

# Genetic circumscription of deep-water coral species in Canada using 18S rRNA

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**Abstract.** Many deep-water coral species have very broad global distributions and are eurybathic from depths of meters to kilometers. Such ecological breadth may be confounded by the presence of cryptic species. We are currently comparing the genetic distances between *Paragorgia* sp. and *Primnoa* sp. across their distribution and depth range in Canada using 18S ribosomal DNA (rDNA) sequences. Initial results show a confusing picture amongst the geographically distant *Paragorgia* taxa. Specimens of *P. arborea* from the Canadian Atlantic are very divergent from the specimen from the Canadian Pacific. The placement of *Pennatulula* and *Anthomastus* relative to these taxa is also unexpected. We expect this topology to alter with the addition of more taxa and further testing.

**Keywords.** *Paragorgia*, *Antipatharian*, *Anthomastus*, *Pennatulula*, molecular genetics, 18S rDNA, Atlantic Canada

## Introduction

Globally, five major taxonomic groups of deep-water corals (DWCs) have been identified (Cimberg et al. 1981), including: Alcyonacea (soft corals), Gorgonacea (sea fans, bamboo corals, and tree corals), Scleractinia (cup corals, stony corals, or true corals), Stylasterina (hydrocorals), and Antipatharia (black corals), many which appear to be concentrated in the northern latitudes. Along the eastern coast of North America, 35 species of DWCs are believed to exist (Breeze et al. 1997) at depths ranging between 100 and 1,300 m but have been observed at depths greater than 4,000 m (Tendal 1992; Cairns and Chapman 2001; Gass 2002), and include seven soft corals (alcyonaceans), ten horny corals (gorgonians), eight sea pens (pennatulaceans), and ~ten stony corals (scleractinians). Approximately ten species of gorgonian corals are known to occur off the coast of Nova Scotia in the Northwest Atlantic (Breeze et al. 1997). Of these, *Paragorgia arborea* (Linnaeus, 1758) is the largest, attaining heights of greater than 2 m. It is eurybathic, with a global depth range of between 50 and 3000 m (Horridge 1956; Broch and Horridge

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1957; Tendal 1992). *P. arborea* is frequently found with a second gorgonian coral, *Primnoa resedaeformis* (Gunnerus, 1763). It is known that *P. resedaeformis* (the red tree coral) appears to occur in “forests/thickets”, but little more is known about these corals (Riley 2001). In Atlantic Canada, this species has a broad distribution and depth range. Given the apparently patchy distribution of these corals and their broad depth range, their taxonomic and reproductive biology is of great interest.

Molecular analyses of tropical corals have revealed substantial genetic differences between otherwise taxonomically cryptic species (Knowlton 1993). We speculate that *P. arborea* and *P. resedaeformis* harbour cryptic taxa. These could occur geographically between oceanic boundaries, and/or vertically with depth.

Our goal is to build a comparative data set of *Paragorgia* and *Primnoa* from different localities and depth ranges, based initially on 18S rDNA gene sequences for comparison with published coral sequences (Berntson et al. 1999, 2001; Le Goff-Vitry et al. 2004).

## Methods

Specimens of DWCs and associated fauna used for this study were primarily collected from Stone Fence, an area located east of Sable Island along Canada’s eastern coast (~44°47’N and 57°17’W). Samples from distant locations (e.g., the Bahamas - *Paragorgia johnsoni*) were provided by the Smithsonian Institute. All samples used were specimens preserved in ethanol.

### DNA extraction from corals

To eliminate ethanol and surface contaminants, each sample was thoroughly washed in phosphate buffered saline solution (PBS; Fischer Scientific, USA). DNA was obtained by dissecting approximately 100 mg of tissue and extracting the DNA using a DNeasy tissue kit (Qiagen, Canada) according to the manufacturer’s protocol.

### Polymerase chain reaction (PCR) amplification

Coral 18S rDNA was amplified by PCR using a modified method following Berntson et al. (1999, 2001). The 18S region was amplified using two overlapping PCR products (primers A<sub>1</sub> and B<sub>1</sub>; Table 1). PCR reactions consisted of 1 nmole of A<sub>1</sub> or B<sub>1</sub> primers and a corresponding internal oligonucleotide primer (primers A<sub>2-5</sub>, B<sub>2-5</sub>; see Table 1), 200 nmoles of each dNTP, 1 unit of Taq DNA polymerase (MBI Fermentas, Canada), and approximately 50 to 100 ng of DNA template, in a final volume of 50 µl.

