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DNA Barcoding the Medusozoa using mtCOI

Brian D. Ortman [a,*], Ann Bucklin [a], Francesc Pagès [b,1], Marsh Youngbluth [c]

[a] Department of Marine Sciences, University of Connecticut, Groton, CT, USA
[b] Institut de Ciències del Mar (CSIC), Barcelona, Spain
[c] Harbor Branch Oceanographic Institution, Fort Pierce, FL, USA

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ABSTRACT

The Medusozoa are a clade within the Cnidaria comprising the classes Hydrozoa, Scyphozoa, and Cubozoa. Identification of medusozoan species is challenging, even for taxonomic experts, due to their fragile forms and complex, morphologically-distinct life history stages. In this study 231 sequences for a portion of the mitochondrial Cytochrome Oxidase I (mtCOI) gene were obtained from 95 species of Medusozoans including; 84 hydrozoans (61 siphonophores, eight anthomedusae, four leptomedusae, seven trachymedusae, and four narcomedusae), 10 scyphozoans (three coronatae, four semaeostomae, two rhizostomae, and one stauromedusae), and one cubozoan. This region of mtCOI has been used as a DNA barcode (i.e., a molecular character for species recognition and discrimination) for a diverse array of taxa, including some Cnidaria. Kimura 2-parameter (K2P) genetic distances between sequence variants within species ranged from 0 to 0.057 (mean 0.013). Within the 13 genera for which multiple species were available, K2P distance between congeneric species ranged from 0.056 to 0.381. A cluster diagram generated by Neighbor Joining (NJ) using K2P distances reliably clustered all barcodes of the same species with $\geq$99% bootstrap support, ensuring accurate identification of species. Intra- and inter-specific variation of the mtCOI gene for the Medusozoa are appropriate for this gene to be used as a DNA barcode for species-level identification, but not for phylogenetic analysis or taxonomic classification of unknown sequences at higher taxonomic levels. This study provides a set of molecular tools that can be used to address questions of speciation, biodiversity, life-history, and population boundaries in the Medusozoa.

1. Introduction

1.1. Medusozoan species diversity and identification

The Medusozoa are a subphylum of the Phylum Cnidaria comprising approximately 3800 species in three classes: Hydrozoa, Cubozoa, and Scyphozoa (Daly et al., 2007). Medusozoans encompass a diversity of forms, including benthic and pelagic life-history stages with many species having both phases within their life-cycle. They are distinguished from their sister group, the Class Anthozoa (Bridge et al., 1995; Collins, 1998, 2003), by a medusoid body form in the life-cycle of some species and a linear mitochondrial genome (Bridge et al., 1992). There has been renewed interest in these species due to dramatic population fluctuations, bloom formation, and biological invasions (reviewed in Mills, 2001; Purcell, 2005, 2007; Graham and Bayha, 2007), as well as increased concern over envenomations affecting human health (Burnett, 2001). Ecologically, medusozoans are voracious predators of zooplankton and can have dramatic effects on ecosystem structure and functioning (Mills, 1995).

Accurate identification of medusozoans is complicated by many factors. These organisms are fragile and easily damaged during traditional collection with nets, due to the high water content of their tissues. As a result, species-level identification of specimens collected in this way, which may depend on subtle morphological characters for many species, is often not possible. Members of the colonial hydrozoan order Siphonophora frequently fragment during net collection with zooids necessary for identification being lost (Totton, 1965). Phenotypic plasticity in size and shape of hydropolyps (e.g. Schuchert, 2001) and scyphistomae (Willcox et al., 2008) as well as morphological divergence associated with geographic distance (Bolton and Graham, 2004) can render morphological characters ambiguous. To further complicate matters, species assignment of medusozoan larval stages (e.g., planulae, actinulae, polyps, and ephyrae) is difficult as frequently these stages are unknown or undescribed. Some hydrozoan polyps have no known distinguishing morphological characters (e.g. Govindarajan et al., 2005b), and some

Abbreviations: mtCOI, mitochondrial cytochrome oxidase I; K2P, Kimura 2-parameter; NJ, neighbor joining; MOTU, molecular operational taxonomic unit

* Corresponding author. Present address: The University of British Columbia, Departments of Botany and Zoology, #3529-6270 University Blvd. Vancouver, Canada BC V6T 1Z4. Tel.: +1 604 910 8407; fax: +1 604 822 6089.
E-mail addresses: bortman@interchange.ubc.ca (B.D. Ortman), Ann.bucklin@uconn.edu (A. Bucklin), Youngbluth@yahoo.com (M. Youngbluth).

