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Slow Mitochondrial *COI* Sequence Evolution at the Base of the Metazoan Tree and Its Implications for DNA Barcoding

Danwei Huang · Rudolf Meier · Peter A. Todd · Loke Ming Chou

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Abstract The evolution rates of mtDNA in early metazoans hold important implications for DNA barcoding. Here, we present a comprehensive analysis of intra- and interspecific *COI* variabilities in Porifera and Cnidaria (separately as Anthozoa, Hydrozoa, and Scyphozoa) using a data set of 619 sequences from 224 species. We found variation within and between species to be much lower in Porifera and Anthozoa compared to Medusozoa (Hydrozoa and Scyphozoa), which has divergences similar to typical metazoans. Given that recent evidence has shown that fungi also exhibit limited *COI* divergence, slow-evolving mtDNA is likely to be plesiomorphic for the Metazoa. Higher rates of evolution could have originated independently in Medusozoa and Bilateria or been acquired in the Cnidaria + Bilateria clade and lost in the Anthozoa. Low identification success and substantial overlap between intra- and interspecific *COI* distances render the Anthozoa unsuitable for DNA barcoding. Caution is also advised for Porifera and Hydrozoa because of relatively low identification success rates as even threshold divergence that maximizes the "barcoding gap" does not improve identification success.

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D. Huang · R. Meier · P. A. Todd · L. M. Chou
Department of Biological Sciences, National University of Singapore, 14 Science Drive 4, Singapore 117543, Singapore

D. Huang (✉)
Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA 92093, USA
e-mail: huangdanwei@nus.edu.sg

Introduction

Most biologists assume that metazoan mitochondrial DNA evolve up to 10 times faster than nuclear DNA (e.g., Dawid 1972; Brown et al. 1979, 1982; Brown 1983; Avise et al. 1987; Moritz et al. 1987). Recent evidence, however, has suggested that this feature of mitochondrial sequences has evolved within the Metazoa. In particular, Anthozoa and Porifera have slow-evolving mtDNA (e.g., Watkins and Beckenbach 1999; France and Hoover 2002; McFadden et al. 2004, 2006; Smith et al. 2004; van Oppen et al. 2004; Lavrov et al. 2005; Tseng et al. 2005; Calderón et al. 2006; see also Neigel et al. 2007). Two hypotheses have been proposed by Shearer et al. (2002) to explain the rate variation in "basal" Metazoa. First, sequence evolution was slow in the metazoan ancestor and later gathered speed in the Bilateria. The second hypothesis posited fast-evolving mitochondrial DNA in the metazoan ancestor and a secondary slowdown in Anthozoa. Here, we use *COI* as a model to test these hypotheses based on our own and GenBank data. We, furthermore, add outgroup information and summarize a large amount of new evidence. Finally, we discuss the implications of our results for DNA barcoding.

Ideally, an analysis of mitochondrial sequence evolution should include all "basal" metazoan taxa as well as the putative sister group of Metazoa. Unfortunately, there are insufficient data for some of these taxa. For example, very few sequences are available for the putative sister group of Metazoa, the choanoflagellates, as well as Placozoa and Ctenophora. However, with regard to the outgroup of Metazoa a recent analysis of the next closest outgroup, Fungi, revealed slow *COI* evolution given that many species of *Penicillium* shared the same *COI* sequence (Seifert et al. 2007). Similarly, recent studies on

the Porifera suggested slow-evolving mtDNA. To date, three analyses of the sponge *COI* gene have been performed. Schröder et al. (2003) found that *COI* sequences of four species from the family Lubomirskiidae were identical, and only 1–2% different from a confamilial species. Duran et al. (2004) demonstrated few differences in the *COI* gene of western Mediterranean and Atlantic *Crambe crambe* populations despite their being separated by up to 3000 km. Wörheide (2006) also showed similar results for the Indo-Pacific desmosponge *Astrosclera willeyana* over a range of 20,000 km. For Cnidaria, Hebert et al. (2003b) noted that interspecific *COI* variability was also unusually low. They found that 94.1% of all pairwise interspecific distances between congeneric cnidarian species were <2%, while most other animal taxa in a broad survey of major metazoan groups had much higher interspecific variability. However, Hebert and coworkers' (2003b) conclusion was based on only 17 species pairs of Cnidaria, while an average of 950 pairs were included for the other major animal taxa. Furthermore, all three Cnidaria genera (10 spp.) in the survey (*Corallium*, *Narella*, and *Urticina*) belonged to the Anthozoa. Thus, the status of the remaining Cnidaria remained uncertain. Subsequently, Hellberg (2006) confirmed low sequence divergence for Anthozoa, and Shearer and Coffroth (2007) found both intra- and interspecific distances to be limited in Scleractinia. On the other hand, Govindarajan et al.'s (2005, 2006) work implied that the class Hydrozoa has more conventional patterns of interspecific *COI* distances. Research on the other major cnidarian class, the Scyphozoa, also suggested a fast-evolving *COI* gene. For example, interspecific divergences varied from 13% to 24% in the cosmopolitan genus *Aurelia* (Dawson and Jacobs 2001), 11.8–15.3% between two putative species of *Cyanea* (Dawson 2005), and 10.9–23.4% in *Cassiopea* (Holland et al. 2004).

