in other kingdoms, which will facilitate universal comparisons and empower algal systematists to take advantage of emerging technologies. To facilitate this process, sequences from the four divergent red algal taxa in GenBank were acquired and used to modify the original barcoding primers published (Hebert *et al.* 2003*a*) for animals. To date these primers have successfully amplified *cox1*-5′ from *ca* 250 individuals spanning 15 families in six orders of the Florideophyceae. In this report the utility of *cox1*-5′ for species level discrimination is tested for three problematic species complexes in two orders, the Gigartinales and Rhodymeniales. These three test cases were selected because of their previous investigation with other DNA marker systems. In all cases *cox1*-5′ species assignments matched the earlier studies.

2. MATERIAL AND METHODS

Samples sequenced in the current study are identified in table 1. DNA was extracted with a protocol modified from Saunders (1993) (instead of the final agarose gel cleaning procedure, the DNA was purified with the Wizard® DNA Clean-Up System, Promega Corp., Madison, WI). The *cox1* sequences for *Cyanidium caldarium* (Tilden) Geitler (Z48930), *Cyanidioschyzon merolae* P. De Luca, R. Taddei *et* L. Varano (NC 000887), *Chondrus crispus* Stackhouse (NC 001677), and *Porphyra purpurea* (Roth) C. Agardh (NC 002007) were acquired from GenBank and aligned by eye in MacClade 4 (v. 4.06) for OSX (Maddison & Maddison 2003). These sequences were used, in conjunction with the previously published *cox1* barcoding primers developed for animals (Hebert *et al.* 2003*a*), to devise specific primers to amplify this gene region for red algae (GazF1 5′ TCAACAAATCATAAAGATATTGG 3′ and GazR1 5′ ACTTCTGGATGTCCAAAAAAYCA 3′; GWS000209 used the forward primer GazF2 5′ CCAACCAYAAAGATATWGGTAC 3′; GWS002199 used the reverse primer DumR1 5′ AAAAAYCARAATAAATGTTGA 3′). The PCR amplification profile followed Hebert *et al.* (2003*a*), but using an annealing temperature of 50 °C. Amplified products were gel purified using a glasswool column procedure (Saunders 1993). Sequencing used the PE Applied Biosystems Big Dye (v 3.0) kit and followed the manufacturer's protocol (ABI, Foster City, CA). Forward and reverse sequence reads from the respective PCR primers were edited and aligned using Sequencher™4.2 (Gene Codes Corporation, Ann Arbor, MI, USA), and a multiple sequence alignment was constructed with MacClade. The final alignment included 101 taxa (table 1; plus the four taxa from GenBank) with 664 nucleotide positions. All analyses and sequence comparisons were conducted in PAUP* 4.0b10 (Swofford 2002). Distances were corrected with a general time reversible model

(a variety of corrections were used, but had no effect on species assignment) and neighbour-joining and UPGMA clustering algorithms were used to provide a visual display of *cox1*-5′ variation within and between species.

3. RESULTS AND DISCUSSION

Using the primers described above *ca* 250 individuals spanning 15 families in six orders of the Rhodymeniophycidae (Florideophyceae) have had *cox1*-5′ barcodes successfully determined. The size of the amplified product is 710 base pairs (bp) of which 46 are complementary to the PCR primers and thus of no value for comparisons. In this report 97 of these sequences are presented that focus on three problematic species complexes.

Of the 101 aligned sequences, there were 87 individuals from within 16 species (from 2 to 20 isolates depending on the species; table 1) for which within species variation was between 0 and 1(2) bp out of the 664 positions or 0–0.3% divergence. Between species comparisons within genera generally ranged between 30 and 90 changes or 4.5 and 13.6% divergence with notable exceptions for the closely related *Mazzaella linearis* and *M. splendens* (0.8–1.2%) and *Dilsea carnosa* and *D. integra* (1.1%), which are discussed below. There was thus a clear distinction in divergence within versus between species observable with *cox1*-5′ for the red algae studied—clearly a necessary attribute for a marker system to be considered useful for the task of species assignment. Below three species complexes are considered in turn.

