

DNA Barcoding of Marine Metazoa

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Abstract

More than 230,000 known species representing 31 metazoan phyla populate the world's oceans. Perhaps another 1,000,000 or more species remain to be discovered. There is reason for concern that species extinctions may outpace discovery, especially in diverse and endangered marine habitats such as coral reefs. DNA barcodes (i.e., short DNA sequences for species recognition and discrimination) are useful tools to accelerate species-level analysis of marine biodiversity and to facilitate conservation efforts. This review focuses on the usual barcode region for metazoans: a ~648 base-pair region of the mitochondrial cytochrome c oxidase subunit I (COI) gene. Barcodes have also been used for population genetic and phylogeographic analysis, identification of prey in gut contents, detection of invasive species, forensics, and seafood safety. More controversially, barcodes have been used to delimit species boundaries, reveal cryptic species, and discover new species. Emerging frontiers are the use of barcodes for rapid and increasingly automated biodiversity assessment by high-throughput sequencing, including environmental barcoding and the use of barcodes to detect species for which formal identification or scientific naming may never be possible.

INTRODUCTION

What Is—and Is Not—DNA Barcoding?

DNA barcoding is the derivation of short DNA sequence(s) that enables species identification, recognition, and discovery in a particular domain of life (e.g., Hebert et al. 2003a). The primary purpose of DNA barcoding is to identify an unknown specimen in terms of known classification (Miller 2007, Stoeckle & Hebert 2008), and thus DNA barcoding will complement—not supplant or invalidate—existing taxonomic practices.

It is important to discriminate the goals of DNA barcoding from DNA taxonomy. The latter involves the circumscription and delineation of species based on evolutionary species concepts and uses DNA sequences as the primary and diagnostic characteristics to identify and classify species (Vogler & Monaghan 2006). In contrast, DNA barcoding provides a means of identifying known species by sequence similarity and does not equate species identity, formally or informally, with a particular DNA sequence.

Ideally, barcoding and species discovery can be seen as complementary, with DNA sequences providing diagnostic characters that complement, accelerate, and revive traditional morphological taxonomy but do not replace it (DeSalle et al. 2005). Barcoding studies can help detect overlooked species with subtle or complex morphological traits and stimulate collection of additional genetic, morphological, ecological, geographical, and behavioral data (Bucklin & Frost 2009, Hebert et al. 2004a, Smith et al. 2008b, Steinke et al. 2009b).

The most frequently used gene for metazoans is the mitochondrial cytochrome c oxidase subunit I (COI) (Hebert et al. 2003a). The COI barcode region is located at the 5' end of the gene—usually 648 base pairs long—flanked by conserved regions that are the usual basis for design of conserved PCR (polymerase chain reaction) primers, such as the widely used Folmer primers (Folmer et al. 1994). This barcode region has been shown to exhibit a marked divergence between genetic distance within metazoan species (typically <3%) versus that between species (typically 10–25%; Hebert et al. 2003b).

Although the principal of DNA barcoding can be extended to various gene regions, the scope of this review is restricted almost entirely to the COI barcode region usually determined for metazoans. Exceptions are for taxa, described here as “problem children,” for which COI is not a suitable barcode (e.g., corals, sea anemones, and sponges). This review examines the utility (i.e., accuracy, precision, and reliability) of COI for the primary purposes of a DNA barcode in the recognition, discrimination, and discovery of species of metazoans.

History of DNA Barcoding

The basis for DNA barcoding is the decades-long use of DNA sequences in taxonomy and systematics for identifying and classifying organisms. The recent explosion of barcoding interest may be traced to Paul D. N. Hebert from the University of Guelph, Ontario, Canada, who proposed the compilation of a public library of DNA barcodes that would be linked to named specimens (Hebert et al. 2003a). In 2004, the Consortium for the Barcode of Life (CBOL; <http://barcodeindexing.si.edu>) was launched with a mission of developing DNA barcoding as a global standard for the identification of biological species, promoting the rapid compilation of high-quality DNA barcode records in a public library of DNA sequences, and developing new instruments and processes to make barcoding cheaper, faster, and more portable (Savolainen et al. 2005). CBOL now comprises more than 170 member organizations in 50 countries. In partnership with the Census of Marine Life (CoML; <http://www.coml.org>), CBOL recently initiated an international campaign for barcoding marine

biodiversity (MarBOL; <http://www.marinebarcoding.org>). MarBOL is working to ensure that barcodes are available for members of all key marine groups, with good coverage for groups of highest scientific or societal importance.

The extraordinarily rapid advances of DNA barcoding have not been without controversy. The sole use of COI barcodes as characters for species identification was met with initial skepticism (e.g., Will & Rubinoff 2004). Some applications of barcodes have engendered controversy and complaint within the taxonomic community, including using barcodes to delimit species boundaries and reveal cryptic species within known taxa (Wiens 2007), as well as barcode analysis for discovery of new or undescribed species (DeSalle 2006, Rubinoff et al. 2006). An emerging frontier is the construction of barcode libraries for analysis of species biodiversity without the expectation of formal identification and naming of the cataloged species (e.g., Blaxter et al. 2005).

Why DNA Barcoding?

There are an estimated 230,000 described species of marine metazoans and perhaps another 1,000,000 or more species awaiting discovery and description. Our knowledge of life in the oceans is still fragmentary and—for organisms that are tiny, rare, fragile, and/or found in obscure habitats—rudimentary. The diversity of life in the oceans can be analyzed and understood at many levels of organization, including higher taxonomic levels, functional groups, species, populations, and individuals. The careful process of collecting, classifying, and identifying known species and describing novel species will continue for decades to come. In the meantime, new methodologies are needed to expedite and facilitate the work of documenting current biodiversity levels and distribution patterns in the ocean.

Marine species may have extensive biogeographic ranges, spanning multiple ocean basins and reaching from pole to pole. Oceanographers and marine biologists are still working to map the oceans and describe the diversity, distribution, and abundance of metazoan species. Marine biodiversity can be examined using morphological characters and classical taxonomy, molecular systematic approaches (e.g., DNA taxonomy), and various other remote observation technologies, such as optical and acoustical imaging. Yet the recognition and discrimination of species is one of the foundations of biodiversity research, and it is exactly this step that is the most time-consuming and difficult—frequently forming a bottleneck in the analytical chain of sample and data collection and analysis.

DNA sequence analysis offers many opportunities for accurate, reliable, rapid, and eventually remote identification of specimens of all metazoan groups. DNA barcodes may be particularly useful for species identification of organisms that are rare, fragile, and/or small, especially when morphological identification is difficult and mistakes are likely due to simple or evolutionarily conserved body plans. Also, many taxa have circumglobal or disjunct geographic distributions; barcodes can reveal taxonomically significant geographic variation and cryptic species (see Ten Reasons for Barcoding sidebar, below).

DNA barcode libraries will lead to rapid molecularly based analysis of samples for known species. DNA barcodes are tools for understanding species diversity for global ocean/earth observing initiatives. Neither morphology nor genetics should be oversimplified; both can assess variation within and between populations and species, evolutionary relationships among species and higher taxa, and processes associated with speciation. Much may be lost in translation, but the goals of morphologists and geneticists studying marine organisms are the same: to gain genuine knowledge of species diversity of life in the ocean.

