Cryptogamie, Algologie, 2010, 31 (4): 435-449
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DNA barcoding in the red algal order Gelidiales: comparison of COI with *rbc*L and verification of the "barcoding gap"

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Abstract – Mitochondria-encoded COI and plastid-encoded *rbc*L sequences were used to assist with the identification or "barcoding" of a variety of red algal species. The *rbc*L locus has been extensively analyzed within the Gelidiales and general levels of intraspecific and interspecific sequence divergences are established. Complementary COI and *rbc*L data sets were compared to explore the utility of COI for Gelidiales species identification and verify the presence of a barcoding gap between closely related species. There was no difference between the two loci in the clustering of specimens into species, but the COI sequences were more variable than *rbc*L and exhibited a larger barcoding gap between closely related sister species. These results indicate that COI barcoding is a useful tool for the molecular assisted identification of Gelidiales species, especially in cases of closely related species where the more conserved *rbc*L may be uninformative. The presence of cryptic species within the widely distributed taxon, *Pterocladiella caerulescens* was also revealed, and taxonomic changes are proposed including the description of *Pterocladiella australafricanensis* sp. nov.

COI / DNA barcoding / Gelidiales / *Gelidium* / *Pterocladiella* / *Pterocladiella australafricanensis* / *rbc*L

Résumé – Les séquences du gène mitochondrial codant la cytochrome c oxydase I COI et du gène plastidial *rbc*L ont été testé comme marqueurs code-barres ADN pour l'identification d'une série d'espèces d'algues rouges. La *rbc*L a été intensivement analysé dans les Gelidiales dont le niveau de divergence intraspécifique et interspécifique à été établi. Des jeux de données complémentaires du COI et de la *rbc*L ont été comparés afin d'explorer l'utilité du COI pour l'identification des espèces de Gelidiales et pour vérifier la présence d'un espace code-barre entre les espèces jumelles. Il n'y avait aucune différence entre les deux marqueurs dans le groupement des spécimens par espèces, mais les séquences du COI étaient plus variables que celles de la *rbc*L et démontraient un plus grand espace code-barre entre les espèces jumelles. Ces résultats indiquent que le code-barre COI est un outil utile pour aider à l'identification moléculaire des espèces de Gelidiales, particulièrement des espèces étroitement liées où le *rbc*L plus conservateur n'est pas informatif. La présence des espèces énigmatiques dans le taxon largement distribué, *Pterocladiella caerulescens* a été également indiquée, et des modifications taxonomiques sont proposes comprenant la description de *Pterocladiella australafricanensis* sp. nov.

COI / code-barres ADN / Gelidiales / *Gelidium* / *Pterocladiella* / *Pterocladiella australafricanensis* / *rbc*L

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INTRODUCTION

The use of "barcoding" to identify species has become increasingly common in studies of marine algae. The mitochondria-encoded COI locus has been used extensively in animals as a barcoding sequence, but whether this locus is also appropriate for barcoding in vascular plants and algae has been debated (e.g. Presting, 2006; Kress & Erickson, 2007). Saunders (2005) and Robba *et al.* (2006) presented the first evidence that COI can be used as a barcode marker in red algae, and subsequent COI based studies have continued to reveal its usefulness for identifying species (Saunders, 2008; 2009; Yang *et al.*, 2008; Clarkston & Saunders, 2010; Le Gall & Saunders, 2010).

The DNA sequence of the large subunit of RuBisCO (*rbc*L) is another locus that has been employed to identify red algae following a barcoding strategy. Sequences of *rbc*L have been used to help with the identification of Gelidiales species for over 15 years (e.g. Freshwater *et al.*, 1995; Millar & Freshwater, 2005; Shimada *et al.*, 1999). Early *rbc*L analyses by Freshwater & Rueness (1994) were able to compare the sequence divergence of specimens known to interbreed and consequently an estimate of the amount of sequence divergence to be expected within a species (per the biological species concept) could be made. In general *rbc*L sequence divergences of $< 1\%$ signify that the specimens in question represent the same species; divergences of $> 2\%$ signify that the specimens in question represent different species, and divergences between 1-2% may, or may not, represent the same species (e.g. Freshwater & Rueness, 1994; Freshwater *et al.*, 1995; Millar & Freshwater, 2005). Additional evidence is needed to make accurate determinations in the latter case. Subsequent studies have shown that although these values are routinely reliable, they may not be accurate for the assessment of recently diverged species. For example, Shimada *et al.* (2000) found $< 1\%$ sequence divergence between two sets of *Gelidium* sister species, *G. allanii* Chapman and *G. koshikianum* Shimada, Horiguchi *et* Masuda, and *G. linoides* Kützing and *G. tenuifolium* Shimada, Horiguchi *et* Masuda.