(a) Mazzaella *in the Pacific Northeast*

Mazzaella is a genus of the red algal family Gigartinaceae, Gigartinales, its species common along the coast of British Columbia (BC), Canada. Species of this genus are notoriously difficult to distinguish in the Northeast Pacific (Hommersand *et al.* 1994; Gabrielson *et al.* 2000; *cf.* Ross *et al.* 2003). *Mazzaella splendens* from sheltered habitats (figure 1*a*) can be discerned from wave-exposed populations of *M. linearis* (figure 1*b*), but a continuum of morphological intermediates (e.g. figure 1*c*) traverse the intervening wave-exposure gradient (Shaughnessy 1996). *Mazzaella flaccida* is also difficult to distinguish (compare figure 1*d* to *e*, *f*) from *M. splendens* (Hommersand *et al.* 1994) such that the 'Keys to the Benthic Marine Algae…of British Columbia…' (Gabrielson *et al.* 2000) indicate that 'the northern distribution limit of *M. flaccida* has not been established, but it may be present in southern British Columbia'. Gabrielson *et al.* (2000) further indicate a difficulty in distinguishing between some morphologies of *M. oregona* (as *M. heterocarpa*) (figure 1*g*) and *M. splendens*, and I have collected plants that defy identification based on morphology (e.g. figure 1*h*). Using sequence of the large subunit of rubisco (*rbcL*), Hommersand *et al.* (1994) were able to establish clearly genetic differences between *M. flaccida* and *M. splendens*, but not strongly between two samples of the latter and an isolate of *M. linearis* included in their study. A series of reciprocal transplant experiments, however, support recognition of *M. linearis* and *M. splendens* as distinct species, and indicate that plants of intermediate morphology were exposed variants of the latter (Shaughnessy 1996; Shaughnessy & DeWreede 2001).

Ross *et al.* (2003) outlined possible scenarios to explain the apparent morphological continuum observed in the field for the *M. linearis*/*splendens* complex: (i) a single species exists with substantial morphological plasticity in response to wave exposure; (ii) two species exist, but one or both display phenotypic plasticity such that the intermediate plants are strictly of one species or the other, or a mixture of plants from both; (iii) the intermediate plants may be tetrasporophytes of *M. splendens*, which was reported to have a possible heteromorphic aspect to its alternation of generations (Shaughnessy *et al.* 1996); (iv) the intermediates are hybrids between the two species; and (v) combinations of the previous could also explain these individuals. To evaluate the previous hypotheses, as well as the published ecological results (Shaughnessy 1996; Shaughnessy & DeWreede 2001), Ross *et al.* sequenced a variable region (ITS) of the nuclear genome for 17 isolates each of classic *M. splendens* (figure 1*a*) and *M. linearis* (figure 1*b*), and 20 isolates of intermediate morphology (figure 1*c*). They concluded that all of the intermediate plants were *M. splendens*, which had a broader morphological and ecological range than the strictly exposed and highly lanceolate *M. linearis*. The ITS varied from 0 to 4 nucleotides within a species, and was only 8–12 nucleotides different between *M. linearis* and *M. splendens*, these 32–38 nucleotides different from the next closest species, *M. sanguinea*. One plant field identified as *M. linearis* (GWS001173E; figure 1*c*) had ITS sequence consistent with placement in *M. splendens*, a result which was consistent with a morphological re-evaluation of the voucher (Ross *et al.* 2003); and another that was identified as *M. flaccida* (figure 1*d*), based on their interpretation of the identification keys in Gabrielson *et al.* (2000), also proved to be *M. splendens*, leaving uncertainty as to whether or not *M. flaccida* extends into BC. The ITS was thus a powerful tool for resolving an outstanding species issue in the flora adjacent to the Bamfield Marine Station, but it had shortcomings. The common occurrence of mononucleotide runs (poly C for example) and/or heterogeneity in the multiple copies of the ITS within an individual made it difficult to obtain sequence from both strands across the entire ITS for many of the individuals. This has serious implications for data quality and for using this marker for rapid species assignments. Additionally, the common occurrence of indels made it virtually impossible to compare the entire ITS from the *M. linearis*/*splendens*/*sanguinea* clade to the other species included (variable regions had to be excluded), rendering the estimation of nucleotide differences beyond the most closely related species inaccurate. Finally, results from this study could not be compared directly to the earlier study of Hommersand *et al.* (1994) because different marker systems were employed.

The *Mazzaella linearis*/*splendens* complex described above was used to test the *cox1*-5′ marker for its utility in distinguishing closely related species of red algae. This gene was successfully sequenced for an isolate of