TEN REASONS FOR BARCODING^a

1. Works with fragments. Barcoding can identify a species from bits and pieces. Once established, barcoding will quickly identify undesirable material in processed foodstuffs and detect commercial products derived from regulated species (e.g., protected whale species served as sushi).
2. Works for all stages of life. Barcoding can identify a species in its many forms (e.g., eggs and larvae of marine fishes).
3. Unmasks look-alikes. Barcoding can distinguish among species that look alike, uncovering dangerous organisms masquerading as harmless ones and enabling a more accurate view of biodiversity (e.g., sibling species swarms of planktonic copepods).
4. Reduces ambiguity. Written as a sequence of four discrete nucleotides—CATG—along a uniform locality on genomes, a barcode of life provides a digital identifying feature, supplementing the more analog gradations of words, shapes, and colors. A library of digital barcodes will provide an unambiguous reference that will facilitate identifying species invading and retreating across the ocean and through centuries.
5. Makes expertise go further. The bewildering diversity of approximately 230,000 known species of 31 phyla confines even an expert to morphological identification of only a small portion of marine metazoa. Foreseeing perhaps 1,000,000 more species to go, scientists can equip themselves with barcoding to speed identification of known organisms and facilitate rapid recognition of new species.
6. Democratizes access. A standardized library of barcodes will empower many more people to call by name the marine species they observe. It will make possible identification of species abundant and rare, native and invasive, and engender appreciation of marine biodiversity locally and globally.
7. Opens the way for an electronic handheld field guide. Barcoding links biological identification to advancing frontiers in DNA sequencing, miniaturization in electronics, and computerized information storage. Integrating those links will lead to portable desktop devices and ultimately to handheld barcoders.
8. Sprouts new leaves on the tree of life. Barcoding the similarities and differences among marine metazoan species already named will provide a wealth of genetic detail, helping to draw the tree of life on Earth.
9. Demonstrates the value of collections. Compiling the library of barcodes begins with the millions of specimens in museums, aquaria, and other biological repositories. Barcoding shines a spotlight on these institutions and their collections and will strengthen their ongoing efforts to preserve marine biodiversity.
10. Speeds writing the encyclopedia of life. Compiling a library of barcodes linked to vouchered specimens and their binomial names will enhance public access to biological knowledge, helping to create an online encyclopedia of marine animals, with a Web page for every species.

^aModified from the Consortium for the Barcode of Life (<http://barcode.sil.edu>).

Why Mitochondrial Cytochrome C Oxidase Subunit I?

Today's gold standard metazoan barcode is a 658-base pair region at the 5' end of the mitochondrial COI gene (Hebert et al. 2003a). In the search for a universal metazoan barcode gene, the mitochondrial genome of metazoans was considered to have numerous advantages over the nuclear genome: lack of introns, limited exposure to recombination, high copy numbers in every cell, haploid character, and a generally strict maternal mode of inheritance. Many previous studies have used mitochondrial 12S and 16S rRNA genes for systematic analysis, but they share a disadvantage in the prevalence of insertions and deletions (indels) that greatly complicates sequence alignment and comparative analysis. The 13 protein coding genes in the metazoan mitochondrial genome generally lack indels, as these would result in codon reading frame shift mutations. COI was chosen over other mitochondrial protein coding genes because (a) conserved "universal" primers for

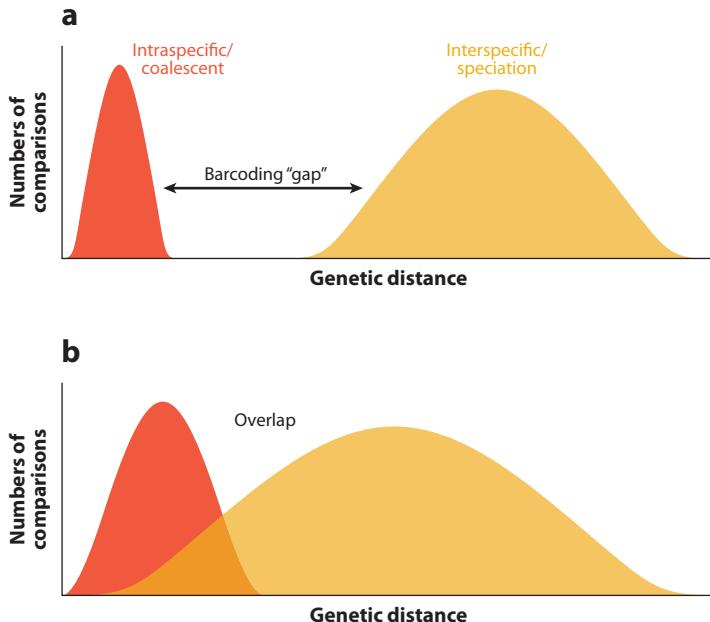


Figure 1

Diagrammatic hypothetical frequency distributions of genetic distances within and between species for two cases: (a) the barcode gap (Meyer & Paulay 2005) formed by the discrete distributions of intraspecific variation (shown in red) and interspecific variation (shown in yellow), and (b) overlapping distributions between intra- and interspecific variation and no barcode gap. Figure reproduced from Meyer & Paulay (2005).

a sizable region of the variable 5' end of the gene had proved useful for species from a broad range of metazoan phyla (Folmer et al. 1994), and (b) COI, compared with other mitochondrial genes, appeared to possess a phylogenetic signal useful over a broader range of taxonomic levels. Similar to other protein coding genes, third codon position nucleotides show a high incidence of base substitutions, leading to a rate of molecular evolution nearly three times greater than that of mitochondrial rRNA genes. Importantly, COI evolution is sufficiently rapid to allow the discrimination of very closely related species in most groups, as well as taxonomically significant intraspecific variation associated with geographic structure.

The primary reason for the selection of COI as the gold standard barcode gene is the typical pattern of variation observed for numerous species, with marked divergence and lack of overlap between intraspecific (i.e., between individuals of the same species) and interspecific (i.e., between individuals of different species) genetic distances (Hebert et al. 2003b). Dubbed the “barcode gap” by Meyer & Paulay (2005) (Figure 1), the lack of overlap between intra- and interspecific variation has been deemed of paramount importance for the accuracy and reliability of barcode genes. Consistent findings through many studies of diverse metazoans, including analysis of intraspecific variation of geographically widespread species, have led to the adoption of COI as the gold standard marker for the barcoding community (Stoeckle & Hebert 2008). This choice is clearly reflected in the large public databases such as the National Center for Biotechnology Information (NCBI) GenBank database (<http://www.ncbi.nlm.nih.gov/genbank>) and the Barcode of Life Data Systems (BOLD; <http://www.boldsystems.org>).

Exceptions to the resolving power of COI and other mitochondrial genes include the phyla Porifera (sponges) and Ctenophora (comb jellies) and the cnidarian class Anthozoa (corals and sea

anemones). The evolutionary rate of mitochondrial DNA (mtDNA) of these animals was found to be 10–20 times lower than other metazoans (Shearer et al. 2002, Huang et al. 2008) and usually insufficient to allow reliable discrimination between closely related species.

Standardization has resulted in a flood of COI sequences into public repositories such as BOLD and GenBank. The exceptional diversity and density of the data have allowed examination of molecular evolutionary hypotheses, including evolutionary changes in nucleotide base composition, such as GC shifts in animals (Min & Hickey 2007), as well as plants and fungi (Clare et al. 2008). Although the COI barcode region is not an especially effective sentinel sequence in predicting nucleotide composition (and likely no different from any region of the mitochondrial genome), barcode libraries will likely contain many replicates, with dense sampling across diverse taxa and thus offer many opportunities for analysis not possible for more limited mitochondrial whole-genome databases.

Other Genes

Numerous studies have compared COI and other gene sequences in terms of usual barcode functions: identifying species, discriminating closely related species, revealing cryptic species, and discovering new species. Erpenbeck et al. (2006) suggested the inclusion of another partition of COI (I3-M11, 23 base pairs downstream of the barcode region) to enable discrimination of species of cnidarians. The internal transcribed spacers (ITS) of nuclear rRNA genes have been evaluated as barcodes for many marine groups, especially those for which COI has proved problematical. A review (Vollmer & Palumbi 2004) of more than 500 studies published in 2000–2004 that used ITS as the main marker found many reports of intraindividual variability, suggesting that caution is required when analyzing these sequences. Calderón et al. (2006) compared patterns of COI and ITS variation within and between species of gorgonian corals in the Mediterranean; the gene regions used both identified species but did not resolve population structure. Chu et al. (2009) compared COI, mitochondrial 16S rRNA, and nuclear 18S rRNA sequences as barcodes for birds, fishes, nematodes, and crustaceans; they concluded that 18S barcodes were advantageous for some analytical methods. Whether or not the COI barcode region alone is sufficient for species assignments—a sometimes contentious issue—it is widely viewed as advantageous to use sequences from multiple loci (Neigel et al. 2007).