The COI locus has generally revealed greater interspecific sequence divergence relative to *rbc*L and may therefore provide better evidence for the separation of closely related species (Yang *et al.*, 2008, though an exception is noted for *Gracilaria vermiculophylla* (Ohmi) Papenf.). Wiriyadamrikul *et al.* (2010) have recently examined both *rbc*L and COI sequence data for the Gelidiales species, *Gelidiella fanii* S.-M. Lin. Although their data sets did not include complementary *rbc*L and COI sequences for all sampled specimens, COI variation ($\leq 2.67\%$) was greater than *rbc*L variation ($< 0.39\%$). Interspecific COI sequence divergences between *G. fanii* and its sister species *G. acerosa (*Forsskål) Feldmann *et* G. Hamel (10.1-10.9%) were also at least twice as much as those for *rbc*L (3.80-4.97%) despite there only being two *G. acerosa* COI sequences available for these comparisons (Wiriyadamrikul *et al.*, 2010).

To better assess the utility of COI as a red algal barcoding locus, complementary COI sequences were generated for Gelidiales specimens from which *rbc*L sequences were already available. Comparisons of the intraspecific and interspecific sequence divergences of the two loci are made with an emphasis placed on the verification of a barcoding gap between closely related species. The "barcoding gap" is herein defined as the difference between the minimum interspecific and maximum intraspecific sequence divergences (Meier *et al.*, 2008) and not the difference between the mean interspecific and intraspecific sequence divergences (e.g. Meyer & Paulay, 2005). These analyses also reveal the presence

of cryptic species within the widely distributed taxon, *Pterocladiella caerulescens* (Kützing) Santelices *et* Hommersand, and taxonomic changes including the description of a new species are proposed.

MATERIALS AND METHODS

Specimen collection information and GenBank accession numbers for sequences analyzed in this study are included in Table 1. Total genomic DNA was extracted from specimens using the methods outlined in Freshwater & Rueness (1994) and Hughey *et al.* (2001), and new sequences generated as follows. The COI and *rbc*L loci were amplified and cleaned using the PCR and thermocycling protocols outlined in Freshwater & Rueness (1994), Freshwater *et al.* (2000; 2005) and Stuercke & Freshwater (2008). Amplification products were used as templates in BigDye v. 3.1 (Applied Biosystems, Foster City, CA, USA) sequencing reactions and determined on an ABI 3130*xl* genetic analyzer (Applied Biosystems, Foster City, CA, USA). Sequence reaction results were edited and assembled using Sequencher (GeneCodes Corp., Ann Arbor, MI, USA). Primers utilized in amplification and sequencing reactions were GazF1, GHalF and GazR1 of Saunders (2005; 2008). Sequences were aligned using MacClade (v. 4, Maddison & Maddison, 2000), and sequence divergences calculated and neighbor-joining trees and UPGMA cluster diagrams generated using PAUP* (v. 4.b10, Swofford, 2002) and MEGA (Kumar *et al.*, 2008). All sequence divergences were calculated as simple distances with no corrections applied. Barcoding gap values between species are presented as how many times greater the minimum interspecific sequence divergence is as compared to the maximum intraspecific sequence divergence. This value is calculated by dividing the minimum interspecific sequence divergence (minTER) by the maximum intraspecific sequence divergence (maxTRA), i.e. minTER / maxTRA. A barcode gap is present when this calculated value is >1, and values reported in this manner are comparable across different loci and evolutionary lineages.

RESULTS AND DISCUSSION

Gelidiales COI amplification trials were not universally successful with currently published primers. The GazF1, GHalF and GazR1 primers of Saunders (2005; 2008) were used to successfully amplify COI from two *Gelidiella* Feldmann & Hamel, three *Pterocladiella* Santelices & Hommersand, ten *Gelidium* Lamouroux, and one *Ptilophora* Kützing species (data not shown). Although this set of species represents nearly the full range of Gelidiales evolution, other species within the genera would not amplify. For example, DNA sequence analyses indicate that *Gelidium spinosum* (S. Gmelin) P. Silva [as *G. latifolium* (Greville) Bornet *et* Thuret] and *G. pulchellum* (Turner) Kützing are closely related (e.g. Freshwater & Rueness, 1994; Freshwater *et al.*, 1995), but only specimens of *G. spinosum* amplified using the GazF1-GazR1 primer pair. Wiriyadamrikul *et al.* (2010) amplified and sequenced a 1300+ bp section of COI from four Gelidiales

species (two *Gelidiella*, one *Pterocladiella* and one *Gelidium*) using the primers published by Geraldino *et al.* (2006), but this fragment is larger than that used for barcoding in red algae (e.g. Clarkston & Saunders, 2010). A systematic study of COI amplification in the Gelidiales is needed to develop additional COI primers that will work universally throughout the order.

COI and *rbc*L sequences from multiple specimens (3-11) of six *Gelidium* and two *Pterocladiella* species (Table 1) were compiled into alignments including 42 sequences and 664 (COI) and 1356 (*rbc*L) sites. Two hundred twenty-six (34.0%) of the COI and 334 (24.6%) of the *rbc*L sites were variable. Whereas the upper limit of COI intraspecific sequence variation in most species was higher than that for *rbc*L, this was not always the case (Table 1). Intraspecific *rbc*L divergences were higher in *Gelidium coulteri*, *G. spinosum* and *G. pusillum*. However, the majority of *rbc*L sequences for these three species were generated by manual sequencing (Freshwater & Rueness, 1994; Freshwater *et al.*, 1995), and some of the variation may be a result of PCR and sequencing error inherent to manual methods. The upper level of intraspecific COI sequence divergence was greater than that of *rbc*L in all other tested species and over twice as much in most.