Thermal cycling was as follows: denaturation at 94°C for 60 s, annealing at 57°C for 30 s, and extension at 72°C for 60 s, repeated 39 times, followed by one cycle with a 10 min extension at 72°C. The instrument used was a MJ Dyad (MJ Research, USA). PCR products were purified using a QIAquick PCR Purification (Qiagen) kit according to the manufacturer’s protocol. To determine the molecular size of the PCR products, all samples were run on 1.0 % agarose gels. For samples that did not amplify or for aged archival specimens, 1 µl of an initial PCR reaction

**Table 1** Primers used to amplify and sequence 18S rDNA from deep-water fauna. Primers are listed as: “A<sub>1</sub>-A<sub>5</sub>” representing forward primers and “B<sub>1</sub>-B<sub>5</sub>” representing reverse primers. Primers A<sub>1</sub> and B<sub>1</sub> were located according to the DNA sequence of the 18S rDNA gene from *Paragorgia* sp. (AF052917; Berntson et al. 2001); the remaining primers were constructed. Primer locations (PL) are given with respect to the sequence given in AF052917

Primers					
Forward (A <sub>1</sub> – A <sub>5</sub> )	PL <sup>1</sup>	Reverse Compliment (B <sub>1</sub> – B <sub>5</sub> )	PL <sup>1</sup>		
A <sub>1</sub>	AACCTGGTTGATCCTGCCAGT	2-22	B <sub>1</sub>	TGATCCTTCTGCAGGTTCCACCTAC	1776-1799
A <sub>2</sub>	TGAAACTGCGAATGGCTCAT	81-100	B <sub>2</sub>	TTGACCAACTTCTCGGCGG	1715-1734
A <sub>3</sub>	TAATTCAGCTCCAATAGCG	582-601	B <sub>3</sub>	CTGGACCTGGTAAGGTTTCC	1202-1221
A <sub>4</sub>	ACGATGCCGACTAGGGATGA	1031-1050	B <sub>4</sub>	CCTGCTTTGAACACTCTAATTCT	759-782
A <sub>5</sub>	CGTCGTGATGGGAATAGATC	1531-1550	B <sub>5</sub>	GGGACTTTTCGCATGTATTAGC	163-184

using universal eukaryotic coral primers A<sub>1</sub> and B<sub>1</sub> (see methods of Elwood et al. 1985; Lane et al. 1985; Weekers et al. 1994; Takabayashi 2000; Berntson et al. 2001) was purified and used as template for the reactions using our constructed internal primers (A<sub>2</sub>-A<sub>5</sub>, B<sub>2</sub>-B<sub>5</sub>; Table 1).

### Sequencing of amplified 18S rDNAs

PCR amplicons (~40-80 ng) were sequenced using the BigDye Terminator Cycling Sequencing Ready Reaction Kit v 3.1 (Applied Biosystems, CA, USA) following the manufacturer’s recommendations. Eight to ten 18S rDNA-specific primers were used in the sequencing reactions (Table 1). Sequencing reactions were analyzed using an MJ Base Station (MJ Research, USA) automated DNA sequencer and Cartographer software (MJ Research, USA). The sequence of the amplicons was assembled using Contig Express from Vector NTI Advance (Informax, USA).

Evolutionary analyses were done using GeneStudio Pro<sup>®</sup>. A blast search using GeneStudio Pro<sup>®</sup> was performed in the National Centre for Biotechnology Information (NCBI) GenBank site and matching homologous 18S rDNA sequences were retained for multiple alignment using CLUSTAL W (Thompson et al. 1994). A data set of 46 sequences was constructed using aligned nucleotides based on sequences with a minimum length of 1700 nucleotides. Two out-groups, *Anemonia sulcata* and Hormathiidae gen. sp. (Table 2), were used following Berntson et al. (1999, 2001).