BIOINFORMATICS AND STATISTICAL ANALYSIS

The bioinformatics of DNA barcoding can be divided into two distinct approaches: (*a*) diagnostic identification of species (i.e., the correct assignment of an unknown sample to a known species), and (*b*) detection and classification of novel sequences for previously unsampled species. Diagnostic identification requires that an individual's DNA sequence can be unambiguously associated with the sequences of a group of organisms, ideally at the taxonomic level of species. Establishing this association is complicated for several reasons, including patterns of intraspecific variation of the reference sequence and possible incongruence between the evolutionary histories of the gene and species.

Most recently published studies have used analysis of genetic distances to infer the species identification based on the barcode designation. One approach is a simple NCBI BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) query (Altschul et al. 1990), which uses a raw similarity score to determine the nearest neighbor to the query sequence. Although BLAST searches are rapid, they have a number of significant drawbacks, including high rates of false-positive results

inherent to the BLAST algorithm and frequent sampling density-dependent accuracy (Koski & Golding 2001).

Other methods such as TaxI (Steinke et al. 2005) and TaxonDNA (Meier et al. 2006) apply linear searches based on distances of pairwise comparisons to infer the most similar sequence and identify the species based on the barcode sequence.

BOLD was initially developed as an informatics workbench for a single, high-volume DNA barcode facility (Ratnasingham & Hebert 2007). BOLD uses an identification (ID) engine that collects nearest neighbors from all reference sequences by aligning the query to a global alignment through a Hidden Markov Model (HMM) profile of the COI protein (Eddy 1998), followed by a linear search of the reference library. BOLD, currently the most commonly used species ID system based on barcodes, has evolved into a community resource and is used by CBOL barcoding campaigns for fishes and birds, as well as MarBOL.

It should be noted that the accuracy of the BOLD algorithm (and all other distance-based methods) depends on the degree of disparity between intra- and interspecific sequence variation (i.e., the barcode gap discussed above). Whereas the existence of such a gap has been successfully demonstrated in a variety of studies (Hajibabaei et al. 2006a, Kerr et al. 2007, Ward et al. 2005), other studies have shown that young species may lack a distinctive barcode gap (e.g., up to 8% of the gastropod group cowries; Meyer & Paulay 2005). Furthermore, the analysis of species affiliation and the inference of species identity require an *a priori* threshold similarity value; it remains questionable whether universal thresholds exist, even among congeneric species (Ferguson 2002, Hickerson et al. 2006, Whitworth et al. 2007).

Recently, statistically more sophisticated methods of species identification have been developed. Matz & Nielsen (2005) described a likelihood-ratio test (LRT) to query species identity. Nielsen & Matz (2006) and Abdo & Golding (2007) used coalescent-based approaches in the context of Markov Chain Monte Carlo (MCMC) methods to assign unknowns to identified species. All these methods are computationally demanding, and in the case of the LRT, corrections for multiple testing remain uncertain (Ross et al. 2008). Furthermore, the Bayesian method currently implemented (Nielsen & Matz 2006) cannot handle more than two species at a time, thereby limiting its use for DNA barcoding. Although the decision-theory method (Abdo & Golding 2007) uses more information in the sequence data than distance-based methods, the statistical power comes with a huge computational expense, limiting its usefulness. None of these methods is a panacea, yet each offers potential improvement over previously used methods.

Character-based methods have been proposed to identify classification rules based on an existing hierarchical organization and then to classify unknown data (DeSalle et al. 2005, Sarkar et al. 2008). A first application is based on the Characteristic Attributes Organization System (CAOS) algorithm developed for discovery of conserved character states from cladograms or groups of categorical information (Sarkar et al. 2002a,b). Although this method has proved to be fast, accurate, and sufficiently sensitive to distinguish sibling species pairs (Kelly et al. 2007, Lowenstein et al. 2009, Rach et al. 2008), it has not been tested with very large and diverse data sets, such as GenBank and BOLD. CAOS avoids some problems of distance-based methods, such as the reliance on a barcoding gap (Meyer & Paulay 2005, Moritz & Cicero 2004) and is thought by some researchers to be compatible with classical character-based taxonomy (DeSalle 2005, Rubinoff et al. 2006). Another character-based approach adopted a logic mining method based on two optimization models; Bertolazzi et al. (2009) reported results for two data sets where COI barcodes were used to identify individuals to different species.

A recent method by Zhang et al. (2008) is based on existing back-propagation neural networks and uses both differences between sequences and their patterns (e.g., relative positions of variable sites). Neural networks have the disadvantage that they are governed by supervised training and

the parameter(s) responsible for an assignment may remain unknown. Also, input sequences must be assigned to a known species, requiring a complete set of predefined taxa, so the method is not useful for uncertain species identifications or species discovery.

Indicator vectors have been used by Sirovich et al. (2009, 2010) to assign sequences to known species and groups of organisms. Sequences are transformed into vectors; species- and group-distinguishing vectors are determined by contrast with other vectors. The results are presented in visually intuitive scalable displays, called Klee diagrams because of their resemblance to modern art (**Figure 2**).

The second bioinformatic approach of barcoding is the classification of new data based on the distribution of sequence variation within and between species. A specific goal of this approach is to determine whether barcodes can be used to identify previously unrecognized species. The proposed mechanism for the evaluation of unknowns within a partially sampled phylogeny is through the implementation of thresholds, chosen to separate intraspecific variation from interspecific differences. Thus, if two specimens putatively belonging to the same species diverge by ten times the average divergence of that group, then one specimen is likely to be a new species (Hebert et al. 2004b) that warrants further taxonomic examination. This rule may be a useful guide but will detect cryptic species only if they are quite divergent from recognized species (Hickerson et al. 2006, Ward 2009). Currently, classification of barcodes is usually done using a clustering algorithm (e.g., distance-based Neighbor Joining, or NJ) that evaluates genetic distances within and between species (Bucklin et al. 2007, Steinke et al. 2009b, Ward et al. 2005). The limits to this method include undersampling due to poor taxonomy (Meyer & Paulay 2005), ancestral polymorphism, and incomplete lineage sorting. Also, mtDNA sequence divergence does not necessarily track species boundaries (Avise 2000, 2004). For these reasons, some studies have used nuclear markers to corroborate barcode analyses (Bucklin & Frost 2009, Raupach et al. 2009, Smith et al. 2007, Sonnenberg et al. 2007).

DNA barcoding analytical methods, algorithms, and bioinformatic approaches have not yet come of age. Distance-based methods are now widely used to assign an unknown specimen to a known species, detect novel sequences, and determine an unknown specimen to be a distinct species. They are computationally fast and yield consistent results for the many species that exhibit the necessary disparity between intra- and interspecific variation. The barcoding community increasingly acknowledges that distance-based approaches are inconsistent with classical character-based taxonomy (DeSalle et al. 2005) and that valuable information is lost by converting sequences into genetic distances (Zhang et al. 2008). New algorithms and methods are in development that represent the next generation of barcode analysis for both correct assignment of barcodes to known species and classification of novel sequences.

PROGRESS IN BARCODING MARINE METAZOA

The numerous and diverse metazoan phyla found in marine environments provide an excellent opportunity to examine the broad taxonomic utility of COI barcodes for species identification, discrimination, and discovery. We report here a sampling of the barcoding studies that have used COI to examine important taxonomic groups of marine metazoa (**Table 1, Figure 3**).