There was no difference in the clustering of specimens into species with either locus or clustering method (UPGMA or NJ), but there were some differences in the groupings of species clusters (Figs 1, 2). The greater level of interspecific sequence divergence in COI versus *rbc*L data is clearly seen in the longer connections between species in the COI UPGMA cluster diagram. This increase in the level of interspecific sequence variation suggests that the COI locus could provide better resolution for distinguishing species within a barcoding framework. The utility of any barcoding locus depends upon both practical considerations such as universality and appropriate length (e.g. Hajibabaei *et al.*, 2007), and also functional considerations such as level of sequence variation and presence of a clear barcoding gap between closely related species (e.g. Meier *et al.*, 2008). Three closely related species pairs are included in the complementary COI and *rbc*L alignments so that the presence of a barcoding gap within Gelidiales species can be verified.

A sister relationship between the southern African species *Gelidium pristoides* (Turner) Kützing and *G. foliaceum* (Okamura) E.M. Tronchin, was established by Tronchin *et al.* (2002; see also Fig. 2). COI and *rbc*L sequences were generated for three specimens of each species and compared (Table 2). The general level of variation seen in COI sequences was greater than that for *rbc*L, however, the range of intraspecific sequence divergences overlap. Interspecific sequence divergences for COI were almost four times greater than that found in *rbc*L. The *rbc*L barcoding gap for the *G. pristoides/G. foliaceum* comparisons was 3.69x, while for COI it was 5.58x.

A second set of comparisons was made between specimens of *Gelidium crinale* (Turner) Gaillon and *Gelidium coulteri* Harvey. These two species are closely related (e.g. Fig. 2) but whether they represent sister species has not been established. Past phylogenetic analyses resolve them together in a clade that also includes *G. capense* (Gmelin) Silva and an unidentified *Gelidium* species, but the relationships within the clade are variable in the different analyses (e.g. Freshwater *et al.*, 1995; Millar & Freshwater, 2005). Whereas *G. coulteri* has a relatively restricted distribution from southern British Columbia to Baja California within the eastern Pacific (Dawson, 1953; Renfrew *et al.*, 1989), *G. crinale* is widely distributed around the world (e.g. Shimada *et al.*, 1999; Thomas, 2000; Tronchin, 2003; Millar & Freshwater, 2005) making its intraspecific divergence range of particular interest.

Table 1. Specimen collection information/source, GenBank accession numbers and intraspecific sequence divergence ranges for species included in *rbc*L and COI comparisons

Specimens	*Collection information/source*	*Accession #*		*Intraspecific divergence (%)*	
		rbc*L*	COI	rbc*L*	COI
Gelidium coulteri	Balboa Peninsula, Orange County, CA, USA (Freshwater *et al.*, 1995)	U00105	HQ412468	0.22 – 0.83	0.00-0.30
	Pacific Grove, Monterey County, CA, USA (Freshwater *et al.*, 1995)	U01815	HQ412469		
	Scripps Institute, La Jolla, San Diego County, CA, USA, coll. J. Rueness	HQ412494	HQ412470		
	Yaquina Bay, OR, USA, coll. Gail Hansen, 05.v.2005	HQ412495	HQ412471		
Gelidium crinale	Masonboro Inlet, New Hanover County, NC, USA (Freshwater *et al.*, 1995)	U00981	HQ412457		
	Beaufort Inlet, Carteret County, NC, USA (Thomas, 2000)	AF308795	HQ412458		
	Radio Island Jetty, Carteret County, NC, USA, coll. Freshwater & Hommersand, 16.ii.1991	HQ412488	HQ412459		
	Bogue Sound, Carteret County, NC, USA, coll. Freshwater & Hommersand, 16.ii.1991	HQ412489	HQ412460		
	Fort Fisher, New Hanover County, NC, USA, coll. Freshwater, 14.v.1991	HQ412490	HQ412461		
	Stump Sound, Onslow County, NC, USA, coll. Freshwater & Montgomery, 11.vii.2003	HQ412491	HQ412462	0.00-1.12	0.00-2.65
	Playa Esperanza, Manati, Puerto Rico (Freshwater *et al.*, 1995)	U00983	HQ412463		
	Green Island, Rottnest Island, WA, Australia, coll. Freshwater, Yee & Millar, 14.viii.2002	HQ412492	HQ412464		
	Green Island, Rottnest Island, WA, Australia, coll. Freshwater, Yee & Millar, 14.viii.2002	HQ412493	HQ412465		
	Old Gulch, Lord Howe Island (Millar & Freshwater, 2005)	AY350781	HQ412466		
	Summer Cloud Bay, Bhewerre Peninsula, Jervis Bay, NSW, Australia (Millar & Freshwater, 2005)	AY350781	HQ412467		
Gelidium foliaceum	Port Edward, KwaZulu-Natal Prov., South Africa (Tronchin *et al.*, 2002)	AF501284	HQ412455	0.00-0.22	0.15-0.44
	East London, Eastern Cape Prov., South Africa (Tronchin *et al.*, 2002)	AF501286	HQ412456		
	Breezy Point, Eastern Cape Prov., South Africa (Tronchin *et al.*, 2002)	AF501285	HQ412454		