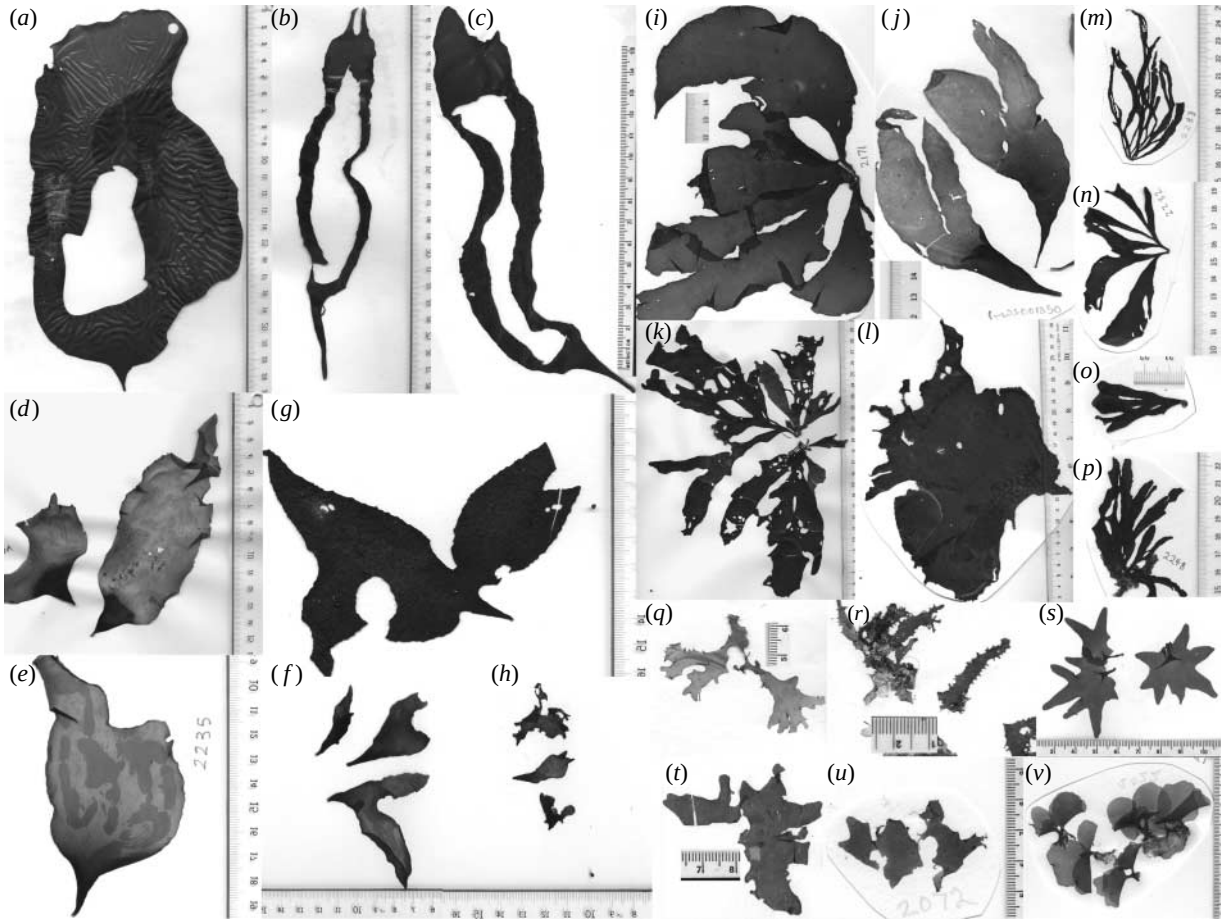


Figure 1. Gross morphology of red blades discussed in this report (Scale = centimetre ruler). Typical sheltered and exposed morphs for *Mazzaella splendens* (*a*; GWS1175I) and *M. linearis* (*b*; GWS001173D), respectively, and an isolate of intermediate morphology (*c*; GWS001173E) (central region removed for DNA extraction). (*d*) Sample (GWS001128) field identified as *M. flaccida*, but subsequently considered *M. splendens*. Two collections of *M. flaccida* (*e*, GWS002235; *f*, GWS002245) considered as unknown during field identification. *Mazzaella oregona* (*g*; GWS002258) from the outer coast of Vancouver I., and a carpet-forming morph from a sheltered habitat (*h*; GWS002199). Typical habit for *Dilsea californica* (*i*; GWS002171), *Dilsea integra* (*j*; GWS001850), *Dilsea carnosa* (*k*; GWS001216), and *Neodilsea borealis* (*l*; GWS002176), as well as morphs field identified as '*Dilsea*(?) exposed' (*m*; GWS002283), '*Neodilsea* exposed' (*n*, GWS002282; *o*, GWS002281), and '*Dilsea* exposed' (*p*, GWS002248). *Asteromenia peltata* from: North Carolina (*q*; NC2987K—not included in barcode analyses); Bermuda (*r*; GWS001252); Western Australia (*s*; HA703—not included in barcode analyses); and Lord Howe Island, LHA (*t*; GWS001062. *u*; GWS002072) and LHB (*v*; GWS002022—representative of GWS002050).