Taxon-by-Taxon Barcoding

Sponges. The lack of complex morphological characters and high degree of homoplasy have confounded classification within the phylum Porifera (sponges), making DNA barcoding particularly useful in this group. Sponges are highly speciose, ecologically important, and

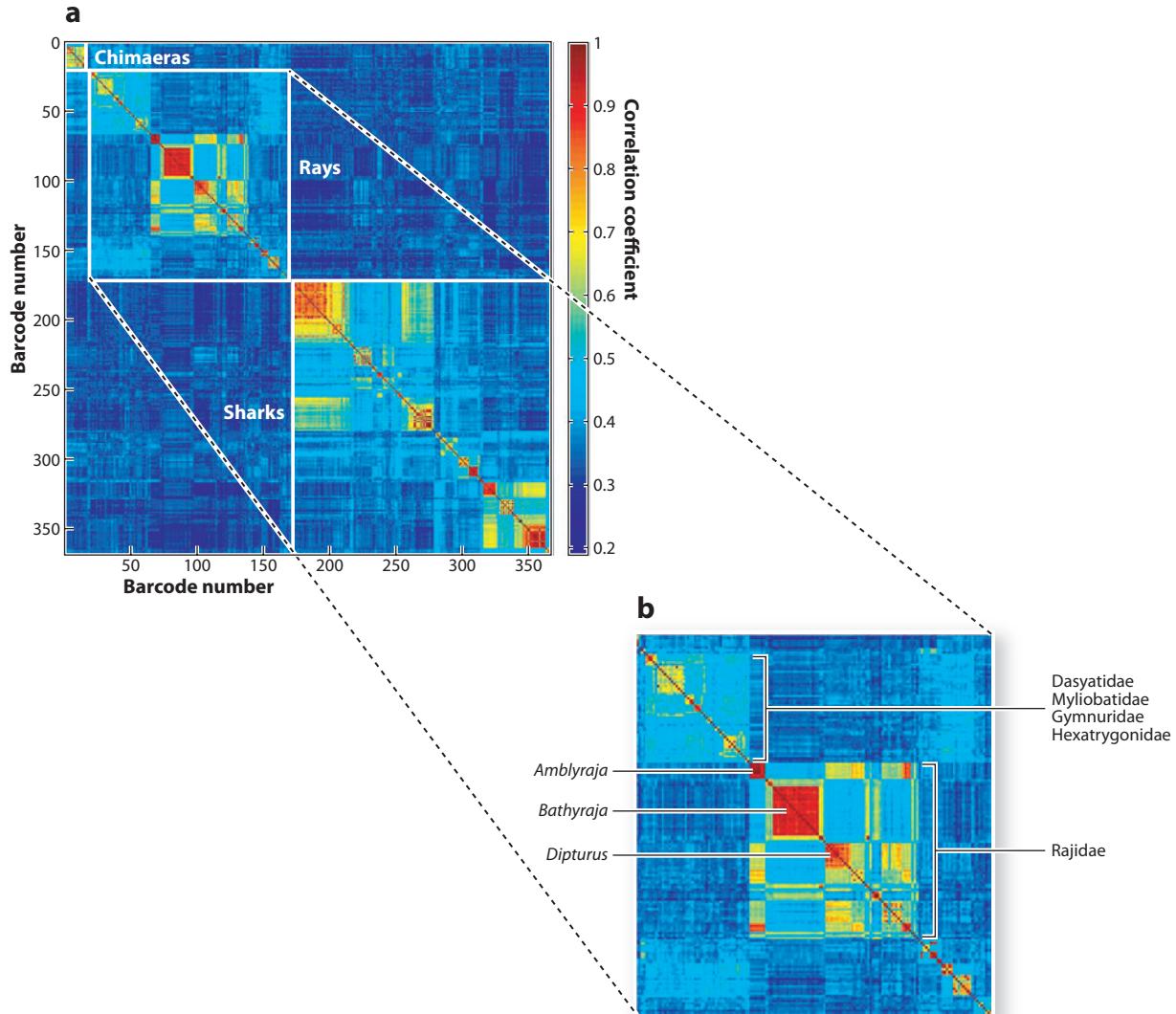


Figure 2

Vector analysis of 4,176 barcodes of 367 species of elasmobranchs (chimaeras, sharks, and rays) using the methods of Sirovich et al. (2009, 2010). Only species with three or more individuals were used. Results are shown as a Klee diagram, so-named for the resemblance to modern art. (a) Major elasmobranch groups are clearly defined by high correlation coefficients from the vector analysis. (b) Enlargement of a portion of the diagram showing families and genera of rays with highly correlated barcodes and demonstrating the zoom function of vector analysis and Klee diagrams. (Unpublished data and figure from D. Steinke, Univ. of Guelph, Canada.)

commercially valuable to the pharmaceutical and biomaterials industries. The Sponge Barcoding Project (Wörheide & Erpenbeck 2007) aims to examine all species. To date, barcoding has worked reasonably well (Blanquer & Uriz 2007, Erpenbeck et al. 2007, Raleigh et al. 2007). However, the resolving power of COI in sponges appears not to correspond to their higher metazoan counterparts (Cardenas et al. 2009, Heim et al. 2007, Park et al. 2007, Wörheide 2006).

Table 1 Progress in barcoding marine metazoa by phylum^a

Phylum	No. of known species	No. of barcoded species	Barcoded species (%)
Acanthocephala	600	10	1.7
Annelida	12,148	635	5.2
Arthropoda	47,217	3,580	7.6
Brachiopoda	550	35	6.4
Bryozoa	5,700	20	0.4
Chaetognatha	121	23	19.0
Chordata	21,517	7,279	33.8
Cnidaria	9,795	594	6.1
Ctenophora	166	0	0.0
Cycliophora	1	1	100.0
Echinodermata	7,000	771	11.0
Echiura	176	2	1.1
Entoprocta	170	0	0.0
Gastrotricha	400	0	0.0
Gnathostomulida	97	8	8.2
Hemichordata	106	2	1.9
Kinorhyncha	130	0	0.0
Loricifera	18	0	0.0
Mollusca	52,525	4,813	9.2
Nematoda	12,000	180	1.5
Nematomorpha	5	0	0.0
Nemertina	1,230	81	6.6
Orthonectida	24	0	0.0
Phoronida	10	0	0.0
Platyhelminthes	15,000	124	0.8
Porifera	5,500	67	1.2
Priapulida	8	1	12.5
Rhombozoa	82	0	0.0
Rotifera	50	20	40.0
Sipuncula	144	15	10.4
Tardigrada	212	9	4.2
TOTALS	192,702	18,270	9.5

^aNumbers of known species are from Bouchet (2006). Numbers of barcoded species are as reported in the National Center for Biotechnology Information (NCBI) GenBank and the Barcode of Life Data Systems (BOLD) databases. Percentages of barcoded species are determined from numbers as shown.

COI divergence rates are 10–20 times lower for sponges than for Bilateria (Shearer et al. 2002, Wörheide 2006), and the barcode region typically does not distinguish between species. Some studies have used alternative barcode regions, such as the I3-M11 region of COI (Erpenbeck et al. 2006) and ITS (Park et al. 2007, Pöpke et al. 2010).

Cnidarians. Very low levels of COI polymorphism within the cnidarian class Anthozoa (including sea anemones, corals, and sea pens) have meant that the barcode region is of limited usefulness

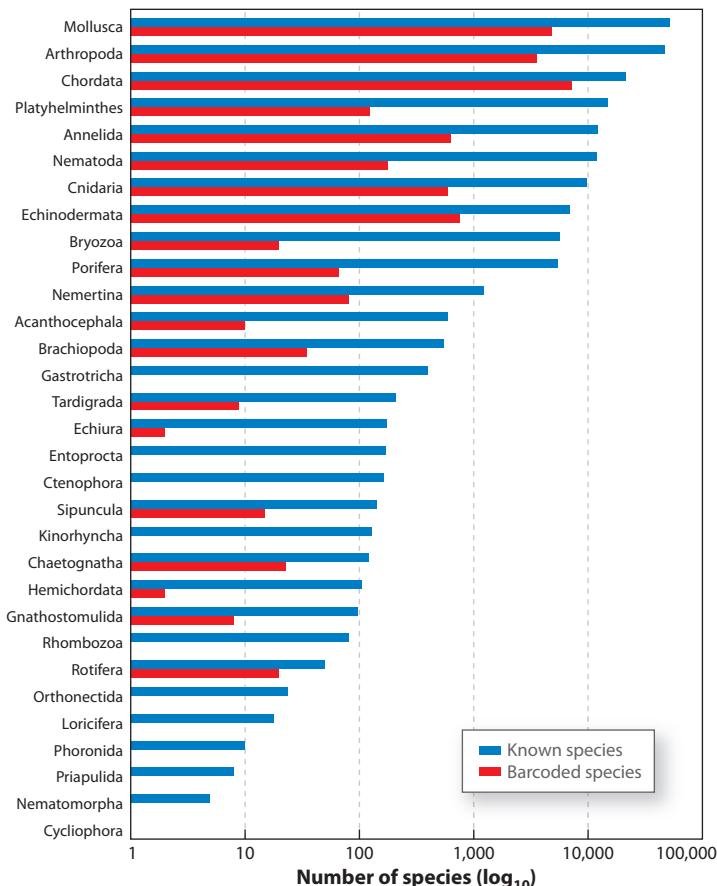


Figure 3

Numbers of known and barcoded marine metazoan species per phylum. The phyla are ordered by relative numbers of marine species; the nine most speciose phyla (*top*) contain 95% of all marine metazoan species. Blue bars indicate numbers of known species; red bars indicate numbers of barcoded species. The scale is logarithmic. See Table 1 for actual numbers. Numbers of known species are based on data from Bouchet (2006).

for identifying or discriminating species of this group when they are relatively closely related (Shearer et al. 2002). The underlying evolutionary dynamics may include very slow nucleotide substitution rates for mitochondrial genes, although nuclear genes may vary (Hellberg 2006). Shearer & Coffroth (2008) suggest that the lack of COI divergence among many species indicates the need for alternate markers. There are exceptions to this general conclusion, however. COI discriminated four species of gorgonian corals in the Mediterranean (Calderón et al. 2006). COI discriminated species of the hexacorallian order Zoantharia (Sinniger et al. 2008); genetic distances differed among species, genera, and families (Sinniger et al. 2010), indicating that COI may be useful for zoanthid classification.