To reduce the likelihood of poor tree representation given the fact that different tree methods are designed on the basis of different evolutionary theories (Steel and Penny 2000), and therefore different algorithms, we chose 3 commonly used tree-building criteria to compare our data. Data were analysed using maximum likelihood (ML), minimum evolution (ME), and Bayesian likelihood analysis (BL). In ML methods, a search for the topology that maximizes the chance of observing a given probabilistic model of nucleotide substitutions was used (Felsenstein 1981). Data sets comprised of more than 7 sequences, however, are computationally demanding and time-consuming (DeBry and Abele 1995) and as a result, we chose to use the quartet-puzzling algorithm (PHYLIP; Felsenstein 1990), setting the bootstrap function to 1,000. MEGA (Molecular Evolutionary Genetics Analysis V2.1;

**Table 2** List of 18S rDNA gene sequences of deep-water taxa used in this study consisting of >1700 nucleotides. Accession numbers are related to National Centre for Biotechnology Information (NCBI) coding. Corals analysed in this study are given in bold. Some authors (\*) listed under 'Citation' submitted gene sequences to NCBI which may not be published; controls used for tree formation are denoted using the symbol “†”

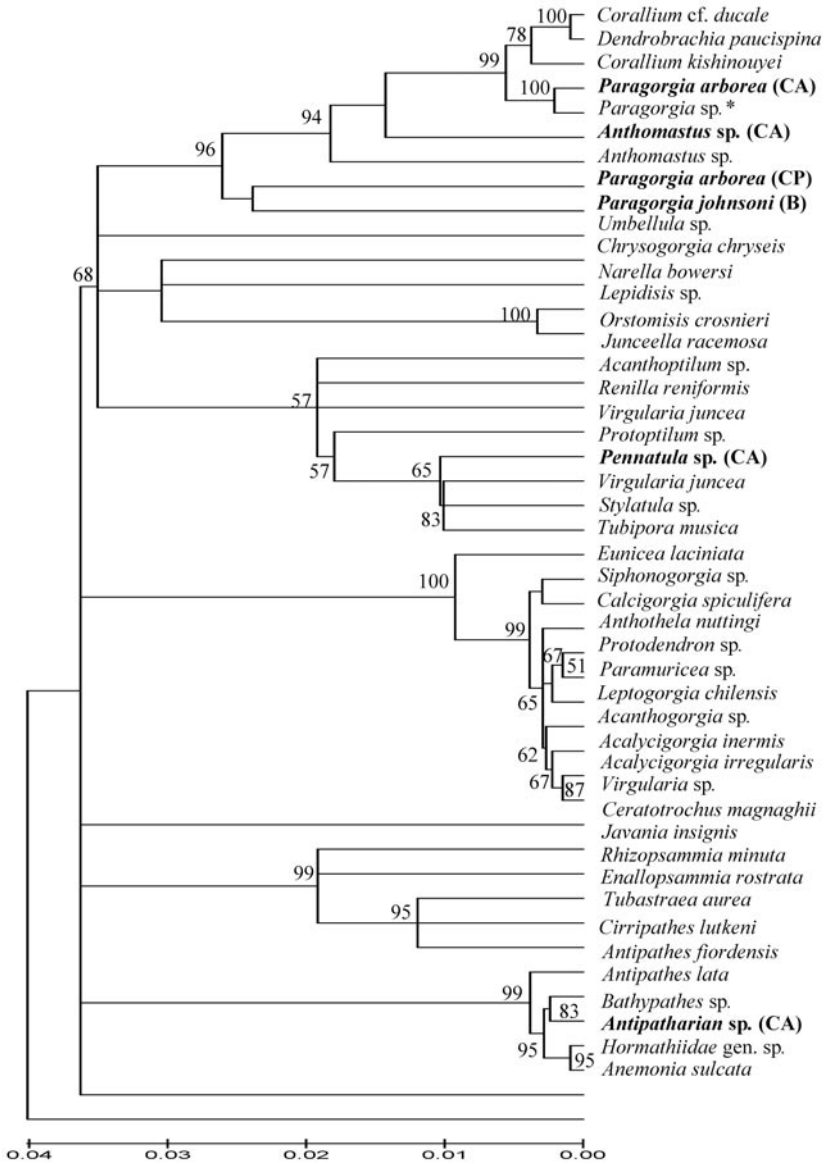
Scientific name	Accession #	Nucleotides (bp)	Citation
<i>Junceella racemosa</i>	AF052937	1800	Berntson et al. 2001
<i>Acalyigorgia inermis</i>	AJ133545	1822	Won et al. 2001
<i>Acalyigorgia irregularis</i>	AJ133546	1821	Won et al. 2001
<i>Acanthogorgia</i> sp.	AF052907	1852	Berntson et al. 1999
<i>Acanthoptilum</i> sp.	AF052910	1802	Berntson et al. 1999
<i>Anthomastus</i> sp.	AF052881	1798	Berntson et al. 2001
<i>Anthothela nuttingi</i>	AF052922	1822	Berntson et al. 2001
<i>Antipathes fiordensis</i>	AF052900	1798	Berntson et al. 1999
<i>Antipathes lata</i>	Z92908	1798	Song and Won 1997
<i>Bathypathes</i> sp.	AF052901	1798	Berntson et al. 1999
<i>Ceratotrochus magnaghii</i>	F052886	1814	Berntson et al. 1999