Chondrus crispus (resolves within *Mazzaella* in phylogenetic studies; Hommersand *et al*. 1999) and multiple individuals ($n = 42$) from nine species of *Mazzaella*. Included were 10 *M. linearis*, nine *M. splendens* (including previously misidentified GWS001128; figure 1*d*), and 11 individuals of intermediate morphology (including previously misidentified GWS001173E; figure 1*c*), and three additional collections (figure 1 *e*, *f*, *h*) of *Mazzaella* spp. that could not be identified based on Gabrielson *et al*. (2000). The *cox1*-5′ barcode echoed exactly the results of the previous ITS study (figure 2). *Mazzaella linearis* and *M. splendens* were resolved as independent lines, and all of the intermediates were assigned to the latter. The previously misidentified samples GWS001128 and GWS001173E were unequivocally included in *M. splendens*. Among the 10 species sampled, there were seven with multiple isolates (between 2 and 20 per species), and the within species variation was limited to 0–2 nucleotides (0–0.3% divergence)(figure 2). Between species comparisons ranged from 35 to 91 changes (5.3–13.7% divergence) with a notable exception for the closely related *Mazzaella linearis* and *M. splendens* at only 5–8 nucleotide differences (0.8–1.2% divergence). This lower range is comparable with values obtained for the most closely related species of Lepidoptera (Hebert *et al*. 2003*a*), and presumably also represents an exceptional case among red algal species in light of the ecological and ITS studies discussed previously. Collection GWS002199 (figure 1*h*) was an odd carpet forming morph, midintertidal on rock from a sheltered locality on the northern end of Vancouver Island, which differed by only two nucleotides in its *cox1*-5′ from a typical collection of *Mazzaella oregona* (GWS002258; figure 1*g*) from a semi-exposed site—these collections were apparently morphological/ecological extremes of a single species (figure 2). Two collections from the northern end of Vancouver Island, GWS002235 (figure 1*e*) and GWS002245 (figure 1*f*), had a novel *cox1*-5′ (figure 2). Based on Gabrielson *et al*. (2000) the collections were either *M. flaccida*, only questionably extending into southern BC, or *M. volans* (C. Agardh) Fredericq, which is not reported north of Oregon. GenBank was searched to determine

	Chondrus 1	*M. lamin.*	*M. affin.*	*M. parksii*	*M. rosea*	*M. oregona*	*M. sanguin.*	*M. flacc.*	*M. splen.*	*M. lin.*
Chondrus										
M. lamin.	78–79									
M. affin.	55–56	91	0							
M. parksii	52–53	73	68	0						
M. rosea	47–48	78	53	52	0					
M. oregona	53–54	90–91	47	56	47	2				
M. sanguin.	41–43	78–79	48–49	60–61	48–49	49–52	1			
M. flacc.	46–47	75	44	56	44	44–46	41–42	0		
M. splen.	49–51	81–83	48–49	57–58	47–49	49–50	39–42	37–38	0–2	
M. lin.	48–51	81	47–49	53–55	48–49	46–48	35–38	36–38	5–8	0–2