COI evolution in the cnidarian classes Hydrozoa and Scyphozoa is more typical of other metazoans (Huang et al. 2008). Multiple cryptic hydrozoan species were discovered based on COI by Folino-Rorem et al. (2009) and Moura et al. (2008). In concert with other genes, COI was used to resolve species and systematic relationships of the hydrozoan family

Campanulariidae (Govindarajan et al. 2006). Within the Scyphozoa, COI has been widely used as a barcode to reveal cryptic species, confirm species identification, and examine evolutionary relationships (Dawson 2005a,b,c; Dawson & Jacobs 2001; Holland et al. 2004). Across the Medusozoa (a cnidarian subphylum comprising of ~3,800 species in the classes Hydrozoa, Cubozoa, and Scyphozoa), COI was shown by Ortman et al. (2010) to be broadly useful as a barcode for species identification and discovery.

Barnacles. Barcoding studies of barnacles in the western Pacific have provided evidence of cryptic species within the acorn barnacle *Chthamalus* (Tsang et al. 2008) and the symbiotic coral barnacle *Wanella* (Tsang et al. 2009) as well as lack of genetic divergence between species of *Tetraclita* (Tsang et al. 2007).

Copepods. Numerous barcoding studies have sought to reexamine morphologically based descriptions of copepod sibling species swarms, which present persistent challenges to expert taxonomists. Among the calanoid copepods, the COI barcode region has been determined and confirmed to be diagnostic for sibling species of *Calanus* (Hill et al. 2001, Unal et al. 2006), *Clausocalanus* (Bucklin & Frost 2009), *Neocalanus* (Machida et al. 2006), and *Pseudocalanus* (Bucklin et al. 2003). Among cyclopoid copepods, species of Oncaeidae—with more than 100 species and many sibling species, a challenge for taxonomists—were examined for genetic distinctiveness of COI and other genes by Böttger-Schnack & Machida (2010). Although copepods are frequently thought of as taxonomically well known, comprehensive analyses based on DNA barcodes are revealing considerable hidden diversity and cryptic speciation across the suborder. Dippenaar et al. (2010) examined the cosmopolitan elasmobranch symbiotic copepod *Nesippsus orientalis* (Siphonostomatoidea) off South African coasts and found two divergent clades related to host species; they expect further discoveries of cryptic species within this group. DNA barcoding of another siphonostomatoid copepod, the common sea louse or parasite of fish, revealed species relationships and evidence of cryptic species (Øines & Heuch 2005).

Stomatopods. Barcodes have been used to identify stomatopods (mantis shrimp) from the South China Sea by Tang et al. (2010), who successfully matched unknown larvae to known adult species from the region. In contrast, Barber & Boyce (2006) used barcodes to discriminate larval stomatopods in the Coral Triangle and the Red Sea, but few could be successfully matched to known adults. These results demonstrated the marked underestimation of biodiversity in this well-studied group and highlighted taxonomic limitations of the reference sequence database.

Mysids. Remerie et al. (2006) found evidence of a complex of cryptic species within the mysid *Mesopodopsis slabberi*, with marked divergences between Atlantic and Mediterranean populations.

Amphipods. Analysis of systematic relationships within the amphipod genus *Gammarus*—a highly speciose genus found to be monophyletic using COI and other molecular markers (Hou et al. 2007)—has revealed strong evidence based on the COI barcode region of geographic divergence (Krebes et al. 2010) and cryptic speciation (Costa et al. 2009, Hou et al. 2009). Havermans et al. (2010) used COI to examine the systematics of Antarctic lysianassoid amphipods, finding incongruence between the molecular phylogeny and morphological classification. Radulovici et al. (2009) found cryptic and invasive amphipod species in the Estuary and Gulf of St. Lawrence, in the northwest Atlantic Ocean.

Euphausiids. Frequently considered to be easily recognizable, euphausiids are ecologically important and may occasionally dominate pelagic communities in terms of abundance and biomass. DNA barcodes for euphausiids are useful for species identification, especially of larval and juvenile forms that can be genuinely cryptic. COI reliably discriminated 40 species of euphausiids in a barcoding study by Bucklin et al. (2007). COI proved useful to examine species-specific evolutionary patterns for *Nyctiphanes* (D'Amato et al. 2008).

Decapods. Many decapods are clearly distinguishable as adults but have larval and juvenile forms that are difficult—or impossible—to identify to species. In some instances, the larvae are distinguishable but not easily matched to the correct adult form. New species descriptions of the East Pacific galatheid squat lobster *Munidopsis* included DNA barcodes, which also revealed high gene flow among distant conspecific populations (Jones & Macpherson 2007). Barcodes clearly discriminated species of the fiddler crab *Uca* in the northwestern Indian Ocean (Shih et al. 2009) and the hermit crab *Clibanarius* of the Ryukyu Archipelago of Japan (Hirose et al. 2010). Although barcodes for coral reef decapods could not be matched to known sequences in the GenBank database, they could be used to count numbers of species (Plaisance et al. 2009). A barcoding study of megalops larvae of *Cancer* crabs in the southeastern Pacific Ocean resulted in the description of diagnostic morphological characters for species identification (Pardo et al. 2009). Phylogeographic analysis of decapods using COI has revealed cryptic species within the brachyuran mangrove crab *Perisesarma guttatum* on the eastern coastline of Africa (Silva et al. 2010).

Mollusks. DNA barcoding has been widely used for marine mollusks, including chitons (Kelly et al. 2007), gastropods (Hunt et al. 2010; Jennings et al. 2010a; Krug et al. 2007; Meyer & Paulay 2005; Puillandre et al. 2009a,b,c), bivalves (Feng et al. 2010, Lorion et al. 2009, Mikkelsen et al. 2007, Nuryanto et al. 2007), and cephalopods (Allcock et al. 2010, Undheim et al. 2010). Barcodes discriminated frequently confused species and revealed species relationships of the gastropod *Nerita* throughout the southwestern tropical Pacific Ocean (Spencer et al. 2007). Johnson et al. (2008) found 14 nominal species and numerous cryptic species of deep-sea lepetodrilid limpets, the most abundant and diverse gastropod group at hydrothermal vents. Barcodes have also revealed lack of genetic differentiation among some species of mollusks: sympatric intertidal limpets (Siphonariidae) off coastal southeast Africa lacked barcode differences, suggesting that they are morphotypes of a single species (Teske et al. 2007b). Two clams of the genus *Donax* showed no significant barcode variation and were found to represent one species (Carstensen et al. 2009). Puillandre et al. (2009c) clearly demonstrated the ability of barcodes to identify gastropod larvae, although barcode data are sparse and taxonomic coverage is biased toward shallow water species. Meyer & Paulay (2005) also emphasized problems associated with limited sampling in delineating closely related species in taxonomically understudied groups.