Table 1. Specimen collection information/source, GenBank accession numbers and intraspecific sequence divergence ranges for species included in *rbc*L and COI comparisons *(cont'd)*

Specimens	*Collection information/source*	*Accession #*		*Intraspecific divergence (%)*	
		rbc*L*	COI	rbc*L*	COI
Gelidium pristoides	False Bay, Western Cape Prov., South Africa (Freshwater *et al.*, 1995)	U01044	HQ412451	0.00-0.52	0.30-1.36
	Port Edward, KwaZulu-Natal Prov., South Africa (Tronchin *et al.*, 2002)	AF501282	HQ412452		
	Kidds Beach, Eastern Cape Prov., South Africa (Tronchin *et al.*, 2002)	AF501283	HQ412453		
Gelidium pusillum	Cancale, Brittany, France (Freshwater & Rueness, 1994)	U01000	HQ412446	0.15-0.68	0.00
	Fedje, Hordaland, Norway (Freshwater & Rueness, 1994)	U00999	HQ412445		
	Masonboro Inlet, New Hanover County, NC, USA, coll. Freshwater, Duncan & Duncan, 30.xii.2006	HQ412487	HQ412447		
Gelidium spinosum	Plouguerneau, Brittany, France (Freshwater & Rueness, 1994)	U00112	HQ412448	0.30-0.54	0.00
	Portstewart, County Londonderry, Northern Ireland (Freshwater & Rueness, 1994)	U10821	HQ412449		
	Bergen, Hordaland, Norway (Freshwater & Rueness, 1994)	U00108	HQ412450		
Pterocladiella caerulescens	Coconut Is., Oahu, Hawaiian Islands (Thomas & Freshwater, 2001)	AF305805	HQ412475	0.00-2.22	0.00-5.58
	Sandy Beach Park, Oahu, Hawaiian Islands (Tronchin & Freshwater, 2007)	EF190250	HQ412476		
	Ribbon Reef, Sodwana Bay, KwaZulu-Natal, South Africa (Tronchin & Freshwater, 2007)	EF190246	HQ412472		
	Doodles Reef, Punta Do Ouro, Mozambique (Tronchin & Freshwater, 2007)	EF190247	HQ412473		
	Texas Reef, Punta Do Ouro, Mozanbique (Tronchin & Freshwater, 2007)	EF190248	HQ412474		
	Cahuita, Limón, Costa Rica (Thomas & Freshwater, 2001)	AF305811	HQ412477		
	Boca del Drago, Bocas, Panama, coll. Freshwater, 27.viii.2009	HQ412496	HQ412478		
	Boca del Drago, Bocas, Panama, coll. Freshwater, 27.viii.2009	HQ412497	HQ412479		
	Boca del Drago, Bocas, Panama, coll. Freshwater, 27.viii.2009	HQ412498	HQ412480		
	Long Bay Point, Isla Colon, Bocas, Panama, coll. Freshwater, 29.viii.2009	HQ412499	HQ412481		

Table 1. Specimen collection information/source, GenBank accession numbers and intraspecific sequence divergence ranges for species included in *rbc*L and COI comparisons *(cont'd)*

Specimens	*Collection information/source*	*Accession #*		*Intraspecific divergence (%)*	
		rbc*L*	COI	rbc*L*	COI
	Old Point, Isla Bastimento, Bocas, Panama, coll. Freshwater, 31.viii.2009	HQ412500	HQ412482		
Pterocladiella psammophila	Ribbon Reef, Sodwana Bay, KwaZulu-Natal, South Africa (Tronchin & Freshwater, 2007)	EF190255	HQ412483		
	4-Buoys Reef, Sodwana Bay, KwaZulu-Natal, South Africa (Tronchin & Freshwater, 2007)	EF190256	HQ412485	0.00-0.11	0.00-0.15
	9-Mile Reef, Sodwana Bay, KwaZulu-Natal, South Africa (Tronchin & Freshwater, 2007)	EF190255	HQ412484		
	9-Mile Reef, Sodwana Bay, KwaZulu-Natal, South Africa (Tronchin & Freshwater, 2007)	EF190255	HQ412486		

Eleven *Gelidium crinale* specimens were included from the western Atlantic, eastern Indian and southwest Pacific for these comparisons (Table 1). All pairwise *rbc*L divergences except those between one of the North Carolina and Indo-Pacific specimens (max. value = 1.12%) were < 0.8% despite the comparison of specimens from opposite sides of the globe. Similarly, the maximum intraspecific COI divergence was only 2.65%. The intraspecific divergence ranges for *rbc*L and COI from both *G. crinale* and *G. coulteri* overlap (Table 3). The range of interspecific sequence divergences was greater for COI and at least twice as great as that found for *rbc*L. This is reflected in the barcoding gap of 4.83x for COI and only 2.52x for *rbc*L.