The evolution of divergence levels in *COI* is not only interesting from an evolutionary point of view, but also important for the prospects of DNA barcoding (see Hebert et al. 2003a). The success of this species identification tool is often thought to depend on the presence of a barcoding "gap"; i.e., the separation between intra- and interspecific variation of *COI* (Meyer and Paulay 2005). Here, we test the data for the presence of a barcoding gap and assess identification success using three threshold values for species delimitation—3% as initially proposed by barcoding advocates (Hebert et al. 2003a), 10× mean intraspecific variability suggested by Hebert et al. (2004) and Kerr et al. (2007), and a taxon-specific value that minimizes overlap (or maximizes separation) between intra- and interspecific distances (Lefébure et al. 2006). In order to test Shearer et al.'s (2002) hypotheses with regard to *COI* divergence levels at the base of the Metazoa tree, as well as to assess DNA barcoding identification success, we summarize GenBank data and examine intra- and interspecific variabilities for Porifera and Cnidaria, the latter of which is analyzed separately for Anthozoa, Hydrozoa and Scyphozoa. We also sequenced *COI* for 30 species of Cnidaria to assemble a data set totaling 619 sequences from 224 species, the largest thus far to examine *COI* variation in "basal" metazoans.

Materials and Methods

All poriferan and cnidarian mtDNA *COI* sequences available in GenBank, including those from mitochondrial genome sequences, were downloaded (as of September 2006). In addition, 66 scleractinian (class Anthozoa) samples representing 30 species were collected from Singapore to expand on the taxon coverage in GenBank. From these samples, genomic DNA was extracted from tissue digested in twice their volume of CHAOS solution (4 M guanidine thiocyanate, 0.1% *N*-lauroyl sarcosin sodium, 10 mM Tris pH 8, 0.1 M 2-mercaptoethanol) (Sargent et al. 1986) for at least 3 days at room temperature before DNA extraction using the phenol-chloroform method with a phenol extraction buffer (100 mM TrisCl pH 8, 10 mM EDTA, 0.1% SDS) (Fukami et al. 2004a). *COI* was amplified with Scleractinia-specific primers M*COI*F (5′–TCT ACA AAT CAT AAA GAC ATA GG–3′) and M*COI*R (5′–GAG AAA TTA TAC CAA AAC CAG G–3′) using the following protocol: 95°C for 2 min, 35 cycles at 94°C for 45 s, 55°C for 45 s, and 72°C for 1.5 min, ending with 72°C for 5 min (Fukami et al. 2004b). PCR products were purified with SureClean (BIOLINE) following the manufacturer's protocol, and cycle sequencing reaction was carried out using the BigDye Terminator kit (Perkin Elmer). Sequences were obtained from the ABI 3100 capillary genetic analyzer and deposited in GenBank under accession numbers EU371658–EU371723.

Sequences were placed in four data sets—Porifera, Anthozoa, Hydrozoa, and Scyphozoa—using TaxonDNA (Meier et al. 2006). They were aligned using AlignmentHelper 1.2 (McClellan and Woolley 2004), which translates the nucleotide sequences into amino acid sequences, aligns them using ClustalW, and translates the results back to DNA data. Sequences not identified to species and those with >30% sequence divergence for all pairwise comparisons were excluded. Uncorrected and K2P pairwise distances for the sequences within each data set were calculated in TaxonDNA (Meier et al. 2006). Data were then separated into three categories: (i) intraspecific, (ii) congeneric interspecific, and (iii) closest congeneric interspecific match. Due to vast disparity in numbers of

pairwise combinations among various groups, the data did not conform to assumptions required for parametric analysis of variance (ANOVA). Hence, nonparametric Kruskal-Wallis ANOVA (for multiple independent groups) and Mann-Whitney *U*-test (for two independent groups) were used to test for significant differences in pairwise distances among the four taxa. All statistical analyses were performed using the software STATISTICA 6.0 (StatSoft).

One concern with using Genbank data is that some specimens whose sequences were submitted to the database could have been misidentified (Harris 2003; Vilgalys 2003). The best way to avoid such errors is to use only sequences that were submitted by researchers with a track record in the respective taxon. We determined the taxonomic expertise of the author team that submitted the data to Genbank by obtaining the number of ISI-listed articles that they had published on the group in question (i.e., Porifera, Anthozoa, Hydrozoa, or Scyphozoa). We then carried out two analyses. In the first analysis, we only included sequences in our survey for which at least one of the submitting researchers had at least five publications on the target taxon. In a second analysis we used the full data set. We found that both data sets yielded similar results (see Supplementary Material 1). Hence, only those of the full data set are reported here. In all, 103 poriferan sequences from 57 species, 288 anthozoan sequences from 129 species, 96 hydrozoan sequences from 28 species, and 132 scyphozoan sequences from 10 species were analyzed (total, 619 sequences and 224 species). Information regarding each sequence examined, including GenBank accession number, location of voucher specimen, submitting authors, and frequency of taxon-specific publications, is given in Supplementary Material 2.

To assess overlap between the intra- and interspecific variabilities of *COI*, relative abundances of sequences at each range of intraspecific and closest matched congeneric interspecific divergences were plotted as histograms. Identification success for each taxon was also examined using the "best close match" strategy: assigning to a query sequence the best-matching barcode from all the other sequences and designating it the name of the barcode if the distance was smaller than a particular reference value (Meier et al. 2006). Identification success rates were tested using three thresholds, 3%, 10× mean intraspecific distance, and a taxon-specific value that minimized intra- and interspecific distance overlap, measured using the mean of two parameters: (i) proportion of intraspecific pairwise divergence values greater than the threshold and (ii) proportion of interspecific pairwise distances smaller than the threshold (Lefébure et al. 2006).