[1] Deceased

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scyphozoan polyps can be identified only through microscopic analysis of nematocyst complement (e.g. Calder, 1971). Ephyrae from different species may be differentiated by nematocyst complement (Calder, 1977) or by subtle, but easily damaged, features (e.g. Gröndahl and Hernroth, 1987). In some cases, they may differ solely as a result of environmental conditions (Hernroth and Gröndahl, 1983). Calycophoran siphonophores can have a biphasic life-cycle consisting of polygastric and eudoxid stages that are morphologically distinct or unknown (e.g. Pagès and Pugh, 2002). Other life-history stages such as cysts (planulocysts or podocysts), plannulae, and actinulae cannot be accurately identified using morphological characters. Using morphological characters to accurately assign species based on taxonomy would require comprehensive anatomical studies of every phase in each medusozoan's life-cycle. Cryptic species (i.e., morphologically indistinguishable, yet genetically and evolutionarily distinct) can best be discovered through the use of molecular techniques (Knowlton, 1993, 2000). Cryptic species of scyphozoans have been uncovered using genetic techniques (Dawson and Jacobs, 2001; Dawson and Martin, 2001; Holland et al., 2004).

1.2. DNA Barcoding

An ideal DNA barcode (i.e., a short DNA sequence for species recognition and discrimination) must meet certain empirically-determined criteria. For many metazoan animals, a 660 base-pair region of the mitochondrial Cytochrome Oxidase I (mtCOI) gene has been used as a DNA barcode (Hebert et al., 2003a, 2003b). DNA barcoding initially relies on expert taxonomists to provide positive identification of specimens used for DNA sequencing. Barcodes should be based on DNA sequences from numerous individuals from multiple locations across a species range to accurately characterize levels of intra-specific variation (DeSalle et al., 2005). DNA barcodes should have low levels of intra-specific diversity, yet significant inter-specific divergence. Ideally, levels of intra-specific variation need to be significantly lower than the distance between closely related sister lineages with no overlap between levels of intra- and inter-specific genetic distance leaving a "barcoding gap" (Meyer and Paulay, 2005). An idealized application of barcoding would be a system in which the sequence variants found within a species group together excluding all other species in a cluster diagram based on genetic distance.

A DNA sequence can be used as a molecular operational taxonomic unit (MOTU), which can be indicative of a species or other empirically determined taxon (Blaxter et al., 2005). The utility of the mtCOI gene for species level discrimination has been demonstrated in many marine taxa including bivalves (Hare et al., 2000; Mikkelsen et al., 2007), bryozoans (McGovern and Hellberg, 2003), chitons (Kelly et al., 2007), gastropods (Meyer and Paulay, 2005) and fish (Ward et al., 2005; Ward and Holmes, 2007), as well as important zooplankton groups such as euphausiids (Bucklin et al., 2007) and copepods (Bucklin et al., 1998, 1999, 2003; Hill et al., 2001; Rocha-Olivares et al., 2001).

The discovery of low levels of inter-specific mtCOI variation within the Anthozoa suggested that it would not be useful for barcoding this group. For example, mtCOI has been ineffective in barcoding corals (Shearer and Coffroth, 2008), due to slow mitochondrial evolution within the Anthozoa (France and Hoover, 2002; Shearer et al., 2002; Hellberg, 2006). However, sufficient levels of mtCOI variation indicative of speciation have been shown for medusozoan species of the scyphozoan genera *Aurelia* (Dawson and Jacobs, 2001), *Cassiopea* (Holland et al., 2004), *Cyanea* (Dawson, 2005a), subspecies of *Mastigias* (Dawson, 2005b), and the hydrozoan genera *Obelia, Eugymnanthea* (Govindarajan et al., 2004) and *Hydra* (Hemmrich et al., 2007), suggesting mtCOI may be useful within the Medusozoa for purposes of species-level identification. Furthermore, an analysis of pre-existing mtCOI sequences available in GenBank has shown that sequence variation within the classes Hydrozoa and Scyphozoa are comparable to those of typical metazoans (Huang et al., 2008).

For this study, 95 species of Medusozoa were collected from the Gulf of Maine, Gulf of Alaska, Gulf of Mexico, Sargasso Sea, and Norwegian fjords, and are representative of each class and most of the orders. Our goal was to determine whether the patterns of mtCOI variability within and between species will allow mtCOI to be used as a DNA barcode for species discrimination within the Medusozoa. Further, we seek to verify that DNA barcodes can provide the means for a non-specialist to discriminate taxa that would otherwise be difficult if not impossible to identify (Savolainen et al., 2005, Schander and Willassen, 2005). We discuss the future applications of DNA barcodes to examine species boundaries, reveal cryptic species, connect life-history stages, and survey biodiversity.

2. Methods

2.1. Specimen and data collection

Specimens of 95 species of medusozoans were collected using dip nets, plankton nets, MOCNESS (Wiebe et al., 1985) or Johnson Sea-Link submersibles (Youngbluth, 1984) in several areas of the Atlantic Ocean (Sargasso Sea, Gulf of Maine, Gulf of Mexico, Icelandic waters, and Norwegian fjords), as well as the Arctic Ocean, and Northeast Pacific Ocean (Gulf of Alaska). All specimens were identified to the lowest possible taxonomic level by morphological experts for this group. Specimens were preserved in 95% ethanol or flash frozen in liquid nitrogen in accordance with established protocols for the molecular analysis of planktonic metazoans (Bucklin, 2000). DNA extractions, PCR amplification, and sequencing for specimens collected during the Census of Marine Zooplankton (CMarZ) 2006 cruise to the Sargasso Sea were all performed aboard ship immediately after collection and identification. Samples preserved from all other cruises were processed at the University of Connecticut Center of Marine Molecular Analysis (COMMA) laboratory.