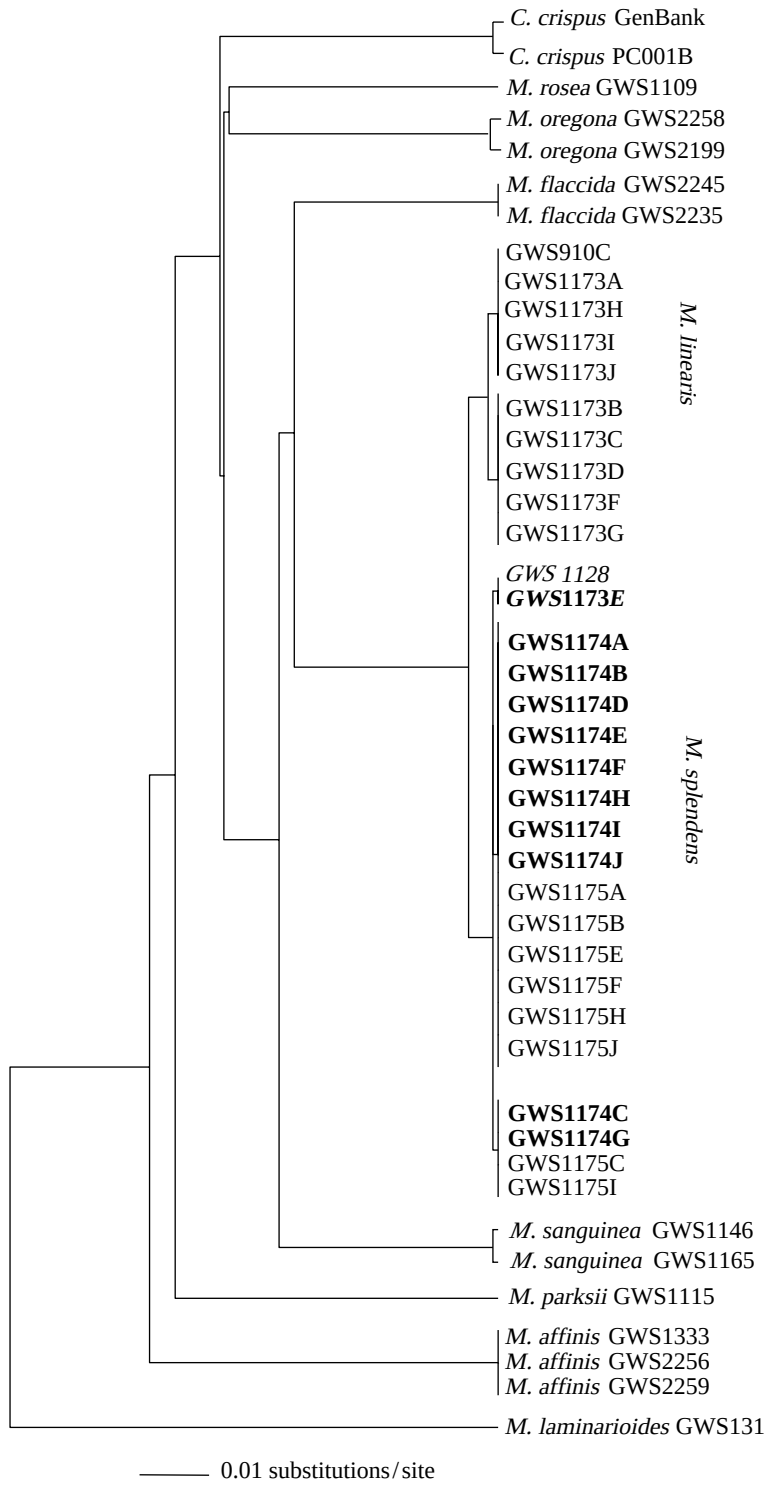
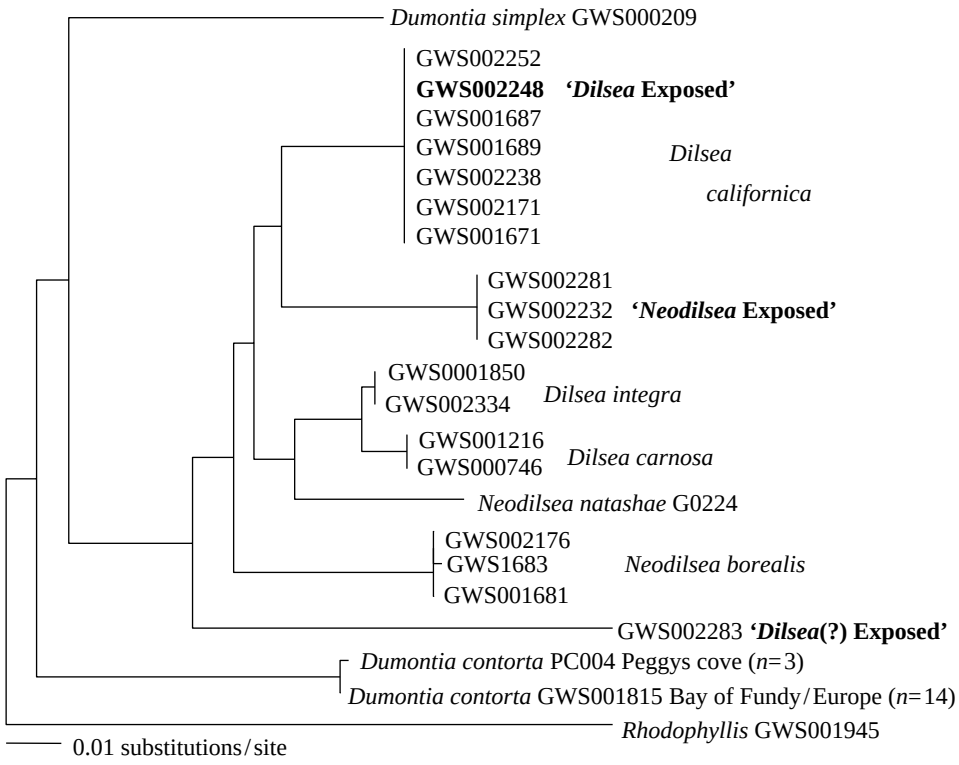


Figure 2. Phylogram (UPGMA) displaying clustering of the included species of *Mazzaella* and *Chondrus crispus*, and a matrix of actual nucleotide differences. Voucher numbers correspond to records in table 1 (central zeros omitted). Bold labels indicate collections intermediate in morphology between *M. linearis* and *M. splendens*; italics indicate two isolates that were misidentified in a previous study (discussed in the text).

that *rbcL* data were available for both species, whereas ITS was reported for only the former. The *rbcL* was thus sequenced for GWS002235 and GWS002245 and confirmed that these isolates are *M. flaccida*, which clearly extends well past southern BC. This represents the first published range extension to result from the application of *cox1*-5′ to species identification among red algae. A standardized system for species diagnosis, as advocated here, would have obviated the need to sequence an additional genetic marker to resolve the identity of these collections.

(b) *Dumontiaceae emphasizing* Dilsea *and* Neodilsea *in the Pacific Northeast*

The Dumontiaceae is also a family of the Gigartinales; although low in species diversity in Canada (*ca* 10), it is a group with known cryptic species (Tai *et al*. 2001). During a variety of collecting trips I have had the