Results

Intraspecific *COI* variation was distinct among taxa: Kruskal-Wallis ANOVA revealed highly significant differences in intraspecific distances among the four data sets ($H = 323.90$, df = 3, $p < 0.001$). The divergence range of Porifera was 0–6%, that of Anthozoa was 0–4%, while the Hydrozoa and Scyphozoa (together termed the Medusozoa) scored intraspecific distances of between 2% and about 20% (Fig. 1). Sequences with divergences of 0–2% accounted for the majority of variation in all taxa, but this was much greater in Porifera and Anthozoa than medusozoans (Porifera, 93.65%; Anthozoa, 99.06%; Hydrozoa, 59.74%; Scyphozoa, 55.73%). The Medusozoa was significantly more divergent than Porifera and Anthozoa, as shown by the Mann-Whitney *U*-test, with Anthozoa recording the lowest intraspecific distances (Table 1a).

Closest congeneric interspecific *COI* distances were more variable than intraspecific divergences in all taxa, with large ranges of about 20%, overlapping with their respective intraspecific distances. A majority of the interspecific distances in the Porifera and Anthozoa were ≤6% or invariant (Porifera, 80.00%; Anthozoa, 98.03%), resulting in substantial overlap between intra- and closest interspecific variation. The Medusozoa generally had greater interspecies distances, i.e., 93.62% of hydrozoan sequences and 82.09% of scyphozoan sequences registered >6% and >10% mean pairwise divergence, respectively. Significant differences existed among taxa (Kruskal-Wallis $H = 301.42$, df = 3, $p < 0.001$). By rank, anthozoan closest congeneric interspecific divergences were the lowest, followed by Porifera, Hydrozoa, and Scyphozoa; distances differed from one another significantly (pairwise Mann-Whitney *U*-test; Table 1b). Mean uncorrected interspecific distances were 4.93% (SE, 0.64%), 1.42% (SE, 0.15%), 12.25% (SE, 0.21%), and 15.27% (SE, 0.34%), respectively, for Porifera, Anthozoa, Hydrozoa, and Scyphozoa.

The threshold values, based on 10× mean intraspecific distances, were 6.0%, 2.0%, 38.4%, and 38.0% for Porifera, Anthozoa, Hydrozoa, and Scyphozoa, respectively. To minimize overlap between intra- and interspecific divergences, distance thresholds employed are 1.2% for poriferans (overlap of 11.32%), 0.4% for anthozoans (overlap of 15.99%), 5.8% for hydrozoans (overlap of 1.31%), and 10.6% for scyphozoans (overlap of 7.45%). Proportions of sequences identified by DNA barcodes using three different threshold values are reported in Table 2. Generally, frequencies of sequences that were correctly identified or ambiguous do not change when different threshold values are used. However, 10× mean intraspecific distance gave the highest frequency of inaccurate species attribution, and the least number without any match closer than the threshold, especially in the Hydrozoa and Scyphozoa. In cases of