DNA was purified from individuals using commercially available Qiagen purification kits (Valencia, CA). A portion of the mtCOI gene was amplified with the universal published primers (Folmer et al., 1994) as well as two additional reverse primers developed for this study (Table 1). PCR protocol was 94 °C for 1 min, 45 °C for 2 min, and 72 °C for 3 min, for 40 cycles. Sequencing was performed directly from purified PCR amplification products on an Applied Biosystems, Inc. (ABI) Model 377 Automated DNA Sequencer or an ABI Prism 3100 4-capillary Genetic Analyzer (Foster City, CA).

2.2. MtCOI sequence analysis

Sequences for multiple individuals per species were generated when possible. All mtCOI sequences were initially aligned using CLUSTAL X (Thompson et al., 1997), and were subsequently adjusted by eye using MacCLADE Ver. 4.0 (Maddison and Maddison, 2000) to ensure correct alignment and placement of insertion/deletion events (indels). GenBank BLAST searches were done on all sequences to confirm the accuracy and validity of the sequences, detect artifactual sequences and any potential pseudogenes. MtCOI sequences were submitted to the National Center for Biotechnology Information (NCBI) GenBank database using the BARCODE submission portal. GenBank Accession numbers include

the nucleotide sequences in text format, amino acid translations, DNA voucher code and specimen ID, collection date, geospatial coordinates of the collection site, PCR primer names and sequences, and names of the persons collecting and identifying specimens. GenBank Accession numbers are provided in Table 2.

Intra- and inter-specific variation was calculated as Kimura 2-Parameter (K2P) genetic distances using MEGA Ver. 4.0 (Tamura et al., 2007). A Neighbor-Joining (NJ) tree was generated from sequences using K2P genetic distances. Genetic distances were calculated between all mtCOI sequences within a species.

Table 1
Name, sequence, size, and source for PCR and sequencing primers used in this study.

Primer Name	Sequence 5′-3′	Estimated size	Reference
LCO-1490	GGTCAACAAATCATAAAGATATTGG		Folmer et al., 1994
HCO-2198	TAAACTTCAGCCTGACCAAAAAATCA	660 bp	Folmer et al., 1994
MedCOIR	GGAACTGCTATAATCATAGTTGC	850 bp	this study
HCO-2607	ACATAGTGGAAATGTGCTACAACATA	1150 bp	Ortman, 2008

Table 2
Taxonomic groups, number of individuals sequenced for mtCOI, number of different sequences, mean intra-specific Kimura 2-Parameter (K2P) distance and S.D., sequence length in number of base pairs (BP), and Genbank Accession numbers for 95 species of Medusozoa analyzed for this study.