Annelids. The vast majority of marine annelids belong to the class Polychaeta. The group is diverse, with over 12,000 known species and undoubtedly many thousands of species awaiting discovery and description. In a comprehensive barcoding study of polychaetes in the Alaskan and Canadian Arctic, Mincks Hardy et al. (2010) estimated that one-fourth of polychaete species were composed of two or more distinct genetic lineages. A second study found cryptic species within the cosmopolitan fireworm *Eurythoe complanata* (Barroso et al. 2010). Similarly, Rice et al. (2008) found a cryptic species complex within *Polydora cornuta* along the Atlantic and Pacific coasts of North America; barcodes revealed cryptic species within the common ragworm *Hediste diversicolor* in the Baltic Sea (Virgilio et al. 2009). COI discriminated closely related sympatric species of the

lugworm *Arenicola* (Luttkhuizen & Dekker 2010) and the oligochaete *Tubificoides* (Erséus & Kvist 2007) in northeast Atlantic and Scandinavian waters.

Echinoderms. Barcodes discriminated 191 species of echinoderms, including representatives of all five classes (ophiuroids, asteroids, echinoids, holothuroids, and crinoids), confirming the usefulness of sequence variation for species discrimination across the phylum (Ward et al. 2008). Barcoding studies have used COI to examine species limits and reveal cryptic species of asteroids (Naughton & O’Hara 2009), holothuroids (Uthicke et al. 2010), and crinoids (Helgen & Rouse 2006, Wilson et al. 2007). Coordinated efforts to accelerate barcoding of echinoderms have yielded new protocols and primers for COI sequencing (Hoareau & Boissin 2010).

Urochordates. Barcodes have allowed accurate species identification for ascideans (sea squirts) that frequently lack diagnostic morphological characters. Pérez-Portela et al. (2007) found cryptic species within the ascidian *Pycnoclavella* in Atlantic and Mediterranean regions; barcodes were used by Hirose & Hirose (2009) to identify species of *Diplosoma*. Pérez-Portela & Turon (2008b) found that COI resolved relationships among ascidian species and genera but not families (Pérez-Portela et al. 2009).

Flatworms. Vilas et al. (2005) used COI for discovery of cryptic species of parasitic flatworms (phylum Platyhelminthes). COI also revealed cryptic species of a free-living flatworm (Sanna et al. 2009). The community is still in search of optimal marker genes and protocols for a broad barcoding effort (Moszczynska et al. 2009).

Nematodes. Morphological identification of individual nematodes to described species is often not possible due to their sheer abundance, small size, and lack of taxonomic experts. Nematode diversity in marine sediments remains essentially unknown; surveys suggest that diversity of marine nematodes may exceed 1,000,000 species (Lambshead 2001). Difficulties in developing COI primers and protocols resulted in the use of the small-subunit (18S) rRNA gene for species identification and diversity estimates (Bhadury & Austen 2006, 2010; Floyd et al. 2005). Recently work has also focused on COI for barcoding (Cross et al. 2007, Elsasser et al. 2009, Ferri et al. 2009). COI variation of the nematode *Thoracostoma trachygaster* revealed cryptic diversity and population genetic structure consistent with dispersal scales of a few 100 km (Derycke et al. 2010). Another study of *Monocelis lineata* showed marked genetic differentiation between the Atlantic and Mediterranean regions and supported the existence a cryptic sibling species complex (Sanna et al. 2009).

Nemerteans. For some taxonomically challenging groups, barcodes alone cannot resolve the systematic questions. Sundberg et al. (2010) barcoded species of the nemertean *Cerebratulus* and were unable to uniquely resolve questions of species identity and relationships based upon the COI barcode region.

Bryozoa. Gomez et al. (2007b) found close correspondence between barcode lineages and reproductive isolation of taxa, despite minimal morphological change and noted the divergence of the cosmopolitan bryozoan *Celleporella* into four major clades (Gomez et al. 2007a).

Pycnogonids. Cryptic lineages within the pycnogonid *Colossendeis megalonyx* were found through COI analysis of Antarctic and sub-Antarctic collections (Krabbe et al. 2010).

Chaetognaths. Subtle morphological differences divide chaetognath species, many of which have extensive biogeographical ranges. Accurate species identification is possible using the COI barcode region, despite high levels of intraspecific variation perhaps resulting from large-scale geographic structuring (Jennings et al. 2010b). Miyamoto et al. (2010) found cryptic species within the deep-sea chaetognath *Caecosagitta macrocephala*.

Fishes. Fishes currently represent the most comprehensively sampled and studied group of marine metazoans. The need for comprehensive and reliable species identification tools, combined with early barcoding success with fishes (Savolainen et al. 2005, Ward et al. 2005), led to the formation of the global initiative Fish Barcode of Life (FISH-BOL; <http://www.fishbol.org>). Launched in 2005, FISH-BOL is gathering DNA barcode records for all 30,000 known species of fishes. To date, barcoding has been completed for 51,906 specimens of 7,437 species, most of which are marine. The barcodes are being used to address issues related to commercial fisheries (e.g., market substitution and quota management) and sampling has been guided by these concerns. FISH-BOL also promises to be a powerful tool for extending the understanding of the natural history and ecological interactions of fish species (Ward et al. 2009).

Recent efforts have developed and optimized analytical protocols for fishes, including DNA extraction and PCR amplification for high-quality barcode sequence data (Ivanova et al. 2006, 2007). Group-specific PCR primers (Paine et al. 2007, Ward et al. 2005) and primer cocktails (Ivanova et al. 2007) are typically used for barcoding fishes. In a few instances (e.g., some elasmobranches and muraenids), more taxonomically specific primers are necessary (Spies et al. 2006).

DNA barcoding can be used to identify whole fish, as well as fillets, fins, fragments, larvae, and eggs. For all but the whole fish, molecular analysis is particularly useful because the morphological traits used for species identification are generally absent. DNA barcoding was used to identify shark fins confiscated from illegal fishers in northern Australian waters (Holmes et al. 2009), based on a comprehensive barcode library assembled in previous years (Ward et al. 2007, 2008). COI barcoding has been successfully used to identify fish larvae taken from the Great Barrier Reef (Pegg et al. 2006), Caribbean (Victor 2007), and Pacific (Hubert et al. 2010, Paine et al. 2008).

The first fish barcode study (Ward et al. 2005) examined a number of taxa that had only gained provisional recognition from morphological examination. In every case, likely species were separable by their barcodes; some have subsequently been named. Species of the dogfish initially referred to as *Squalus* sp. B, C, D, E, and F have now gained formal scientific names (Last et al. 2007b, Ward et al. 2007). A study of fish species thought to bridge South African and Australian waters found that approximately one-third of those species actually represented two taxa (Zemlak et al. 2009). There are numerous examples among fishes of the power of an integrated approach to taxonomy using barcoding. The first vertebrate to include a COI barcode as part of its species description was the goby *Coryphopterus kuna* (Victor 2007). COI barcoding helped validate a number of new fish species, including a goby (Victor 2008), sting ray, Antarctic ray *Bathyraja* (Smith et al. 2008b), handfish *Brachionichthys australis* (Last et al. 2007a), and five new species of damselfish *Chromis* (Pyle et al. 2008).

Larger-scale barcoding studies of fishes of different regions revealed an average 2% overlooked species based on the distance metric proposed by Hebert et al. (2004b), which targets for further study any barcode that diverges from its putative species by ten times the average divergence within that group. These flagged instances have been subsequently validated by morphology, geography, and ecology and are awaiting formal description (**Table 2**). Extrapolating species discoveries to date among 2% of known species, we can expect up to 600 overlooked or cryptic species awaiting discovery through similar studies.

Table 2 Overlooked species of fishes found as a result of large barcoding studies in diverse ocean regions^a

Name of study	No. of species	No. of specimens	Overlooked species (N)	Overlooked species (%)	Reference
Fishes of Australia	207	754	5	2.40	Ward et al. 2005
Sharks and rays of Australia	210	945	4	1.90	Ward et al. 2008
Fishes of Pacific Canada	201	1,225	2	1.00	Steinke et al. 2009a
Reef-associated fishes	390	1,638	7	1.80	Steinke et al. 2009b
Arctic fishes of W. Pacific	106	882	3	2.80	Mecklenburg et al. 2010

^aThe studies listed examined more than 650 specimens of more than 100 species. Analysis resulted in detection of highly divergent barcodes within some putative species. Barcoding evidence of “overlooked” species was corroborated through morphological, geographical, and ecological data.