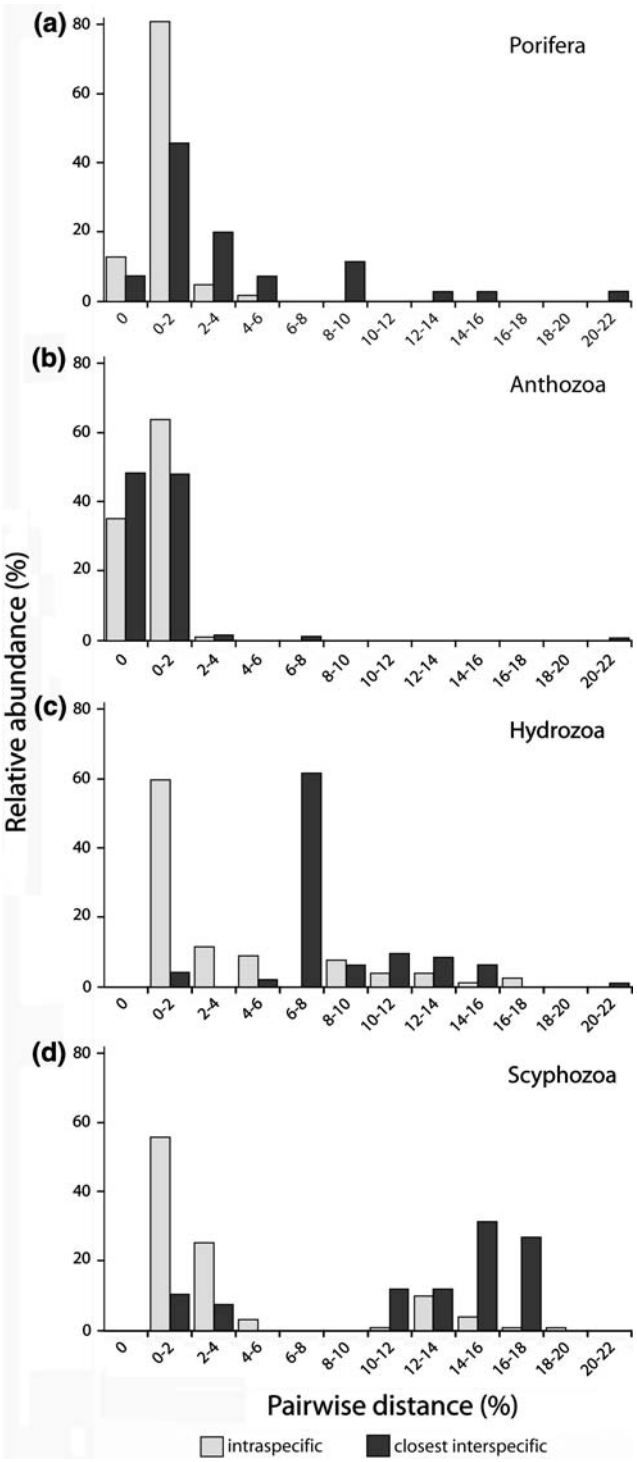

Fig. 1 Bar chart showing proportion of pairwise comparisons of the *COI* gene at each range of sequence divergence. Intraspecific and closest congeneric interspecific matches of the following taxa are represented: (**A**) phylum Porifera, (**B**) class Anthozoa, (**C**) class Hydrozoa, and (**D**) Scyphozoa

minimum overlap, rates of accurate identification were not higher than other thresholds, but many sequences labeled as incorrect otherwise were not named.

Discussion

This study shows that intraspecific variability is much lower in Porifera and Anthozoa compared to the Medusozoa, while interspecific distances are also low in Anthozoa (uncorrected mean, 1.42%) and high in Hydrozoa and Scyphozoa (12.25% and 15.27%, respectively). In Porifera the mean interspecific distance is 4.93%, very similar to the 5.6% registered for the outgroup Fungi (Seifert et al. 2007). Both values are low, given that Hebert et al. (2003b) calculated a divergence mean of 11.3% for congeneric species pairs from 11 Bilateria phyla. Distances in the Medusozoa are therefore comparable to those of bilaterians, while the Anthozoa, Porifera, and Fungi exhibit low interspecific *COI* divergence. It should be noted that Anthozoa and Porifera sequence evolution appears to be unusually slow for other mitochondrial genes as well. For example, in Anthozoa, van Oppen et al. (1999) observed that the cytochrome *b* gene displays a maximum of 0.8% sequence divergence between Caribbean and Pacific *Acropora* species. Fukami et al. (2000) surveyed eight species of *Acropora* and found no differences for cytochrome *b* and only up to 0.46% in ATP6 genes. In Porifera, Lavrov et al. (2005) determined that the variability of mitochondrial SSU-rRNA genes relative to nuclear SSU-RNA genes is about 2.5 times lower in poriferans than in mammals. Conversely, the 16S rDNA in the hydrozoan *Obelia geniculata*, family Campanulariidae, and scyphozoan *Aurelia* seems to mirror the *COI* gene in adhering to evolutionary rates that are similar to those of typical invertebrates (Schroth et al. 2002; Govindarajan et al. 2005, 2006).

To explain changes in divergence levels, we use a metazoan tree as summarized from Kim et al. (1999), Borchiellini et al. (2001), Medina et al. (2001), and Halanych (2004) (Fig. 2). Note that poriferan paraphyly would not alter our interpretation. Our results support two equally parsimonious reconstructions for the origin of slowly evolving mtDNA, both supporting a metazoan ancestor with slow-evolving mitochondrial DNA. We can thus reject Shearer et al.'s (2002) hypothesis of fast evolution at the base of the metazoan tree. Higher rates could have originated twice—in the Medusozoa and Bilateria—possibly resulting from two independent losses of an mtDNA repair mechanism that suppresses substitution rates (van Oppen et al. 1999, 2002; Shearer et al. 2002; Hellberg 2006; Medina et al. 2006). Alternatively, fast mtDNA could also have been acquired before the Cnidaria-Bilateria split and subsequently lost in the Anthozoa. Interestingly, medusozoans have unusual linear genomes, while Porifera and Anthozoa possess typical circular mitochondrial DNA (Bridge et al. 1992; Pont-Kingdon et al. 2000). Proponents of slow mitochondrial sequence evolution in "basal" metazoans have used genome organization to lend support

Table 1 *COI* distances, pairwise Mann-Whitney *U*-statistics, significance tests, and rank of each taxon (largest to smallest distances) among Porifera, Anthozoa, Hydrozoa, and Scyphozoa for intraspecific (a) and closest congeneric interspecific distances (b). Distance values denote means and standard errors

Taxon	Uncorrected distance (%; mean ± SE)	K2P distance (%; mean ± SE)	Porifera	Anthozoa	Hydrozoa	Scyphozoa
(a) Intraspecific distances						
Porifera	0.60 ± 0.10	0.64 ± 0.10	Rank = 2	$U = 3173$	$U = 518$	$U = 617$
Anthozoa	0.20 ± 0.03	0.20 ± 0.03	$p < 0.001$	Rank = 3	$U = 476$	$U = 574$
Hydrozoa	3.84 ± 0.47	4.14 ± 0.53	$p < 0.001$	$p < 0.001$	Rank = 1	$U = 4348$
Scyphozoa	3.80 ± 0.39	4.27 ± 0.46	$p < 0.001$	$p < 0.001$	$p = 0.097$	Rank = 1
(b) Closest congeneric interspecific distances						
Porifera	3.76 ± 0.57	4.10 ± 0.65	Rank = 3	$U = 3028$	$U = 1212$	$U = 675$
Anthozoa	0.71 ± 0.13	0.75 ± 0.15	$p < 0.001$	Rank = 4	$U = 565$	$U = 819$
Hydrozoa	8.49 ± 0.34	9.16 ± 0.39	$p < 0.001$	$p < 0.001$	Rank = 2	$U = 1380$
Scyphozoa	12.52 ± 0.67	14.76 ± 0.78	$p < 0.001$	$p < 0.001$	$p < 0.001$	Rank=1