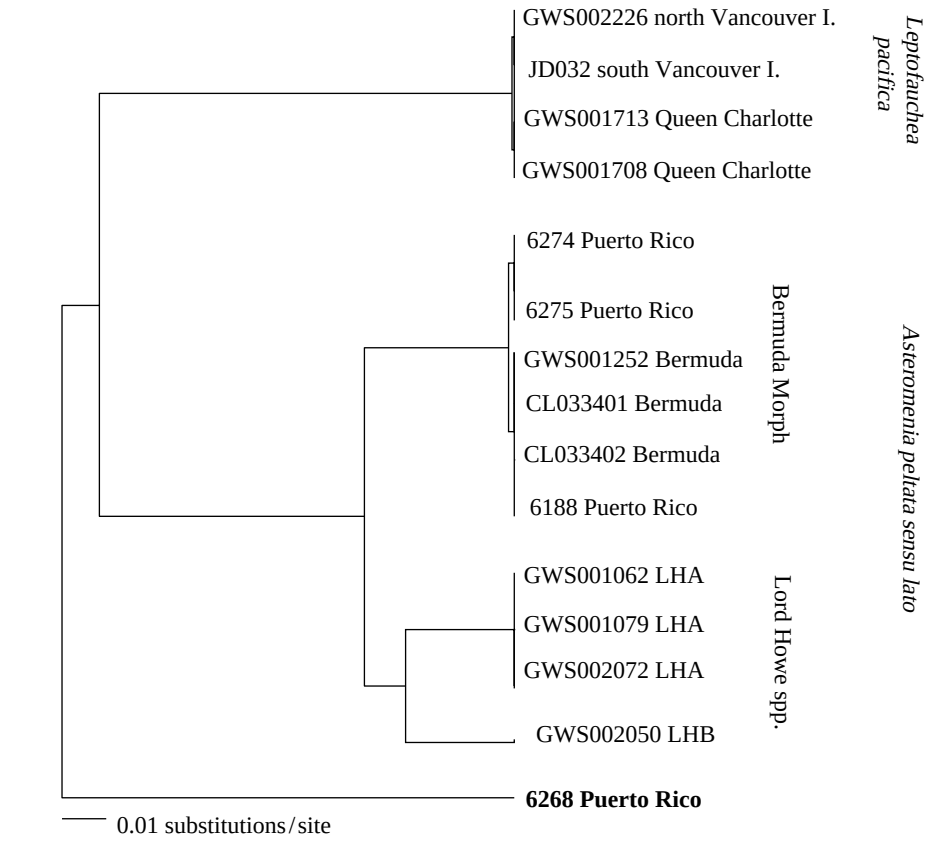
GWS0209	-																						
PC004A	74	(0)	*Dumontia*																				
GWS1815	73	1	(0)	*contorta*																			
GWS2252	72	78	77	-																			
GWS2334	71	71	70	30	-	*Dilsea*																	
GWS1850	71	71	70	30	0	-	*integra*																
GWS2248	72	78	77	0	30	30	-																
G0224	80	81	80	41	30	30	41	-															
GWS2176	66	73	72	42	39	39	42	42	-														
GWS1681	66	73	72	42	39	39	42	42	0	-	*Neodilsea*												
GWS1683	67	74	73	43	40	40	43	43	1	1	-	*borealis*											
GWS2281	78	82	81	36	36	36	36	46	51	51	52	-	*'Neodilsea*										
GWS2232	78	82	81	36	36	36	36	46	51	51	52	0	-	Exposed'									
GWS2282	78	82	81	36	36	36	36	46	51	51	52	0	0	-									
GWS1216	73	69	68	35	7	7	35	31	38	38	39	41	41	41	-								
GWS0746	73	69	68	35	7	7	35	31	38	38	39	41	41	41	0	-	*Dilsea carnosa*						
GWS1687	72	78	77	0	30	30	0	41	42	42	43	36	36	36	35	35	-						
GWS1689	72	78	77	0	30	30	0	41	42	42	43	36	36	36	35	35	0	-	*Dilsea*				
GWS2238	72	78	77	0	30	30	0	41	42	42	43	36	36	36	35	35	0	0	-	*californica*			
GWS2171	72	78	77	0	30	30	0	41	42	42	43	36	36	36	35	35	0	0	0	-			
GWS1671	72	78	77	0	30	30	0	41	42	42	43	36	36	36	35	35	0	0	0	0	-		
GWS2283	93	88	87	66	73	73	66	82	76	76	77	79	79	79	72	72	66	66	66	66	66	-	
GWS1945	100	100	99	105	105	105	105	103	106	106	106	111	111	111	109	109	105	105	105	105	105	121	-

Figure 3. Phylogram (neighbour joining) displaying clustering of the included species of Dumontiaceae, and a matrix of actual nucleotide differences. Voucher numbers correspond to records in table 1 (central zeros omitted in the matrix).

fortune of collecting *Dilsea californica* (figure 1*i*) along the coasts of BC, *D. integra* (figure 1*j*) from the Canadian Arctic, *D. carnosa* (figure 1*k*) from Europe, and *Neodilsea borealis* (figure 1*l*), again from BC. At the same time plants from exposed habitats in BC tentatively identified as '*Dilsea*(?) exposed' (figure 1*m*), '*Neodilsea* exposed' (figure 1*n*, *o*) and '*Dilsea* exposed' (figure 1*p*) were acquired. Part of the uncertainty derives from the similarity of the species (*ca* 11 in total) included in the *Dilsea*/*Neodilsea* complex (Lindstrom & Scagel 1987), the fact that only *D. californica* and *N. borealis* are recorded from BC (Lindstrom & Scagel 1987; Gabrielson *et al*. 2000), and the recovery by Tai *et al*. (2001) of divergent ITS sequences for *D. californica* from Alaska and Oregon indicating cryptic species.