<i>Calcigorgia spiculifera</i>	AF052925	1852	Berntson et al. 2001
<i>Chrysogorgia chryseis</i>	AF052913	1811	Berntson et al. 2001
<i>Cirripathes lutkeni</i>	AF052902	1798	Berntson et al. 1999
<i>Corallium</i> cf. <i>ducale</i>	AF052919	1801	Berntson et al. 2001
<i>Corallium kishinouyei</i>	AF052918	1801	Berntson et al. 2001
<i>Dendrobrachia paucispina</i>	AF052903	1803	Berntson et al. 1999
<i>Enallopsammia rostrata</i>	AF052885	1797	Berntson et al. 1999
<i>Eunicea laciniata</i>	AF052926	1851	Berntson et al. 2001
<i>Javania insignis</i>	AJ133555	1805	Won et al. 2001
<i>Virgularia gustaviana</i>	Z86106	1805	Kim et al.*
<i>Lepidisis</i> sp.	AF052906	1825	Berntson et al. 1999
<i>Leptogorgia chilensis</i>	AF052928	1823	Berntson et al. 2001
<i>Narella bowersi</i>	AF052905	1799	Berntson et al. 1999
<i>Orstomisis crosnieri</i>	AF052916	1828	Berntson et al. 2001
<i>Paramuricea</i> sp.	AF052920	1850	Berntson et al. 2001
<i>Protodendron</i> sp.	AF052921	1823	Berntson et al. 2001
<i>Protoptilum</i> sp.	AF052911	1799	Berntson et al. 1999
<i>Renilla reniformis</i>	AF052581	1800	Berntson et al. 1999
<i>Rhizopsammia minuta</i>	Z92907	1799	Song and Won 1997
<i>Siphonogorgia</i> sp.	AF052927	1823	Berntson et al. 2001
<i>Stylatula</i> sp.	AF052934	1798	Berntson et al. 2001
<i>Tubastraea aurea</i>	Z92906	1797	Song and Won 1997
<i>Tubipora musica</i>	AF052909	1837	Berntson et al. 2001
<i>Umbellula</i> sp.	AF052904	1802	Berntson et al. 1999
<i>Virgularia juncea</i>	AJ133551	1800	Won et al. 2001
<i>Virgularia</i> sp.	AF052935	1804	Berntson et al. 2001
<b><i>Paragorgia arborea</i> (CP)</b>	AY838559	1800	Seq7-2
<b><i>Antipatharian</i> sp. (CA)</b>	AY838560	1763	SeqF-2
<b><i>Pennatula</i> sp. (CA)</b>	AY838561	1806	SeqK-2
<b><i>Paragorgia johnsoni</i> (B)</b>	AY838562	1734	Seq9-2
<b><i>Paragorgia arborea</i> (CA)</b>	AY838563	1802	SeqJ-2
<b><i>Anthomastus</i> sp. (CA)</b>	AY838564	1792	SeqM-2
<i>Anemonia sulcata</i> †	X53498	1799	Hendriks et al. 1990
Hormathiidae gen. sp.†	AF052890	1800	Berntson et al. 1999
<i>Paragorgia</i> sp.	AF052917	1799	Berntson et al. 2001