The third comparison was made between specimens classified as *Pterocladiella caerulescens* (Kützing) Santelices *et* Hommersand and *P. psammophila* Tronchin *et* Freshwater, a closely related species that was recently described from southern Africa (Fig. 2; Tronchin & Freshwater, 2007). The maximum intraspecific *rbc*L variation found in the *P. 'caerulescens'* specimens is at a level considered to usually represent different species, and the intraspecific sequence divergence of some pairwise specimen comparisons is greater than that for some interspecific comparisons (Table 4). This may be in part because these samples are from three very distant sites around the globe — Hawai'i, southern Africa, and Caribbean Panama/Costa Rica (Table 1) and represent what may be better referred to as the *P. 'caerulescens'* complex. The Caribbean Costa Rican specimen was originally described as *P. beachiae* Freshwater (Thomas & Freshwater, 2001), a name subsequently proposed to be a synonym of *P. caerulescens* (Tronchin & Freshwater, 2007). Whereas there was no gap between the intra- and interspecific sequence divergence ranges of *rbc*L for these specimens (barcoding gap < 1.00, Table 4), the intraspecific and interspecific COI sequence divergence ranges did not overlap. However, the barcoding gap of 1.48x for COI is relatively low.

Comparisons were also made between the separate geographic groups within the *P. 'caerulescens'* complex and *P. psammophila* (Table 5). The "interspecific" or between geographic group *rbc*L divergences range between 2-3%

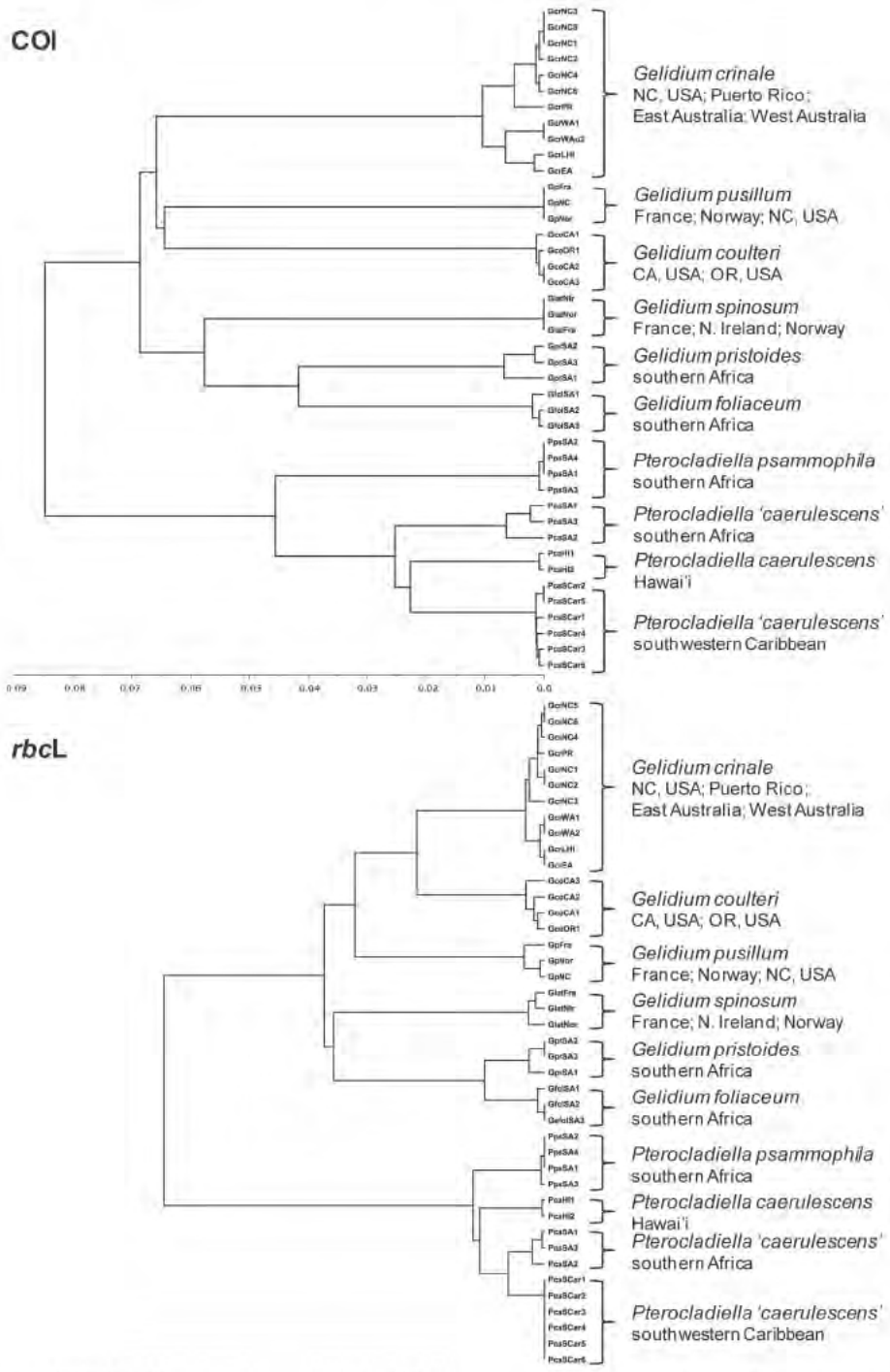

Fig. 1. UPGMA cluster diagrams calculated from uncorrected distances for complementary COI (above scale) and *rbc*L (below scale) sequences of 42 Gelidiales specimens.