As a first step toward a *cox1*-5′ analysis of the *Dilsea*/*Neodilsea* complex in BC, 17 individuals of a related taxon, *Dumontia contorta*, were collected from the Maritime Provinces of Canada ($n=6$) and Europe ($n=11$). The three Peggys Cove isolates were identical and differed from the other collections at only one position, while the isolates of *D. contorta* differed from the congenor *D. simplex* at 73 or 74 positions (figure 3). These results are consistent with those noted above for *Mazzaella* with regards to within species variation (figure 2).

Two isolates each of *Dilsea carnosa* (figure 1*k*) and *D. integra* (figure 1*j*) had identical *cox1*-5′ sequences, with these two closely related species from Europe and the Canadian Arctic/Maritimes, respectively, differing from each other at only seven nucleotide positions



GWS002226	–													
GWS001713	1	–												
GWS001708	1	0	–											
JD032	0	1	1	–										
6268PR	**113**	**114**	**114**	**113**	–									
6274PR	107	108	108	107	**118**	–								
6275PR	107	108	108	107	**118**	0	–							
GWS002152Ber	106	107	107	106	**117**	2	2	–						
CL033401Ber	106	107	107	106	**117**	2	2	0	–					
CL033402Ber	106	107	107	106	**117**	2	2	0	0	–				
6188Pr	106	107	107	106	**117**	2	2	0	0	0	–			
GWS001062LHA	108	109	109	108	**114**	43	43	41	41	41	41	–		
GWS001079LHA	108	109	109	108	**114**	43	43	41	41	41	41	0	–	
GWS002072LHA	108	109	109	108	**114**	43	43	41	41	41	41	0	0	–
GWS002050LHB	102	103	103	102	**115**	43	43	43	43	43	43	31	31	31

Leptofauchea pacifica; *Asteromenia peltata* Berm; *Asteromenia peltata* LHA

Figure 4. Phylogram (neighbour joining) displaying clustering of the included species of the genera *Leptofauchea* and *Asteromenia*, Rhodymeniales, and a matrix of actual nucleotide differences. Voucher numbers correspond to records in table 1. Isolate 6268 in bold represents an anomalous 'Bermuda morph', which is only distantly related to *Asteromenia* spp.

(figure 3). It was expected that these two would represent closely related species (Tai *et al*. 2001), along with *Dilsea socialis* (Postels *et* Ruprecht) Perestenko from Alaska and the Northwest Pacific, which has not been included in the *cox1*-5′ alignment to date. Six isolates of *Dilsea californica* (figure 1*i*) had identical *cox1*-5′ sequences, as did '*Dilsea* exposed' (GWS002248; figure 1*p*), which clearly represents the exposed morphology of this species (figure 3). *Neodilsea borealis* (figure 1*l*) formed a distinct lineage in the *cox1*-5′ analyses with only 0–1 nucleotide differences noted among the three isolates, but these differed substantially (51–52 nucleotide differences) from collections tentatively identified as exposed morphs ('*Neodilsea* exposed'; figure 1*n*, *o*) of this species (figure 3). '*Neodilsea* exposed' resolved as sister (36 nucleotides divergent) to *Dilsea californica* and represents a new species, or a range extension of one of the four Northwest Pacific species (*cf*. Lindstrom 1994). Tai *et al*. (2001) also resolved two divergent entities in their ITS investigation (identified as *D. californica* from Oregon and Alaska), which may correspond to *Dilsea californica* and *Neodilsea* exposed uncovered here. *Neodilsea natashae*, reported from Alaskan waters as far south as the Sumner Strait, Alexander Archipelago, can be excluded as a possible designation for *Neodilsea* exposed because it was included in the *cox1*-5′ analyses (figure 3). Consistent with the earlier ITS studies of Tai *et al*. (2001), *N. natashae* was closest to *D. carnosa* and *D. integra* in its *cox1*-5′ sequence (figure 3).

Collection GWS002283 (figure 1*m*), labelled simply '*Dilsea*(?) exposed' in the field had a divergent *cox1*-5′ sequence (figure 3). Consistent with the previous, a subsequent anatomical investigation revealed it to be

the exposed morph of another dumontiacean species, *Farlowia mollis* (Harvey *et* Bailey) Farlow *et* Setchell (*cf.* Lindstrom & Scagel 1987). A comprehensive barcode alignment would have provided an accurate identification for this collection.

In summary, all six of the species (figure 3) for which multiple isolates were studied in the Dumontiaceae displayed intraspecific *cox1*-5′ divergence of 0–1 nucleotide changes, with the closely related *D. carnosa* and *D. integra* differing at seven sites, but with most interspecific divergences >30 changes.