Kumar et al. 2001) was used to determine a best-fit ME model using p-distance sequence divergence. ME analysis, unlike ML, 'assumes' the shortest evolutionary route. Bootstrap replication (1,000) was used to test relative nodal support in the topology (Felsenstein 1985). Bayesian analysis was implemented using Mr Bayes Version 3.0b4 (Huelsenbeck and Ronquist 2001) utilizing the gamma function, "4-by-4" model, sampling 1,000,000 generations, with Markov chains sampled every 100 iterations. The analysis was conducted three times using identical settings to ensure convergence on the same  $\ln L$  values (Huelsenbeck and Bollback 2001; Leaché and Reeder 2002). Approximately 33,000 random iterations generated prior to stationarity (call "burn-in generations"; Huelsenbeck and Ronquist 2001) were discarded and the subsequent generations used to establish the posterior probabilities. Trees were displayed using TreeView Version 1.6.0 (Page 1996).

## Preliminary results and discussion

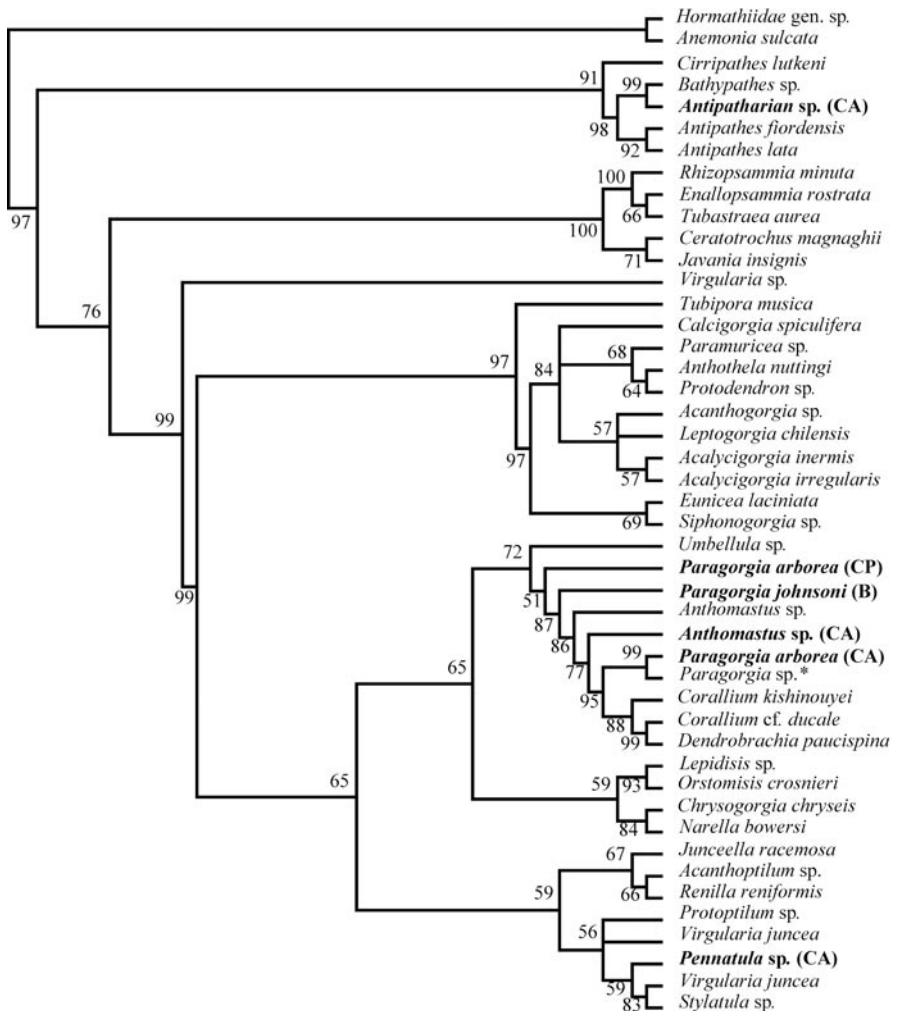
Initial results comparing tree topologies of ME (Fig. 1), ML (Fig. 2) and BL (Fig. 3) show an interesting picture amongst the geographically distant *Paragorgia* taxa. Specimens of *P. arborea* from the North Atlantic (Nova Scotia, Canada) are very similar to *Paragorgia* sp. from the South Pacific (California, USA; >99 % nodal support among all three trees). *P. arborea* from the North Pacific (British Columbia, Canada) however, appear more closely related *P. johnsoni* from Bermuda (Fig. 3; 100 % support). The placement of *Anthomastus* sp. relative to these taxa is also unexpected (Figs. 1-3). Berntson et al. (2001) suggest that *Anthomastus* relative to *Paragorgia*, *Corallium* and *Umbellula* share many morphological features and are dimorphic, however, resemblances are superficial and further comparisons are needed.