Table 2 Frequencies of sequences (percentages in parentheses) with accuracy of species attribution using three threshold values: 3.0%, 10× mean intraspecific distance (10×), and point of minimum overlap (MO)

Taxon	Threshold	Correct	Ambiguous	Incorrect	Unmatched
Porifera	3.0%	54 (52.4%)	8 (7.8%)	21 (20.4%)	20 (19.4%)
	6.0% (10×)	54 (52.4%)	8 (7.8%)	26 (25.2%)	15 (14.6%)
	1.2% (MO)	52 (50.5%)	7 (6.8%)	17 (16.5%)	27 (26.2%)
Anthozoa	3.0%	105 (36.5%)	123 (42.7%)	57 (19.8%)	3 (1.0%)
	2.0% (10×)	105 (36.5%)	123 (42.7%)	54 (18.8%)	6 (2.1%)
	0.4% (MO)	102 (35.4%)	122 (42.4%)	40 (13.9%)	24 (8.3%)
Hydrozoa	3.0%	66 (68.8%)	0 (0.0%)	4 (4.2%)	26 (27.1%)
	38.4% (10×)	68 (70.8%)	2 (2.1%)	26 (27.1%)	0 (0.0%)
	5.8% (MO)	68 (70.8%)	0 (0.0%)	6 (6.3%)	22 (22.9%)
Scyphozoa	3.0%	122 (93.2%)	1 (0.8%)	4 (3.0%)	4 (3.0%)
	38.0% (10×)	125 (94.7%)	1 (0.8%)	6 (4.5%)	0 (0.0%)
	10.6% (MO)	124 (93.9%)	1 (0.8%)	4 (3.0%)	3 (2.3%)

Note. An accurate identification of a sequence is classified as "correct," sequence with best matches to the correct barcode and at least an incorrect one is "ambiguous," erroneous species attribution is "incorrect," and an "unmatched" sequence has no match closer than the threshold

for our first reconstruction, suggesting that genome linearization is related to mtDNA acceleration in Hydrozoa and Scyphozoa (Shearer et al. 2002; van Oppen et al. 2002). On the other hand, the loss of DNA repair genes by medusozoans and bilaterians, e.g., the *mutS* homologue found in octocoral *Sarcophyton glaucum* (Pont-Kingdon et al. 1995, 1998), could also have led to the same outcome (Hellberg 2006).

Slow evolution of mitochondrial sequences at the Metazoa base is currently the best-supported hypothesis, but additional data would be welcome. In particular, more information on Ctenophora and Placozoa is needed and could be used to distinguish between the two equally parsimonious reconstructions in Fig. 2. Furthermore, we note that differences in interspecific distances can be the result of either slow mitochondrial evolution (Shearer et al. 2002)

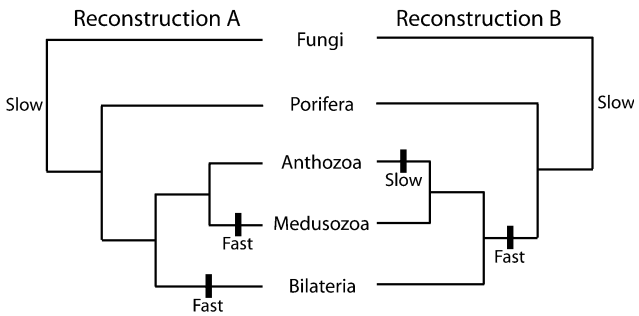

Fig. 2 The two most parsimonious evolutionary scenarios for slow mtDNA evolution in Anthozoa and Porifera. From a slow ancestral mtDNA, (**A**) fast evolution originated in the Medusozoa and Bilateria independently (Hellberg 2006), or (**B**) fast mtDNA evolved in the Cnidaria + Bilateria clade but was lost in Anthozoa. Black bars labeled "fast" and "slow" denote acceleration and deceleration of mitochondrial sequence evolution, respectively

or differences in speciation rates. For example, if Porifera and Anthozoa were to speciate more rapidly, smaller interspecific distances would be expected. However, this explanation for the observed low divergence values appears unlikely because differences in speciation rates would affect both mitochondrial and nuclear genes similarly. Yet in a comparison of both gene types for Porifera and Bilateria, Lavrov et al. (2005) only found lower relative divergences between mitochondrial and nuclear genes in Porifera. Similar studies for other "basal" Metazoa should be carried out to verify Lavrov et al.'s (2005) results for other taxa. A comparison among nonbilaterian and bilaterian groups using relative levels of mtDNA and nDNA distances would confirm that slower mtDNA evolution, rather than faster speciation, is the cause of low mitochondrial sequence divergence values.