(c) *The genus* Asteromenia *from three oceans*

Asteromenia was recently erected in the red algal order Rhodymeniales to include the single species *Fauchea peltata* W. R. Taylor, which was reportedly widely distributed throughout tropical and warm temperate waters (Huisman & Millar 1996). However, Saunders *et al.* (unpublished) have used LSU sequence data (GenBank DQ068294–DQ068301) in combination with anatomical analyses to argue that at least five cryptic species are included in this complex (figure 1*q–v*). The first (figure 1*q*) is distributed throughout the Caribbean, to Bermuda and North Carolina, and is anatomically the most divergent supporting the molecular evidence in resolving it as a separate species. A second species is common in Bermuda, but extends throughout the Caribbean including Puerto Rico, has a distinctive morphology (Bermuda morph; figure 1*r*), and was the most divergent species in LSU analyses (Saunders *et al.*, unpublished). A third species was recognized from Western Australia (figure 1*s*) and is sister in LSU analyses to two species from Lord Howe Island, Australia, LHA (figure 1*t*, *u*) and LHB (figure 1*v*), which only differed by six nucleotides in their LSU sequences (Saunders *et al.*, unpublished).

To assess intraspecific *cox1*-5′ divergence in the Rhodymeniales, *Leptofauchea pacifica* Dawson from the Northeast Pacific was investigated. In vegetative state *L. pacifica* is virtually indistinguishable from *Rhodymenia californica* Kylin, which has contributed uncertainty as to the geographical range of the former species (considered absent from Canadian waters; Hawkes & Scagel 1986). These species are, however, in different families and are easily distinguishable when thalli are reproductive (Saunders *et al.* 1999). In 2001 a number of reproductive plants were collected subtidally in southern BC (e.g. JD032, figure 4) and were unmistakably assignable to *Leptofauchea* extending the range of this taxon into Canadian waters. Subsequent collections from northern Vancouver Island (e.g. GWS002226, figure 4) and the Queen Charlotte Islands (e.g. GWS001708 and GWS001713, figure 4), variously vegetative or reproductive, were similar to the southern BC plants. These four isolates were compared for *cox1*-5′ divergence (figure 4) and had 0–1 nucleotide changes consistent with anatomical observations that these were of a single species and greatly extending the northern range of *Leptofauchea pacifica* in the Northeast Pacific.

Within *Asteromenia*, results echoed the LSU analyses. Three isolates each from Bermuda and Puerto Rico, which had the characteristic anatomy of the Bermuda morph and identical LSU sequences, clustered together in the *cox1*-5′ analysis with 0–2 nucleotide changes (figure 4). Again consistent with the LSU and anatomical observations, a second species encompassed plants assigned to LHA; the *cox1*-5′ sequences identical among the three included samples (figure 4). Lord Howe Island LHB resolved as a third species, again consistent with the earlier observations, and was 31 nucleotides different in *cox1*-5′ from its sister species LHA (figure 4). Isolate 6268 from Puerto Rico was considered *Asteromenia peltata* Bermuda morph based on its gross morphology and was not included in the LSU investigation. The *cox1*-5′ sequence determined from this collection, however, was the most divergent of all the members of the Rhodymeniales studied here (figure 4). A preliminary assessment of the internal anatomy confirmed that this collection is not related to *Asteromenia* (Ballantine & Saunders, unpublished) and further investigation is required to determine its identity. Of importance here, the *cox1*-5′ barcode clearly established the novelty of this collection despite its gross morphological similarity to the Bermuda morph of *Asteromenia*.

4. CONCLUSION

The case studies presented here indicate that *cox1*-5′ barcoding will be a powerful ally in the identification of red algal species. In all cases intraspecific divergence values ranged from 0 to 2 bp, whereas interspecific divergences >30 bp were usually observed. Exceptions were noted for the closely related *Mazzaella linearis* and *M. splendens* (5–8 differences) and *Dilsea carnosa* and *D. integra* (7 differences), but in both cases the marker successfully assigned collections to the correct species. Indeed the *cox1*-5′ system without fail matched the previous anatomical and molecular results. Considering the low number of taxa studied in this report (table 1), the number of new records is astonishing and indicates that considerable taxonomic and biogeographical work remains among the Rhodoplantae. Genetic barcoding will thus not signal the end of taxonomy for phycologists, but will initiate a revolution of molecular-assisted alpha taxonomy that will greatly change the number and distribution of species that are recognized in this lineage. It is not, however, a tool that should be used in isolation, particularly during the development stages when it will be desirable to accompany the molecular results with thorough anatomical observations, and in the case of closely related species where it will be prudent to assess the mitochondrial data with nuclear markers to search for introgression, hybridization and incomplete species boundaries. Further, the utility of *cox1*-5′ for species identification in asexual lines, a common manifestation in protistans including the red algae, has not been adequately assessed and requires detailed investigation.

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