Taxon	N	N Variants	K2P Mean	Distance S.D.	BP	Accession Numbers(s)
HYDROZOA						
Siphonophora						
Abylopsis eschscholtzi	6	1	0	n/a	647	GQ119937
Abylopsis tetragona	2	2	0.008	n/a	606	GQ119938-39
Agalma elegans	4	4	0.014	0.004	507	GQ119940-43
Agalma okeni	6	5	0.008	0.003	554	GQ119944-48
Amphicaryon acaula	2	2	0.057	n/a	752	GQ119949-50
Amphicaryon earnesti	2	2	0.02	n/a	751	GQ119951-52
Amphicaryon polifera	1	1	n/a	n/a	803	GQ119953
Apolemia sp.	2	2	0.043	n/a	845	GQ119954-55
Athorybia rosacea	2	1	0	n/a	871	GQ119956
Bargmannia sp. 1	1	1	n/a	n/a	846	GQ119957
Bargmannia sp. 2	1	1	n/a	n/a	939	GQ119958
Bassia bassensis	2	2	0.005	n/a	830	GQ119959-60
Ceratocymba sagittata	2	2	0.012	n/a	830	GQ119961-62
Ceratocymba sp. 1	1	1	n/a	n/a	846	GQ119963
Chuniphyes multidentata	2	2	0.01	n/a	976	GQ119964-65
Dimophyes arctica	1	1	n/a	n/a	796	GQ119966
Diphyes bojani	4	4	0.009	0.002	719	GQ119967-70
Diphyes dispar	4	3	0.007	0.002	566	GQ119971-73
Enneagonum hyalinum	2	2	0.025	n/a	812	GQ119974-75
Erenna sp.	2	1	0	n/a	744	GQ119976
Eudoxoides mitra	5	4	0.011	0.002	712	GQ119977-80
Eudoxoides spiralis	3	3	0.006	0.001	592	GQ119981-83
Forskalia contorta	1	1	n/a	n/a	886	GQ119984
Forskalia tholoides	1	1	n/a	n/a	820	GQ119985
Frillagalma sp.	2	2	0.005	n/a	623	GQ119986-87
Halistemma amphitridis	3	3	0.002	0.001	600	GQ119990,92, GQ120047
Halistemma sp.	2	2	0.003	n/a	792	GQ119989, GQ119991
Hippopodius hippopus	6	6	0.032	0.017	554	GQ119993-98
Kephyes ovata	1	1	n/a	n/a	754	GQ119999
Lensia achilles	1	1	n/a	n/a	831	GQ120000
Lensia campanella	2	2	0.03	n/a	789	GQ120001, GQ120066
Lensia conoidea	1	1	n/a	n/a	822	GQ120002
Lensia exeter	2	1	n/a	n/a	631	GQ120003
Lensia fowleri	8	4	0.008	0.006	549	GQ120004-07
Lensia grimaldii	1	1	n/a	n/a	635	GQ120008
Lensia hospur	1	1	n/a	n/a	628	GQ120009
Lensia meteori	1	1	n/a	n/a	860	GQ120010
Lensia multicristata type 1	2	2	0.01	n/a	644	GQ120011, GQ120013
Lensia multicristata type 2	2	2	0.007	n/a	599	GQ120012, GQ120014
Lilyopsis fluoracantha	1	1	n/a	n/a	654	GQ120015
Lilyopsis rosea	2	2	0.002	n/a	924	GQ120016-17
Maresearsia praeclara	1	1	n/a	n/a	569	GQ120018
Marrus orthocanna	1	1	n/a	n/a	872	GQ120019
Marrus sp. 1	1	1	n/a	n/a	1047	GQ120020
Marrus sp. 2	1	1	n/a	n/a	936	GQ120021
Nanomia bijuga	2	1	0	n/a	997	GQ120022
Nanomia cara	34	7	0.003	0.001	1008	GQ120023-29
Nectopyramis diomedeae	1	1	n/a	n/a	505	GQ120030
Nectopyramis natans	1	1	n/a	n/a	447	GQ120031
Physalia physalis	2	2	0.003	n/a	1005	GQ120032-33
Physalia sp.	1	1	n/a	n/a	1004	GQ120034

Table 2 (*continued*)

Taxon	N	N Variants	K2P Mean	Distance S.D.	BP	Accession Numbers(s)
Physophora hydrostatica	1	1	n/a	n/a	1028	GQ120035
Praya reticulata	3	2	0.031	n/a	487	GQ120036-37
Rhizophysa eysenhardti	3	3	0.03	0.024	525	GQ120038-40
Rhizophysa filiformis	1	1	n/a	n/a	1021	GQ120041
Rosacia cymbiformis	1	1	n/a	n/a	1013	GQ120042
Rosacia sp. 1	2	2	0.081	n/a	836	GQ120043-44
Rosacia sp. 2	1	1	n/a	n/a	1021	GQ120045
Sphaeronectes gracilis	1	1	n/a	n/a	578	GQ120046
Sulculeolaria quadrivalvis	3	3	0.01	0.003	642	GQ120048-50
Vogtia spinosa	1	1	n/a	n/a	1031	GQ120051
Anthomedusae						
Bougainvillia superciliaris	1	1	n/a	n/a	803	GQ120052
Chromatonema rubrum	2	1	0	n/a	854	GQ120053
Corymorpha nutans	3	3	0.008	0.003	831	GQ120054-56
Leuckartiara octona	1	1	n/a	n/a	1028	GQ120057
Margelopsis hartlaubi	4	2	0.003	n/a	782	GQ120058-59
Porpita porpita	1	1	n/a	n/a	827	GQ120060
Sarsia princeps	1	1	n/a	n/a	1000	GQ120061
Sarsia tubulosa	2	2	0.018	n/a	789	GQ120062-63
Leptomedusae						
Clytia languidum	2	2	0.004	n/a	1026	GQ120064-65
Cyclocanna welshi	3	2	0.004	n/a	713	GQ120067-68
Eutonina indicans	3	2	0.001	n/a	805	GQ120069-70
Melicertum octocostatum	1	1	n/a	n/a	840	GQ120071
Trachymedusae						
Aglantha digitale	1	1	n/a	n/a	821	GQ120073
Aglaura hemistoma	2	1	0	n/a	839	GQ120074
Botrynema brucei	2	1	0	n/a	824	GQ120075
Colobonema sericeum	2	2	0.009	n/a	709	GQ120076-77
Geryonia proboscidalis	1	1	n/a	n/a	847	GQ120078
Pentachogon haeckeli	5	1	0	n/a	810	GQ120079
Rhopalonema velatum	2	1	0	n/a	822	GQ120080
Narcomedusae						
Aeguinura grimaldii	2	1	0	n/a	635	GQ120081
Cunina fowleri	1	1	n/a	n/a	1063	GQ120082
Solmissus incisa	1	1	n/a	n/a	868	GQ120083
Tetraplatia volitans	2	1	0	n/a	741	GQ120091
SCYPHOZOA						
Coronatae						
Atolla vanhoeffeni	5	2	0.001	n/a	808	GQ120084-85
Atolla wyvillei	3	3	0.012	0.009	637	GQ120086-88
Nausithoe atlantica	1	1	n/a	n/a	848	GQ120089
Semaeostomae						
Aurelia sp.	1	1	n/a	n/a	829	GQ120090
Cyanea sp.	2	1	0	n/a	837	GQ120092
Pelagia noctiluca	4	4	0.011	0.003	809	GQ120093-96
Phacellophora ornata	3	3	0.034	0.026	745	GQ120097-99
Rhizostomae						
Cassiopea sp.	1	1	n/a	n/a	865	GQ120100
Phyllorhiza punctata	2	1	0	n/a	785	GQ120101
Stauromedusae						
Craterolophys colvolvulus	1	1	n/a	n/a	813	GQ120102
CUBOZOA						
Chiropsalmus quadrumanus	1	1	n/a	n/a	821	GQ120103