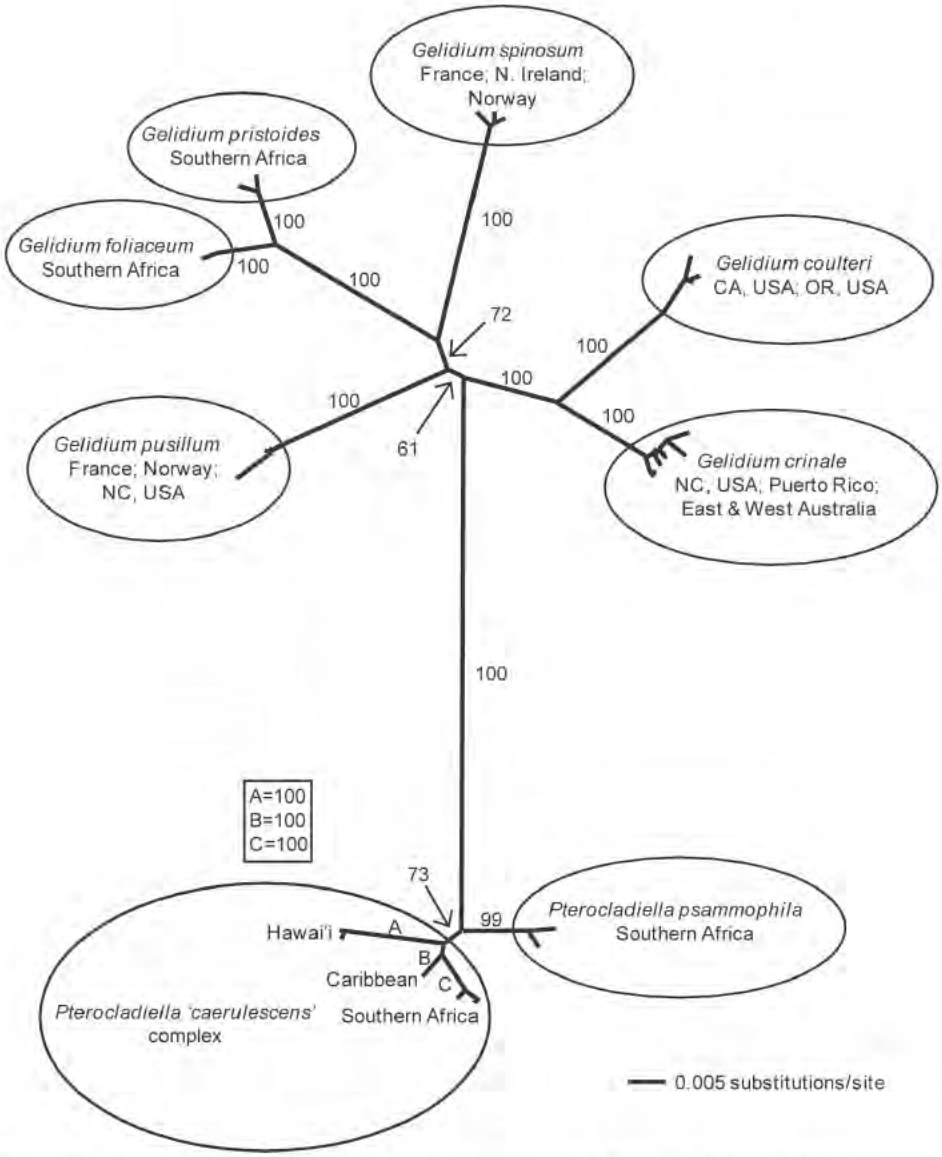

Fig. 2. Neighbor joining distance tree based on uncorrected distances between *rbc*L sequences from 42 Gelidiales specimens. Only bootstrap values (10,000 replications) for major lineages shown. Geographic groups within *Pterocladia caerulescens* indicated by bootstrap values A, B and C are Hawai'i, southwestern Caribbean and southern Africa, respectively.

in all but the *P. 'caerulescens'* southern Africa and *P. 'caerulescens'* Caribbean comparison when the specimens are partitioned in this manner. Although these values are relatively low the "intraspecific" or within geographic group *rbc*L divergences are also low (maximally 0.44%) resulting in barcoding gap values similar to, or much greater than those calculated for the *Gelidium pristoides* –

Table 2. Intra- and interspecific *rbc*L and COI sequence divergence (%) ranges for specimens of *Gelidium pristoides* and *G. foliaceum*. Barcoding gap values are shown above the diagonal.