The generalization by Hebert et al. (2003b) that barcoding the Cnidaria would be problematic appears to be unfounded. In their analysis, the taxon coverage for *COI* in the phylum was biased toward the anthozoans, with their low intra- and interspecific variabilities (69% of all species and 50% of all sequences from GenBank), while Medusozoa has more normal divergence values. However, this does not change our observation that all taxa lack barcoding gaps due to extensive overlap between intra- and interspecific variabilities. All intraspecific distances are nested within the range of interspecific values for Porifera, Anthozoa, and Hydrozoa, while intra- and interspecific distances of Scyphozoa had similar ranges. When the reference divergence value for species recognition was set at 3%, i.e., the proposed species delimitation threshold (e.g., Hebert et al. 2003a; Barrett and Hebert 2005), overlap was extremely high, resulting in many intraspecies pairs scoring distances above the threshold and interspecific variability falling below it. Thus, a threshold value would have to be customized for each taxon in order to minimize overlap. Hebert et al. (2004) stated that a cutoff of 10× mean intraspecific difference could be effective for screening of new animal taxa, but these values fall out of the range of most distances obtained in this study, resulting in high overlap rates. We find that overlap can be reduced most effectively by using taxon-specific thresholds supported by empirical data.

Overall, Scyphozoa has the highest species identification success based on DNA barcodes. However, we advise caution in the use of barcodes for identification in Porifera and Hydrozoa, as they do not perform as well. Low positive identification rate and substantial lack of a DNA barcoding gap in Anthozoa (minimum degree of overlap, 15.99%) strongly suggest that closely related species share DNA barcodes and cannot be identified based on *COI* (Meyer and Paulay 2005). We also found that the barcoding gap has limited predictive power for identification success. The proportion of sequences given the correct identities in each taxon is similar among thresholds of 3%, 10× mean intraspecific distances, and point of minimum overlap. Surprisingly, the latter does not improve accuracy of identification, but is more conservative as it tends not to attribute name of the best matching species to the query sequence unless variability between the sequences is low.

The use of *COI* sequences for taxonomic purposes (i.e., DNA taxonomy) is already stirring controversy in Anthozoa systematics, for example, the gene has been used to examine species classification in the *Montastraea annularis* complex (Medina et al. 1999) and among various Zoanthidea (Reimer et al. 2004). Using *COI* sequence invariance as partial evidence, Medina et al. (1999) concluded that the *M. annularis* species complex is a single species instead of three separate ones, despite clear distinctions in colony and corallite morphology and reproductive characteristics (Knowlton et al. 1997; Fukami et al. 2004a). Fukami and Knowlton (2005) also found that, upon determination of complete mitochondrial DNA sequences of these cryptic species, only 25 of 16,134 base pair positions were variable. The dispute over the taxonomic status of the *M. annularis* complex continues. In zoanthids, Reimer et al. (2004) concluded that four presumed species of the genera *Zoanthus* spp. were conspecific based on the invariance of the *COI* gene. Although their results were supported by some morphological characters (polyp diameter, tentacle count, and mesentery count data), strong reliance on the *COI* barcode without full taxonomic appraisal potentially confounds their findings. In fact, our study reveals that 48.43% of anthozoan *COI* sequences share DNA barcodes, including a currently valid zoanthid species pair (*Palythoa caribaeorum* and *P. tuberculosa*) (Boscolo and Silveira 2005; Reimer et al. 2006). We thus suggest that, in Anthozoa, low *COI* divergence may not be used as conclusive evidence when deciding whether two closely related populations are con- or allospecific.

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References

Avise JC, Arnold J, Ball RM, Bermingham E, Lamb T, Neigel JE, Reeb CA, Saunders NC (1987) Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. Annu Rev Ecol Syst 18:489–522

Barrett RDH, Hebert PDN (2005) Identifying spiders through DNA barcodes. Can J Zool 83:481–491

Borchiellini C, Manuel M, Alivon E, Boury-Esnault N, Vacelet J, Le Parco Y (2001) Sponge paraphyly and the origin of Metazoa. J Evol Biol 14:171–179

Boscolo HK, Silveira FL (2005) Reproductive biology of *Palythoa caribaeorum* and *Protopalythoa variabilis* (Cnidaria, Anthozoa, Zoanthidea) from the southeastern coast of Brazil. Brazil J Biol 65:29–41

Bridge D, Cunningham CW, Schierwater B, DeSalle R, Buss LW (1992) Class-level relationships in the phylum Cnidaria: evidence from mitochondrial genome structure. Proc Natl Acad Sci USA 89:8750–8753

Brown WM (1983) Evolution of animal mitochondrial DNA. In: Nei M, Koehn RK (eds) Evolution of genes and proteins. Sinauer Associates, Sunderland, MA, pp 147–164

Brown WM, George M Jr, Wilson AC (1979) Rapid evolution of animal mitochondrial DNA. Proc Natl Acad Sci 76:1967–1971

Brown WM, Prager EM, Wang A, Wilson AC (1982) Mitochondrial DNA sequences of primates: tempo and mode of evolution. J Mol Evol 18:225–239

Calderón I, Garrabou J, Aurelle D (2006) Evaluation of the utility of COI and ITS markers as tools for population genetic studies of temperate gorgonians. J Exp Mar Biol Ecol 336:184–197

Dawid IB (1972) Evolution of mitochondrial DNA in *Xenopus*. Dev Biol 29:139–151

Dawson MN (2005) *Cyanea capillata* is not a cosmopolitan jellyfish: morphological and molecular evidence for *C. annaskala* and *C. rosea* (Scyphozoa: Semaeostomeae: Cyaneidae) in south-eastern Australia. Invertebr Syst 19:361–370