Sequences were unweighted for all analyses, in order to prevent artificial reduction of genetic distances within a species. Distances between congeneric species were calculated as a range of the distance(s) between every distinct sequence for each species to those of different species in the same genus. Members of the genus *Lensia* were removed from analysis of distances between species and genera, and analyzed separately due to phylogenetic evidence based on 28S rDNA that they do not represent a monophyletic group, but comprise at least three genetically and probably taxonomically distinct clades (Ortman, 2008). Also removed from analysis of intra- and inter- specific variation were any taxa not identified to species (e.g. *Marrus* sp. *and Rosacea* sp.). *Halistemma* sp. and *Physalia* sp. were included as distinct species from *Halistemma amphitridis* and *Physalia physalis* (respectively), based on analysis by Francesc Pagès. Levels of K2P distance between higher taxonomic levels were done by pairwise comparisons between sequences from representatives of different taxonomic groups. Ranges and frequency of K2P variation were

shown as the proportion of comparisons that fell within a specific range of distances, normalized for variations in sample size and number of comparisons within taxonomic groups.

3. Results

A total of 231 mtCOI sequences and 165 distinct sequence variants were generated for 95 species of Medusozoans (Table 2). The lengths of the amplified mtCOI products ranged from 487 to 1008 base-pairs. The Kimura 2-Parameter/Neighbor Joining (K2P/NJ) tree grouped haplotypes assigned to the same species within the same cluster (Fig. 1). In no case was a species haplotype assigned to an incorrect or different species, although higher taxonomic groups were not consistently resolved.

Variation within a species was always less than – and did not overlap with – variation between species, even those of the same genus. That is, a barcoding gap (Meyer and Paulay, 2005) was observed for all taxa. Sequence divergence (measured as K2P distance) between individuals of the same species ranged from 0 to 0.057 (mean 0.013, s.d. $\pm$ 0.013). Intra-specific mtCOI sequence divergence was $\leq$0.01 for more than two-thirds of the species (67.5%) and $<$0.05 for 98.4% of comparison between individuals of the same species (Fig. 2); inter-specific sequence divergence between species of the same genus ranged from 0.056 for *Sarsia* spp to 0.381 for *Lilyopsis* spp (mean 0.176, s.d. $\pm$ 0.073; Fig. 3). Two species, the scyphozoan *Phacellophora ornata* and the siphonophore *Amphicaryon acaula*, showed intra-specific sequence divergence $>$0.05. Although these levels of intra-specific variation were higher than those between species in other genera, there was no overlap between the intra-specific and inter-specific variation within each genus, thus maintaining the barcoding gap for these genera. Cryptic species of the calycophoran siphonophore *Lensia multicristata* were uncovered from the mtCOI analysis. Four specimens of *L. multicristata,* collected and identified from close locations in and around the Sargasso Sea, formed two distinct clusters in the K2P/Neighbor Joining cluster diagram, designated as *L. multicristata* types 1 and 2 (Fig. 1). Intra-cluster variation was 0.011 and 0.007 (types 1 and 2 respectively), whereas inter-cluster variation had a mean of 0.345 (s.d. $\pm$ 0.005).

For the Medusozoa as a whole, mtCOI distance between species of the same order ranged from 0.093 to 0.721 (both within the Siphonophora) with a mean 0.404 (s.d. $\pm$ 0.129, Table 3). MtCOI distance between species of the siphonophore genus, *Lensia,* ranged from 0.150 to 0.676 with a mean of 0.506 (s.d. $\pm$ 0.085). Distance between species within orders of the Class Hydrozoa ranged between 0.133 and 0.750 (Table 3, Fig. 4). The mean within-class distance was 0.346 (s.d. $\pm$ 0.048) for the Scyphozoa and 0.364 (s.d. $\pm$ 0.124) for the Hydrozoa, which was similar to the variation between the classes (mean 0.364, s.d. $\pm$ 0.122). Distance within the Cubozoa could not be computed as only one species, *Chriopsalmus*

Fig. 1. Kimura 2-Parameter/Neighbor Joining cluster diagram of 165 mtCOI sequence variants from 95 medusozoan species including 61 siphonophores, 23 hydromedusae (▼), 10 scyphozoans (●), and one cubomedusae (■). ○ indicates node with bootstrap support $\geq$99%.