		Gelidium pristoides	*Gelidium foliaceum*
Gelidium pristoides	(*rbc*L)	0.00-0.52	3.69x
(n = 3)	(COI)	0.30-1.36	5.58x
Gelidium foliaceum	(*rbc*L)	1.92-2.07	0.00-0.22
(n = 3)	(COI)	7.59-8.61	0.15-0.44

Table 3. Intra- and interspecific *rbc*L and COI sequence divergence (%) ranges for specimens of *Gelidium crinale* and *G. coulteri*. Barcoding gap values are shown above the diagonal.

		Gelidium crinale	*Gelidium coultieri*
Gelidium crinale	(*rbc*L)	0.00-1.12	3.00x
(n = 11)	(COI)	0.00-2.65	4.83x
Gelidium coulteri	(*rbc*L)	3.36-5.65	0.22-0.83
(n = 4)	(COI)	12.80-13.40	0.00-0.30

Table 4. Intra- and interspecific *rbc*L and COI sequence divergence (%) ranges for specimens classified as *Pterocladiella caerulescens* and *P. psammophila*. Barcoding gap values are shown above the diagonal.

		Pterocladiella caerulescens	*Pterocladiella psammophila*
Pterocladiella caerulescens	(*rbc*L)	0.00-2.22	<1.00x
(n = 11)	(COI)	0.00-5.58	1.48x
Pterocladiella psammophila	(*rbc*L)	2.04-2.80	0.00-0.11
(n = 4)	(COI)	8.28-10.69	0.00-0.15

Table 5. Intra- and interspecific *rbc*L and COI sequence divergence (%) ranges for specimens of the *Pterocladiella 'caerulescens'* complex (*Pcaer*) subdivided into geographic groups and *P. psammophila* (*Ppsam*). Barcoding gap values are shown above the diagonal.

		Pcaer Hawai'i	*Pcaer SAfrica*	*Pcaer Caribbean*	*Ppsam*
Pcaer Hawai'i	(*rbc*L)	0.07	4.86x	30.71x	4.64x
(n = 2)	(COI)	0.15	3.54x	14.10x	7.08x
Pcaer SAfrica	(*rbc*L)	2.14-2.22	0.07-0.44	2.84x	20.73x
(n = 3)	(COI)	4.82-5.42	0.45-1.36	3.10x	61.27x
Pcaer Caribbean	(*rbc*L)	2.15-2.21	1.18-1.25	0.00-0.00	18.55x
(n = 6)	(COI)	4.37-4.67	4.22-5.58	0.00-0.31	26.71x
Ppsam	(*rbc*L)	2.28-2.80	2.04-2.43	2.04-2.51	0.00-0.11
(n = 4)	(COI)	9.19-9.49	9.64-10.69	8.28-8.74	0.00-0.15

G. foliaceum and *G. coulteri* – *G. crinale* comparisons. The distinction between the *P. 'caerulescens' complex* geographic groups is even clearer in the COI data, and there is also a greater separation between these groups and *P. psammophila* (Table 5, Fig. 1). Interestingly, the *rbc*L barcoding gap is greater than the COI

value for three of these comparisons. However, this is a product of the low maximum intraspecific divergence values used for this calculation and not greater interspecific variability in *rbc*L vs. COI.

When the *P. 'caerulescens'* complex samples are treated as three different taxa, comparisons among them and the *P. psammophila* samples reflect differences among cryptic species. As previously noted, the Caribbean Costa Rica *P. 'caerulescens'* specimen was originally described as a different species, *P. beachiae*, based on the developmental morphology of the cystocarp and *rbc*L sequence data (Thomas & Freshwater, 2001). Subsequent study of the southern African *P. 'caerulescens'* specimens found the same cystocarp morphology suggesting that it may be a characteristic of *P. caerulescens* not recognized prior to the description of *Pterocladiella beachiae* (Tronchin & Freshwater, 2007). Tronchin & Freshwater (2007) also found no clear distinction between *P. beachiae* and *P. caerulescens* in *rbc*L analyses and consequently proposed that they represented the same species. The increased sampling of Caribbean specimens and current COI analyses indicate that the three *P. 'caerulescens'* geographic groups represent distinct species. More extensive sampling of *P. caerulescens* throughout its reported range, and especially from the type locality in New Caledonia, is needed to determine if additional species are included under this name. *Pterocladiella beachiae* is proposed as the name for the Caribbean taxon identified as *P. caerulescens* and a new species is described for the southern African taxon similarly identified.

Pterocladiella australafricanensis E.M. Tronchin *et* D.W. Freshwater **sp.nov. Fig. 3**