Dawson MN, Jacobs DK (2001) Molecular evidence for cryptic species of *Aurelia aurita* (Cnidaria, Scyphozoa). Biol Bull 200:92–96

Duran S, Pascual M, Turon X (2004) Low levels of genetic variation in mtDNA sequences over the western Mediterranean and Atlantic range of the sponge *Crambe crambe* (Poecilosclerida). Mar Biol 144:31–35

France SC, Hoover LL (2002) DNA sequences of the mitochondrial *COI* gene have low levels of divergence among deep-sea octocorals (Cnidaria: Anthozoa). Hydrobiologia 471:149–155

Fukami H, Knowlton N (2005) Analysis of complete mitochondrial DNA sequences of three members of the *Montastraea annularis* coral species complex (Cnidaria, Anthozoa, Scleractinia). Coral Reefs 24:410–417

Fukami H, Omori M, Hatta H (2000) Phylogenetic relationships in the coral family Acroporidae, reassessed by inference from mitochondrial genes. Zool Sci 17:689–696

Fukami H, Budd AF, Levitan DR, Jara J, Kersanach R, Knowlton N (2004a) Geographic difference in species boundaries among members of the *Montastrea annularis* complex based on molecular and morphological markers. Evolution 58:324–337

Fukami H, Budd AF, Paulay G, Sole-Cava A, Chen CA, Iwao K, Knowlton N (2004b) Conventional taxonomy obscures deep divergence between Pacific and Atlantic corals. Nature 427:832–835

Govindarajan AF, Halanych KM, Cunningham CW (2005) Mitochondrial evolution and phylogeography in the hydrozoan *Obelia geniculata* (Cnidaria). Mar Biol 146:213–222

Govindarajan AF, Boero F, Halanych KM (2006) Phylogenetic analysis with multiple markers indicates repeated loss of the adult medusa stage in Campanulariidae (Hydrozoa, Cnidaria). Mol Phylogenet Evol 38:820–834

Halanych KM (2004) The new view of animal phylogeny. Annu Rev Ecol Evol Syst 35:229–256

Harris DJ (2003). Can you bank on GenBank? Trends Ecol Evol 18:317–319

Hebert PDN, Cywinska A., Ball SL, deWaard JR (2003a) Biological identifications through DNA barcodes. Proc Roy Soc Lond Ser B 270:313–321

Hebert PDN, Ratnasingham S, deWaard JR (2003b) Barcoding animal life: cytochrome *c* oxidase subunit 1 divergences among closely related species. Proc Roy Soc Lond Ser B 270: S96–S99

Hebert PDN, Stoeckle MY, Zemlak TS, Francis CM (2004) Identification of birds through DNA barcodes. PLoS Biol 2:1657–1663

Hellberg ME (2006) No variation and low synonymous substitution rates in coral mtDNA despite high nuclear variation. BMC Evol Biol 6:24

Holland BS, Dawson MN, Crow GL, Hofmann DK (2004) Global phylogeography of *Cassiopea* (Scyphozoa: Rhizostomeae): molecular evidence for cryptic species and multiple invasions of the Hawaiian Islands. Mar Biol 145:1119–1128

Kerr KCR, Stoeckle MY, Dove CJ, Weigt LA, Francis CM, Hebert PDN (2007) Comprehensive DNA barcode coverage of North American birds. Mol Ecol Notes 7:535–543

Kim J, Kim W, Cunningham CW (1999) A new perspective on lower metazoan relationships from 18S rDNA sequences. Mol Biol Evol 16:423–427

Knowlton N, Maté J, Guzmán HM, Rowan R, Jara J (1997) Direct evidence for reproductive isolation among the three species of the *Montastraea annularis* complex in Central America (Panamá and Honduras). Mar Biol 127:705–711

Lavrov DV, Forget L, Kelly M, Lang BF (2005) Mitochondrial genomes of two demosponges provide insights into an early stage of animal evolution. Mol Biol Evol 22:1231–1239

Lefébure T, Douady CJ, Gouy M, Gibert J (2006) Relationship between morphological taxonomy and molecular divergence within Crustacea: proposal of a molecular threshold to help species delimitation. Mol Phylogenet Evol 40:435–447

McClellan DA, Woolley S (2004) AlignmentHelper, version 1.0. Brigham Young University, Provo, UT

McFadden CS, Tullis ID, Hutchinson MB, Winner K, Sohm JA (2004) Variation in coding (NADH dehydrogenase subunits 2, 3, and 6) and noncoding intergenic spacer regions of the mitochondrial genome in Octocorallia (Cnidaria: Anthozoa). Mar Biotechnol 6:516–526

McFadden CS, France SC, Sánchez JA, Alderslade P (2006) A molecular phylogenetic analysis of the Octocorallia (Cnidaria: Anthozoa) based on mitochondrial protein-coding sequences. Mol Phylogenet Evol 41:513–527

Medina M, Weil E, Szmant AM (1999) Examination of the *Montastraea annularis* species complex (Cnidaria: Scleractinia) using ITS and COI sequences. Mar Biotechnol 1:89–97

Medina M, Collins AG, Silberman JD, Sogin ML (2001) Evaluating hypotheses of basal animal phylogeny using complete sequences of large and small subunit rRNA. Proc Natl Acad Sci 98:9707–9712

Medina M, Collins AG, Takaoka TL, Kuehl JV, Boore JL (2006) Naked corals: skeleton loss in Scleractinia. Proc Natl Acad Sci 103:9096–9100