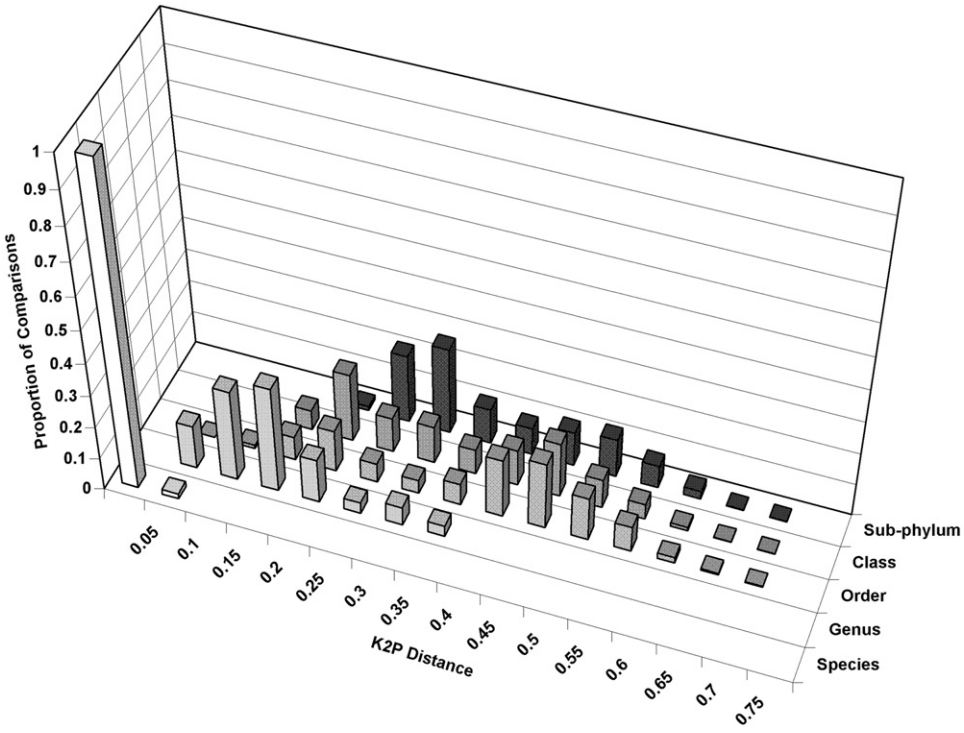

Fig. 2. MtCOI genetic distance for various levels of taxonomic separation, within species, genus, order, class, and sub-phylum. Data are presented in ranges of Kimura 2-Parameter (K2P) distance normalized as a proportion of comparisons between taxa within the designated taxonomic level.

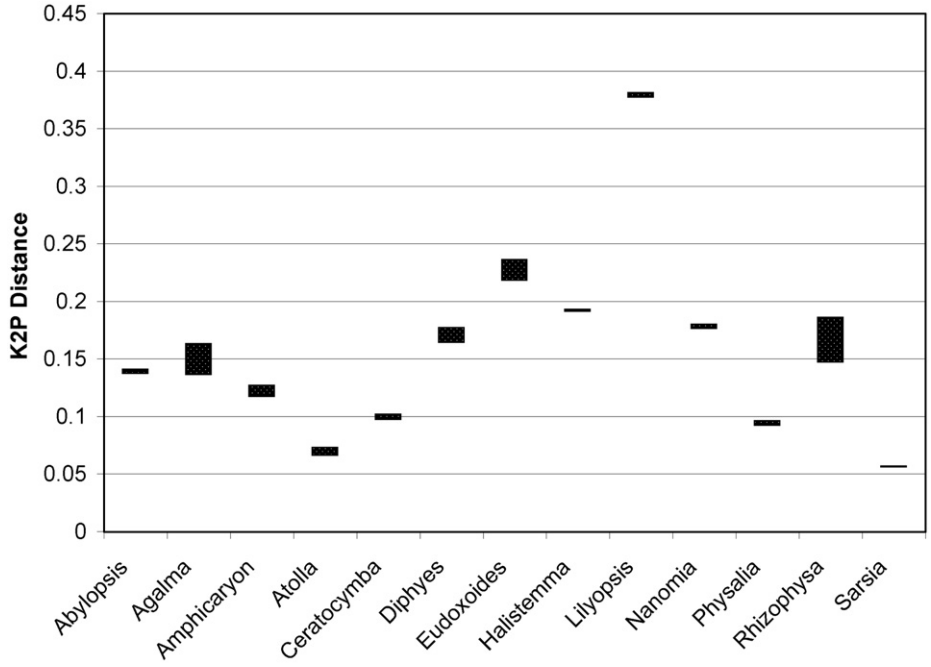

Fig. 3. Patterns of mtCOI sequence variation between species within genera. Histogram shows the range between minimum and maximum Kimura 2-Parameter (K2P) distance between congeneric species.

quadrumanus, was analyzed. Minimum genetic distance between groups did not increase with higher taxonomic levels of orders and above (0.093 - 0.277); the mean (0.182 - 0.406) and maximum distance (0.270 - 0.721) remained relatively constant at taxonomic levels at and above the generic level, a characteristic indication of gene saturation.