Reptiles. DNA barcodes can be useful for conservation and wildlife forensics of sea turtles by identifying turtle meat and eggs illegally traded or carcasses stranded on beaches (Vargas et al. 2009). Although sea turtles represent an ancient group with a slow mutation rate, all species were successfully identified and no cryptic species were revealed based on genetic distances and character-based methods (Naro-Maciel et al. 2010).

Birds. The CBOL All Birds Barcoding Initiative (<http://www.barcodingbirds.org>) includes seabirds. Among 17 sets of species of North American seabirds with overlapping barcodes, Kerr et al. (2007) found 8 species of large white-headed gulls. These may represent well-formed species that are losing genetic identity due to secondary contact and hybridization. The study also showed deep divergence between two lineages of the circum-Arctic species *Fulmaris glacialis*.

Mammals. Marine mammals currently lack a comprehensive library of DNA barcodes. Analysis of beaked whales (Ziphiidae), the least known of all cetacean families, led to significant discoveries, including description of a new species from the north Pacific, *Mesoplodon perrini* (Dalebout et al. 2002); resurrection of a long-forgotten species in the Southern Hemisphere, *M. traversii* (van Helden et al. 2002); confirmation of the identity of the enigmatic tropical bottlenose whale, *Indopacetus pacificus* (Dalebout et al. 2003); and perhaps discovery of a new species of *Mesoplodon* in the tropical Pacific (Dalebout et al. 2007).

Problem children. In two of the most ecologically important groups on coral reefs, the Anthozoa (e.g., corals and sea anemones) and Porifera (sponges), COI sequences have diverged too little to be diagnostic for all species (or even some genera) and hybrids cannot be distinguished from their maternal ancestors (Neigel et al. 2007). COI gene trees are typically paraphyletic (i.e., containing a single ancestor and some, but not all, of its descendants). Whereas in sponges a longer COI fragment than the standard 5' end might be useful (Erpenbeck et al. 2006), cnidarian barcoding might need another gene (Shearer & Coffroth 2008, Shearer et al. 2002).

Evolutionarily young (i.e., recently isolated) species present challenging cases for barcoding. Distinct but sympatric species may have very similar or identical barcodes due to incomplete lineage sorting or introgression (Kempainen et al. 2009, Steinke et al. 2009a). This was an early criticism of barcoding (Rubinoff et al. 2006), although such cases are rare outside the sponges, ctenophores, and anthozoans. Comparative analysis of a secondary nuclear marker can be used to confirm—or refute—the barcode results (e.g., Cardenas et al. 2009, Sonnenberg et al. 2007).

Two other groups bear brief mention here, although the difficulties may be more a matter of methodology than molecular evolution. Very few COI sequences are available for the pelagic

groups Appendicularia (Larvacea) and Thaliacea. Until sufficient COI sequence data are available and can be analyzed, it will remain unclear whether or not COI will be an appropriate barcode gene for these groups.

Barcoding Ocean Realms

Pelagic. Until recently, some pelagic taxa (e.g., copepods, euphausiids, and chaetognaths) were thought to be well known taxonomically. However, DNA barcoding of representatives of even these well-known groups has increasingly altered this perspective, and morphologically cryptic but genetically distinctive species of zooplankton are being found with increasing frequency. This issue is especially relevant for widely distributed species and/or for species with disjoint distributional ranges (McManus & Katz 2009). Interestingly, coastal and neritic environments appear to contain a higher frequency of zooplankton species that are endemic (i.e., occupying a highly restricted or unique habitat or geographic range) than do open ocean regions (e.g., Chen & Hare 2008, Dawson & Hamner 2005, Ueda & Bucklin 2006). DNA barcodes for pelagic assemblages have provided evidence of the power of COI for reliable identification of species across the 14 metazoan phyla and 20+ invertebrate classes occurring in the pelagic realm (Bucklin et al. 2010a,b) (**Figure 4**).

Polar seas. Polar regions are under threat from human activities, ocean acidification, and global warming, with potentially greater impacts than at lower latitudes. Arctic and Antarctic regions are considered to have intermediate levels of diversity (~5,000 species each), but these figures may markedly underestimate actual levels. Recent CoML and International Polar Year (IPY) sampling efforts have yielded new collections for DNA barcoding (Grant & Linse 2009, Mincks Hardy

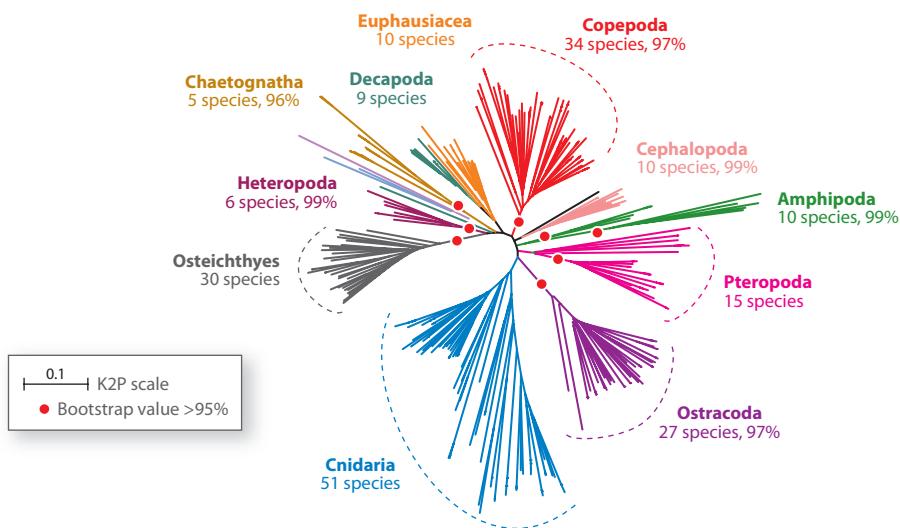


Figure 4

Distance-based analysis of barcodes for a marine zooplankton assemblage of the Sargasso Sea, Northwest Atlantic Ocean. The tree shown was determined using the Neighbor Joining algorithm and Kimura-2-Parameter (K2P) genetic distances and was bootstrapped 1,000 times. A total of 328 individuals of 207 species were barcoded. The tree shows strong support for barcode resolution of species and major functional groups (usually classes) of zooplankton and fish. Figure modified from Bucklin et al. (2010b).

et al. 2010). The result is a growing collection of studies that employed barcodes both in the Arctic (Bucklin et al. 2010a, Mecklenburg et al. 2010) and Antarctic (Rock et al. 2008, Smith et al. 2008b, Webb et al. 2006). Increased sampling efforts in shallow and deep polar waters have uncovered novel species and generated numerous records of previously unrecorded taxa. Some of the new Arctic records may represent northward range expansions of boreal species with a warming climate, but many discoveries likely reflect the access to new samples from poorly sampled deeper areas. Using barcoding, Hunt et al. (2010) found species with bipolar distributions (i.e., occurring at both North and South polar regions) to be considerably more rare than previously proposed. Analysis revealed genetic structuring and cryptic speciation in bipolar species with both disjunct (i.e., absent at midlatitudes) and continuous distributions.

Coral reefs. Coral reefs are among the most endangered marine ecosystems, and dramatic declines in corals and fishes have been well documented (Gardner et al. 2003, Knowlton & Jackson 2008, Pandolfi et al. 2003). Most reef biodiversity lies outside these two groups in what is termed cryptofauna (i.e., hidden biodiversity associated with coral reefs), largely overlooked in biodiversity estimates. A study of the crustacean cryptofauna inhabiting dead coral heads of a single coral species revealed an unexpected high diversity of brachyuran crabs in a small geographic region (Plaisance et al. 2009). Similar findings have been reported for stomatopods (Barber & Boyce 2006) and even for fishes (Steinke et al. 2009b). Lins-de-Barros et al. (2010) used COI to confirm the presence of coral in a study of coral-associated archaea, bacteria, and eukaryotic algal plastid communities. The CoML coral reef project CREEFS has deployed Autonomous Reef Monitoring Structures (ARMS) in most of the world's coral seas and is developing DNA barcode catalogs for all of the species that settle on these structures.