Meier R, Kwong S, Vaidya G, Ng PKL (2006) DNA barcoding and taxonomy in Diptera: a tale of high intraspecific variability and low identification success. Syst Biol 55:715–728

Meyer CP, Paulay G (2005) DNA barcoding: error rates based on comprehensive sampling. PLoS Biol 3:229–2238

Moritz C, Dowling TE, Brown WM (1987) Evolution of animal mitochondrial DNA: relevance for population biology and systematics. Annu Rev Ecol Syst 18:69–292

Neigel J, Domingo A, Stake J (2007) DNA barcoding as a tool for coral reef conservation. Coral Reefs 26:487–499

Pont-Kingdon GA, Okada NA, Macfarlane JL, Beagley CT, Wolstenholme DR, Cavaliersmith T, Clarkwalker GD (1995) A coral mitochondrial *mutS* gene. Nature 375:109–111

Pont-Kingdon GA, Okada NA, Macfarlane JL, Beagley CT, Watkins-Sims CD, Cavalier-Smith T, Clark-Walker GD, Wolstenholme DR (1998) Mitochondrial DNA of the coral *Sarcophyton glaucum* contains a gene for a homologue of bacterial MutS: a possible case of gene transfer from the nucleus to the mitochondrion. J Mol Evol 46:419–431

Pont-Kingdon GA, Vassort CG, Warrior R, Okimoto R, Beagley CT, Wolstenholme DR (2000) Mitochondrial DNA of *Hydra attenuata* (Cnidaria): a sequence that includes an end of one linear molecule and the genes for l-rRNA, $tRNA^{f\text{-}Met}$, $tRNA^{Trp}$, COII, and ATPase8. J Mol Evol 51:404–415

Reimer JD, Ono S, Fujiwara Y, Takishita K, Tsukahara J (2004) Reconsidering *Zoanthus* spp. diversity: molecular evidence of conspecifity within four previously presumed species. Zool Sci 21:517–525

Reimer JD, Ono S, Takishita K, Tsukahara J, Maruyama T (2006) Molecular evidence suggesting species in the zoanthid genera *Palythoa* and *Protopalythoa* (Anthozoa: Hexacorallia) are congeneric. Zool Sci 23:87–94

Sargent TD, Jamrich M, Dawid IB (1986) Cell interactions and the control of gene activity during early development of *Xenopus laevis*. Dev Biol 114:238–246

Schröder HC, Efremova SM, Itskovich VB, Belikov S, Masuda Y, Krasko A, Müller IM, Müller WEG (2003) Molecular phylogeny of the freshwater sponges in Lake Baikal. J Zool System Evol Res 41:80–86

Schroth W, Jarms G, Streit B, Schierwater B (2002) Speciation and phylogeography in the cosmopolitan marine moon jelly, *Aurelia* sp. BMC Evol Biol 2:1

Seifert KA, Samson RA, deWaard JR, Houbraken J, André Lévesque C, Moncalvo JM, Louis-Seize G, Hebert PDN (2007) Prospects for fungus identification using CO1 DNA barcodes, with *Penicillium* as a test case. Proc Natl Acad Sci 104:3901–3906

Shearer TL, Coffroth MA (2007). Barcoding corals: limited by interspecific divergence, not intraspecific variation. Mol Ecol Resour (in press). doi: 10.1111/j.1471-8286.2007.01996.x

Shearer TL, van Oppen MJH, Romano SL, Wörheide G (2002) Slow mitochondrial DNA sequence evolution in the Anthozoa (Cnidaria). Mol Ecol 11:2475–2487

Smith PJ, McVeagh SM, Mingoia JT, France SC (2004) Mitochondrial DNA sequence variation in deep-sea bamboo coral (Keratoisidinae) species in the southwest and northwest Pacific Ocean. Mar Biol 144:253–261

Tseng CC, Wallace CC, Chen CA (2005) Mitogenomic analysis of *Montipora cactus* and *Anacropora matthai* (cnidaria; scleractinia; acroporidae) indicates an unequal rate of mitochondrial evolution among Acroporidae corals. Coral Reefs 24:502–508

van Oppen MJH, Willis BL, Miller DJ (1999) Atypically low rate of cytochrome *b* evolution in the scleractinian coral genus *Acropora*. Proc Roy Soc Lond Ser B 266:179–183

van Oppen MJH, Catmull J, McDonald BJ, Hislop NR, Hagerman RJ, Miller DJ (2002) The mitochondrial genome of *Acropora tenuis* (Cnidaria; Scleractinia) contains a large group I intron and a candidate control region. J Mol Evol 55:1–13

van Oppen MJH, Koolmees EM, Veron JEN (2004) Patterns of evolution in the scleractinian coral genus *Montipora* (Acroporidae). Mar Biol 144:9–18

Vilgalys R (2003) Taxonomic misidentification in public DNA databases. New Phytol 160:4–5

Watkins RF, Beckenbach AT (1999) Partial sequence of a sponge mitochondrial genome reveals sequence similarity to Cnidaria in cytochrome oxidase subunit II and the large ribosomal RNA subunit. J Mol Evol 48:542–554

Wörheide G (2006) Low variation in partial cytochrome oxidase subunit I (COI) mitochondrial sequences in the coralline demosponge *Astrosclera willeyana* across the Indo-Pacific. Mar Biol 148:907–912