4. Discussion

DNA barcoding can be an invaluable tool for taxonomists working with medusozoans, if used in concert with traditional morphological analysis (Dawson, 2005d; Hajibabaei et al., 2007). A DNA barcode needs to be generated from a specimen which has

been accurately identified by an expert taxonomist in this group. Second, a barcode cannot be used to describe a species, it can only identify a species based on sequence similarity in a database; therefore, an unknown species can only be accurately identified if a representative species barcode exists in the database (Ross et al., 2008). Subsequently, DNA barcoding will require a comprehensive searchable database for effective identification of unknowns, which emphasizes the need for expanded sequence data representing all taxonomic groups (Ekrem et al., 2007).

The practice of DNA barcoding may help to bring about a resurgence of interest in taxonomy (Hebert and Gregory, 2005). For medusozoans, DNA barcodes can be used with other information as part of a species description, as is being done with other animals (e.g. Smith et al., 2005). For example, molecules and morphology have been used in concert to describe subspecies of *Mastigias* in marine lakes in Palau (Dawson and Hamner, 2003; Dawson, 2005c).

MtCOI has been used previously as supporting evidence for the existence of cryptic species in the Medusozoa (Dawson and Jacobs, 2001; Dawson and Martin, 2001; Holland et al., 2004). Established barcodes can not only be used to uncover cryptic species (e.g. Dawson and Martin, 2001), but also to connect life-history stages by equating polyps with their corresponding medusa stage (Govindarajan et al., 2005a, 2005b), and by eliminating synonymous species names (Alroy, 2002).

There was no clear relationship between mtCOI divergence and taxonomic separation. Gene saturation results in a plateau of mtCOI divergence between more distant taxonomic groups, e.g. orders, classes, and subphyla. Since gene saturation can obfuscate accurate phylogenetic analysis regardless of the method used, mtCOI was not used in this study to reconstruct evolutionary histories within the Medusozoa. The data are therefore represented by a cluster diagram that graphically displays degrees of sequence similarity (Fig. 1). Although mtCOI has been shown to contain some phylogenetic information between closely related taxa (e.g. Bucklin et al., 2003, 2010), DNA barcodes generally lack sufficient phylogenetic signal at deeper levels (Hajibabaei et al., 2007). Importantly, the fact that mtCOI does not resolve deeper phylogenetic relationships does not lessen its usefulness as a tool for species identification.

There was considerable variability in levels of mtCOI variation between species within a genus. For some genera (*Ceratocymba*, *Atolla*, *Sarsia*, and *Physalia*), sequence differences (K2P distance) among species were <0.10, while variation between others (*Eudoxoides* and *Lilyopsis*) was >0.20. Levels of mean mtCOI variation between species within a genus in this study ranged from 0.056 (*Sarsia*) to 0.381 (*Lilyopsis*). These levels are consistent with levels of mtCOI variability found in *Aurelia* (23.5% Dawson, 2003), *Cassiopea* (23.4% Holland et al., 2004), and *Cyanea* (15.3%; Dawson, 2005a). However, it is important to note that even though we used variation within a genus as a proxy for interspecific genetic variation, representative congeners in this study may not be a given species closest sister lineage. Increased taxon sampling combined with robust phylogenetic data will increase the utility of mtCOI as a barcode in medusozoans.

Closer examination of intraspecific variation is useful to reveal cryptic species and analyze geographic distribution of lineages, or phylogeography (Avise, 2000). MtCOI variation revealed putative

Table 3
MtCOI distance between different taxonomic levels. Columns are number of comparisons within a taxonomic group, minimum, maximum, mean, and S.D. of Kimura 2-Parameter (K2P) distance.

Taxonomic Groups	N Comparisons	Min	Mean	Max	S.D.
Species	127	0	0.013	0.057	0.014
Genera	95	0.056	0.176	0.381	0.073
Orders	6378	0.093	0.403	0.721	0.129
Siphonophora	6240	0.093	0.406	0.721	0.128
Anthomedusae	59	0.185	0.231	0.27	0.021
Leptomedusae	13	0.163	0.182	0.214	0.016
Trachymedusae	27	0.162	0.281	0.404	0.062
Narcomedusae	6	0.277	0.298	0.345	0.026
Coronatae	5	0.274	0.285	0.293	0.008
Semaeostomae	27	0.203	0.265	0.296	0.026
Rhizostomae	1		0.23		
Classes	4015	0.133	0.364	0.75	0.122
Hydrozoa	3914	0.133	0.364	0.75	0.124
Scyphozoa	101	0.256	0.346	0.438	0.048
Medusozoa	2792	0.231	0.393	0.718	0.105