There are several possible reasons which may explain the disparities between the topology of *Anthomastus*, *Corallium* and *Umbellula* relative to *Paragorgia*. All target genes may not amplify with the same efficiency expected using octocoral-specific designed primers. Such biases inherent to natural mixes of rRNA genes are in the early stages of being investigated (see von Wintzingerode et al. 1997). Gene templates with high G+C content, for example, are not as efficiently amplified as are those with low G+C content (Reysenbach et al. 1992). In contrast, Moon-van der Staay et al. (2000) suggest that templates with G/C content, when amplified with degenerated primers, amplify better than those with A/T content (Polz and Cavanaugh 1998). Moon-van der Staay et al. (2000) also suggest that non-complementarity of either PCR primer may decrease PCR yield through inhibition of binding targets during high annealing temperatures. Hansen et al. (1998) further suggest that some amplified sequences may be inhibited by DNA flanking the template region. Further, the presence of excess templates during reannealing may reduce amplification of templates initially present in high abundance (Suzuki and Giovannoni 1996). As a result primer specificity for some copies of the 18S rDNA may be responsible for the unusual results of such phylogenetic analyses. We expect this topology to alter with the addition of more taxa.



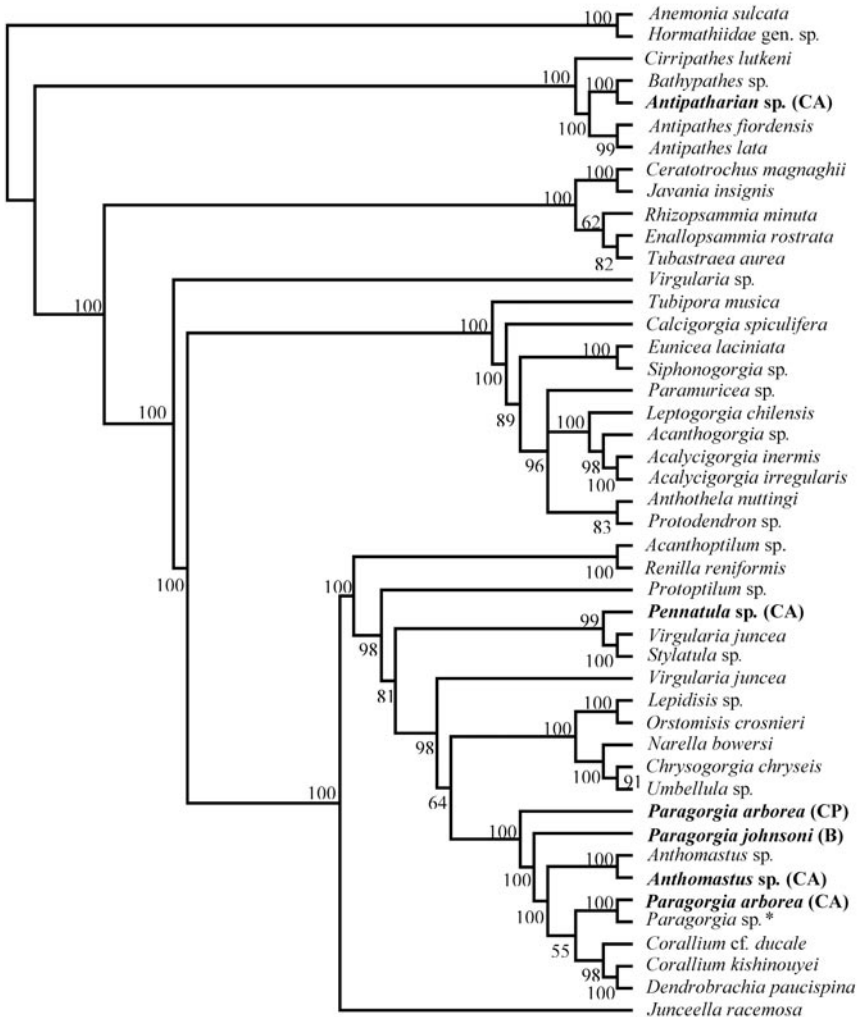
**Fig. 1** Phylogenetic tree from minimum-evolution (ME) analysis. Bootstrap analysis ( $n = 1,000$  replicates) provided an estimate of support for internal nodes ranging from 50 to 100 %. The scale bar indicates 0.05 substitutions per nucleotide position. *Paragorgia* sp.\* represents “control” gene from Berntson et al. (1999, 2001); outgroups used for tree construction were Hormathiidae gen. sp. (AF052890; Berntson et al. 1999) and *Anemonia sulcata* (X53498; Hendriks et al. 1990). Symbols CA = Canadian Atlantic; CP = Canadian Pacific; B = Bermuda