Thalli usque ad 3 cm alt., compositi erectorum axium exorientium compressis prostratisque axibus, 200-400 µm diam., affixi in substrato hapteris coalescentium parallelorum rhizoidalium filamentorum. Erecti axes lanceolati usque ad ligulati, subteretes proxime, complanati distaliter 1.0-1.5 mm wide, cum usque ad duobus ordonibus alternati usque ad pinnati ramificationis. Rami ligulati usque ad lanceolati, acuminati proxime cum obtusatis, saepe emarginatis, apicibus. In transversali sectione externae corticales cellulae anticlinaliter elongata 7-13 × 5.0-7.5 µm; internae corticales cellulae angulares, 9-17 × 6.5-14.5 µm, medullares cellulae elongatae parallelae ad axem, 11-17 µm diam. Rhizinae plerumque in medulla, dispersae in interno cortice. Structurae fertiles productae distaliter in axibus ultimis ramis, multae interdum seriatae super eodem ramum. Carpogonii fecundatio formans cylindricale placentale centrum compositum nutriciarum cellularum filamentorum cingentium axialia filamenta postea circumcinctorum gonimoblasti filamentis producentibus radialiter evolutantia carposporangialia filamenta undique placentalis centri. Placentalis centrum centraliter cystocarpii cavite omnino evolutionem. Cystocarpium maturum unilocularis cum pericarpio elevato plerumque in unilatere paginae ubi unicum ostiolum sine peristomio evolutum. Tetrasporangiales sori sine sterilibus marginibus evoluti in ramorum tumores. Tetrasporangia cruciatim divisa. Spermatangia non visa.

Thalli up to 3 cm tall composed of erect axes arising from compressed prostrate axes, 200-400 µm in diameter, attached to substrate by holdfasts of coalescent parallel rhizoidal filaments. Erect axes lanceolate to ligulate, subterete proximally, flattened distally 1.0-1.5 mm wide, with up to two orders of alternate to pinnate branching. Branches ligulate to lanceolate, tapering proximally and with obtuse, often emarginated, apices. In transverse section outer cortical cells anticlinally elongated 7-13 × 5.0-7.5 µm; inner cortical cells angular, 9-17 × 6.5-14.5 µm, and medullary cells elongated parallel to axis, 11-17 µm in diameter. Rhizines predominantly in medulla, scattered in inner cortex. Fertile structures

Fig. 3. *Pterocladiella australafricanensis* holotype specimen (BOL) from Sodwana Bay, KwaZulu-Natal Province, South Africa. Scale = 2 mm.

produced distally on axes and ultimate branches, multiple fertile structures sometimes produced in series on same branch. Carpogonium fertilization initiates formation of a cylindrical placental core consisting of nutritive cell filaments that surround the axial filament and are subsequently surrounded by gonimoblast filaments that produce radially developing carposporangial filaments on all sides of the placental core. Placental core centrally positioned in cystocarp cavity throughout development. Mature cystocarp unilocular with pericarp elevated mostly on one side of blade where a single ostiole without a peristome develops. Tetrasporangial sori without sterile margins develop in branch swellings. Tetrasporangia cruciately divided. Spermatangia not observed.

Holotype: Four Buoy Reef, Sodwana Bay, KwaZulu-Natal Province, South Africa, -12 m: *Tronchin*# S49 (Coll. E.M. Tronchin) 10.ii.2001 (BOL).

Paratypes: Seven Mile Reef, Sodwana Bay, KwaZulu-Natal Province, South Africa, -17 m: KZN2K4-53 (Coll. D.W. Freshwater) 17.iv.2004 (BOL). Two Mile Reef, Sodwana Bay, KwaZulu-Natal Province, South Africa, -10 m: *Tronchin*# P17 (Coll. E.M. Tronchin) 10.ii.2001, wet preserved (BOL). Bikini Reef, Sodwana Bay, KwaZulu-Natal Province, South Africa, -22 m: KZN2K4-64 (Coll. D.W. Freshwater) 18.iv.2004, wet preserved (BOL). Ribbon Reef, Sodwana Bay, KwaZulu-

Natal Province, South Africa, -17 m: KZN2K4-72 (Coll. D.W. Freshwater) 9.iv.2004, wet preserved (BOL). Doodles Reef, Punta Do Ouro, Mozambique, -18 m: PDO017 (Coll. D.W. Freshwater) 14.iv.2004, wet preserved (BOL). Texas Reef, Punta Do Ouro, Mozambique, -15 m: SA04-095 and WNC2006-028 (Coll. D.W. Freshwater) 15.iv.2004 (WNC).

Etymology: the specific epithet refers to southern Africa from where the species is described.

A detailed description and additional figures of this species are included in Tronchin & Freshwater (2007) under the name *Pterocladiella caerulescens*.

CONCLUSIONS

The results presented here show that COI barcoding is an excellent strategy for distinguishing Gelidiales species, especially when they are very closely related. Substantial COI barcoding gaps were found between the species pairs examined and cryptic species identified. Sequences of *rbc*L can also be used for the identification of Gelidiales species in many cases, but this locus does not fulfill the universality requirement of a barcode marker and distinguishing closely related species may be problematic. Advantages of *rbc*L are the expanding baseline dataset that is already available to researchers and the presence of phylogenetic signal that may be lost in the more variable COI marker. However, the utility of COI for phylogenetic analyses still needs to be assessed, and similar to its advantages over *rbc*L for barcoding closely related species, it may prove better at resolving their evolutionary relationships.

Acknowledgments. The authors gratefully acknowledge S. Fredericq and L. Le Gall for translating the abstract into French, and S. Fredericq for providing the Latin diagnosis. Funding for this research was provided by US National Science Foundation grants DEB-9726170, DEB-0328491 and DEB-0742437, CMS DNA Algal Trust and an award from the Roger Williams University Foundation to Promote Teaching and Scholarship.

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