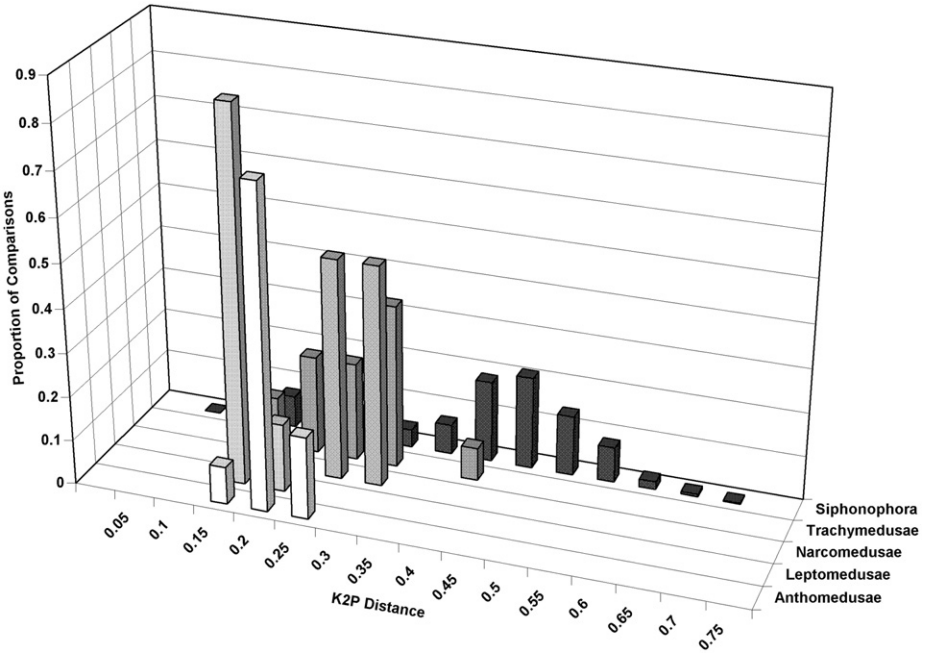

Fig. 4. MtCOI sequence divergence between species for five orders of the Class Hydrozoa. Data are presented in ranges of Kimura 2-Parameter (K2P) distance normalized as a proportion of comparisons between taxa within the designated hydrozoan order.

cryptic species within *Lensia multicristata*, based on patterns of intra- and interspecific mtCOI sequence distances. In contrast, the physonect siphonophore *Nanomia cara* exhibited low levels of intraspecific mtCOI variation, despite its extensive geographic distribution throughout North Atlantic Ocean (Margulis, 1972) and exceptional morphological and behavioral variation (Youngbluth, personal observation). The DNA barcode region was thus useful to assign species identity for widely-distributed populations with phenotypic plasticity. No significant intraspecific variation in mtCOI was observed for 13 other species of medusozoans for which mtCOI sequences were determined from multiple ocean regions (Table 2).

Patterns of interspecific variation for several genera in this study could not be accurately analyzed due to the lack of definitive species identifications. Three specimens of *Marrus* spp. were collected in the Gulf of Maine (*Marrus* sp.1, sp. 2, and *M. orthocanna*). Although they clustered together (Fig. 1), levels of mtCOI divergence among the individuals were larger than levels of variation within any other species, yet smaller than variation between most genera. More ambiguous were the results for four specimens of *Rosacea* spp., only one of which could be unambiguously identified to species (i.e., *Rosacea cymbiformis*). The species formed two distinct clades (*Rosacea* sp.1 versus *Rosacea* sp.2/*R. cymbiformis*). This could imply a significantly large intra-specific variation within these species or a significant degree of variability between species in this genus.

Although this study provides some coverage of most medusozoan groups, it is not a complete analysis of the ~3600 species that encompass this sub-phylum. Taxonomic coverage was uneven, with 61 species of siphonophores, four species of Narcomedusae, and one species of Cubomedusae. An expanded database of medusozoan barcodes will improve the usefulness of DNA barcoding and the accuracy of species identification for this ecologically important and taxonomically challenging group of organisms.

5. Conclusions

DNA sequences for a portion of the mitochondrial Cytochrome Oxidase I (mtCOI) gene was examined to characterize patterns of variation within and among species of the Medusozoa. A total of 231 sequences was obtained for 95 species of medusozoans. Although in most cases true "sister taxa" were unknown, there were significant mtCOI distances between congeneric species, even for species with high levels of intra-specific variation. For medusozoans, DNA barcodes can be used with other information as part of a species description, and are thus invaluable tools for alpha taxonomists. Due to high levels of inter-specific distance within mtCOI, identification or classification of unknown specimens is possible only when there is a close sequence match in a searchable database (e.g. GenBank). The utility of DNA barcoding systems for all metazoans will increase as a more comprehensive library of sequences becomes available and phylogenetic analyses identify relationships between sister lineages within this group.

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