Placement of *Antipatharian* sp. (Canadian Atlantic (CA)) with *Bathypathes*, *Antipathes fiordensis* and *A. lata* was not unexpected since these taxa have similar morphological characteristics (Figs. 1-3). However, given that the *Antipatharian*



**Fig. 2** Phylogenetic tree from maximum likelihood (ML) quartet-puzzling analysis. Bootstrap analysis ( $n = 1,000$  puzzling steps) provided an estimate of support for internal nodes ranging from 50 to 100 %. Note that branch lengths are arbitrary and only the pattern is important. *Paragorgia* sp.\* represents “control” gene from Berntson et al. (1999, 2001); outgroups used for tree construction were *Hormathiidae* gen. sp. (AF052890; Berntson et al. 1999) and *Anemonia sulcata* (X53498; Hendriks et al. 1990). Symbols CA = Canadian Atlantic; CP = Canadian Pacific; B = Bermuda

sp. (CA), *Bathypathes* sp. (Hawaii - Pacific), *Antipathes fiordensis* (New Zealand) and *A. lata* (South Korea) are from geographically distant regions, we question the level of genetic similarity (ME = 99 %, ML = 56 %, BL = 81 %). Similarly,



**Fig. 3** Phylogenetic tree from bayesian-likelihood (BL) analysis using the gamma function, 4-by-4 model, sampling 1,000,000 generations, with Markov chains sampled every 100 iterations. Above node support is the 50 %-majority-rule consensus from the sampled trees. Values <50 % are not displayed. Note that branch lengths are arbitrary and only the pattern is important. *Paragorgia* sp.\* represents “control” gene from Berntson et al. (1999, 2001); outgroups used for tree construction were *Hormathiidae* gen. sp. (AF052890; Berntson et al. 1999) and *Anemonia sulcata* (X53498; Hendriks et al. 1990). Symbols CA = Canadian Atlantic; CP = Canadian Pacific; B = Bermuda

the topology of our *Pennatula* sp. (CA) amongst *Virgularia* sp., *Stylatula* sp. and *Protoptilum* sp. was consistent between our tested methodologies (ME, ML and BL; Figs. 1-3 respectively). These species, however, are geographically scattered amongst the Pacific and Atlantic Oceans, and Yellow Sea (South Korea). As with previous molecular phylogenetic analyses (e.g., Bertson et al. 1999, 2001; Won et al. 2001), our present trees indicate problematic areas in the current classification of corals.

Attempts to explain why deep-water coral isolates from geographically different regions appear closely related have been hampered by a lack of knowledge of transmission of larvae from one host to another. We propose, although speculative, that the isolation of distinct DWC genes, and different clades and subclades as described by Bertson et al. (2001), is the result of shifting oceanic circulation (Benzie 1999) and global warming and cooling trends (Bluemle et al. 1999), where larval transmission is related to changing environments and host-specific signals. During periods of low sea levels and colder water temperatures, geographical barriers may have promoted coevolution of various DWCs to produce one or more dominant clades per geographical location. As sea levels rose due to global warming and polar ice cap melting (Chappell 1983; Digerfeldt and Hendry 1987; Barnett 1988; Jones and Wigley 1990; Monastersky 1994), changing current patterns and environmental stimuli has probably resulted in limited reproductive events, decreasing diversity and increasing the number of hybridization events (and thus the significant similarity observed between geographically distant populations). Under stressful environmental conditions, Lamb and Avise (1986), Lehman et al. (1991) and Wayne et al. (1992) indicate the likelihood of hybridization increases as species become rare. Further testing using polymorphic nuclear markers, such as microsatellites and allozymes, are techniques that could be used to test our speculation.

No information is available yet for *Primnoa*, or from depth-stratified samples from the Scotian Shelf.

In conclusion, the present study demonstrates for the first time that *Paragorgia* sp. from Eastern and Western Canada, although appearing similar in morphology, are genetically dissimilar. Further, other genera appear more closely related to *Paragorgia* than previously speculated. Such new light potentially provides a new assessment of the relative taxonomic distribution and abundance of the various groups of deep-water corals, associated fauna, and cryptic taxa and to their morphologies. It may also reveal new lineages that may have considerable ecological and/or taxonomic importance. It is clear, however, that more molecular analyses are required to reveal such taxonomic implications.

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