Other marine communities. The Moorea Biocode Project is carrying out a comprehensive inventory of all nonmicrobial life of the French Polynesian island (Check 2006), using a barcode reference library as well as high-throughput sequencing for multigene analysis. Initial work on crustaceans uncovered a wealth of unrecognized diversity in the relatively well-known reef dweller *Calcinus* (Malay & Paulay 2010) and in crustacean cryptofauna (Plaisance et al. 2009). Hubert et al. (2010) created a reference collection of barcoded species of the perciform families Acanthuridae and Holocentridae; they found unprecedented levels of resolution in the identification of early stages of fish and provided evidence that conspecific larvae do not always aggregate in large schools.

BARCODE ANALYSIS BEYOND SPECIES IDENTIFICATION

Population Genetic and Phylogeographic Analysis

Population genetic analysis entails the quantitative description and statistical analysis of genetic diversity and structure (i.e., the distribution of genetic variation within and between populations) for a given species. Population genetic characters can also be used for phylogeographic analysis (i.e., the description of the geographical distributions of the genetic lineages within a population or species; Avise 2000). Whereas an ecologist might use archival or data-telemetry tags to track dispersal patterns, and a physical oceanographer might use drifters and dyes to analyze transport in currents, another useful approach to understanding dispersal is to use a tag that the organisms themselves carry—their COI barcode. Recent reviews of larval dispersal and population connectivity (Cowen & Sponaugle 2009) and gene flow (Hellberg 2009) in the ocean have provided comprehensive assessment and analyses for marine organisms. We note here a selection of studies

that have used the COI barcode region for population genetic and phylogeographic analysis of marine species. Indeed, COI sequence variation has proved useful to examine a wide variety of fundamental questions in the ecology and evolution of marine metazoa and has resolved geographic and temporal patterns over a range of scales

Gene flow and population connectivity. COI revealed significant population genetic structuring of marine metazoan species across the taxonomic spectrum, including sponges (Duran et al. 2004b, López-Legentil & Pawlik 2009), flatworms (Sanna et al. 2009), polychaetes (Breton et al. 2003, Xu et al. 2009), gastropods (Duda & Lee 2009, Gruenthal & Burton 2008, Keeney et al. 2009, Kelly & Eernisse 2007), bivalves (DeBoer et al. 2008, Lind et al. 2007), cephalopods (Teske et al. 2007a), crustaceans (Azuma et al. 2008, Liu et al. 2009, Luttkhuizen et al. 2008, Mathews 2007, Unal et al. 2006, Zitari-Chatti et al. 2009), brittle stars (Christensen et al. 2008, Muths et al. 2009), sea urchins (Duran et al. 2004a, Lessios et al. 2003), and ascidians (Pérez-Portela & Turon 2008a). Little or no differentiation for COI among geographic populations—even at global scales—was observed for a coralline sponge (Wörheide 2006), moon jelly (Ki et al. 2008), harpacticoid copepod (Eberl et al. 2007), commensal barnacle on hawksbill turtles (Torres-Pratts et al. 2009), spiny lobster (Inoue et al. 2007, Silberman et al. 1994), fiddler crab (Silva et al. 2010), galatheid shrimp (Samadi et al. 2006), amphidromous gastropod (Crandall et al. 2009), and mussel (Nicastro et al. 2008). Small- and large-scale patterns of connectivity are determined by many factors, including the ecology, life history, and behavior of the species. The rock-pool harpacticoid copepod *Tigriopus* has become a model system for small-scale population differentiation using COI (e.g., Handschumacher et al. 2010, Rawson & Burton 2006). For pelagic copepods, large-scale patterns of connectivity are strongly influenced by vertical migration behavior, which allows position-keeping and generates population differentiation (Blanco-Bercial et al. 2010) (**Figure 5**).

Historical range expansion and glacial refugia. COI is a frequent genetic marker of choice for phylogeographic and population genetic studies aimed at describing historical patterns of range expansion and contraction and at identifying glacial refugia for marine species. A number of North Atlantic marine species have been inferred to have shared patterns of preglacial maxima of demographic expansion, with evidence of glacial refugia and postglacial expansion (see Wares & Cunningham 2001). The European stalked barnacle, *Pollicipes pollicipes*, was found to exhibit this pattern based on COI variation (Campo et al. 2010). Similarly, the penaeid shrimp *Melicertus kerathurus* was hypothesized to have experienced a postglacial recolonization of the Mediterranean Sea from its Atlantic refugium based on COI variation (Pellerito et al. 2009). Species restricted to the nearshore and coastal margins of the North Atlantic have been hypothesized to exhibit marked cladogenesis in COI resulting from range expansion from glacial refugia, including the copepod *Acartia tonsa* (Hill 2004) and the amphipod *Gammarus tigrinus* (Kelly et al. 2006).

Van den Broeck et al. (2008) proposed a stepping stone postglacial range expansion through the Eastern Atlantic to explain marked phylogeographic structuring of COI of the periwinkle *Tectarius striatus*. Elsewhere, Goodall-Copestake et al. (2010) timed population expansion of Southern Ocean krill based on COI variation. Coral Triangle seastars and associated parasitic and commensal species revealed species-specific phylogeographic patterns resulting from differential postglacial range expansions (Crandall et al. 2008). Species-specific phylogeographic patterns for intertidal gastropod (Littorinidae) species throughout the Indo-West Pacific were tied to historical events (Reid et al. 2006).

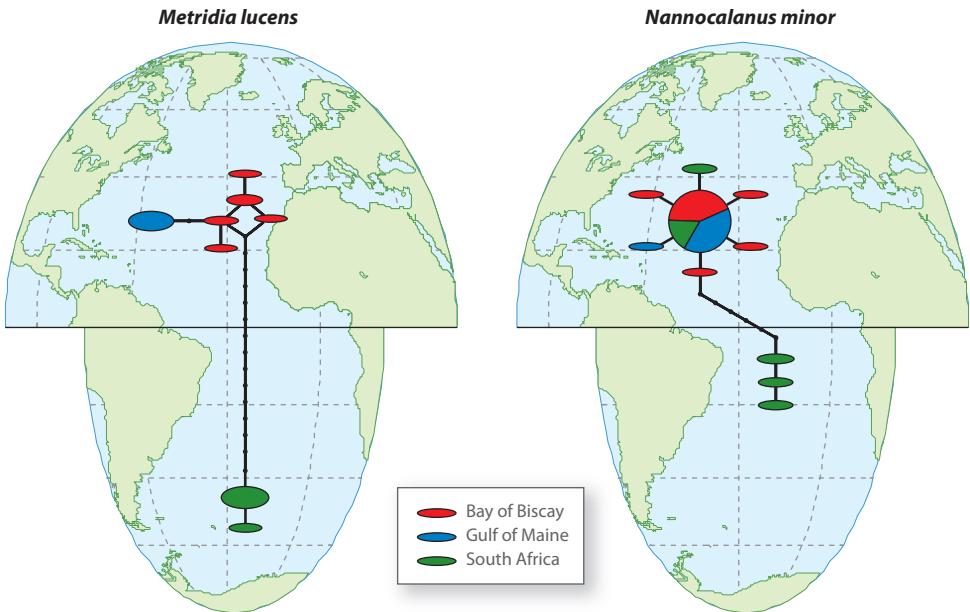


Figure 5

Connectivity of two calanoid copepod species populations in the Atlantic Ocean based on the cytochrome c oxidase subunit I (COI) barcode region. *Metridia lucens* (left) shows lower connectivity; the species' strong vertical migration behavior may aid geographic position-keeping and reduce large-scale dispersal; nonmigrating *Nannocalanus minor* (right) shows greater connectivity throughout the ocean basins; it may be more readily transported in surface currents (Blanco-Bercial et al. 2010). Haplotype network diagrams were determined by TCS, a computer program that estimates gene genealogies (Clement et al. 2000). Colors indicate the geographic source of collections; sizes of pie diagrams and ovals are relative to numbers of haplotypes; dotted lines indicate numbers of nucleotide substitutions between haplotypes. Adapted from unpublished data and figure from L. Blanco-Bercial and A. Bucklin, Univ. of Conn.

Phylogeny

In general, the COI barcode region is a poor choice as a phylogenetic marker. However, on its own, the phylogenetic signal of COI has been widely shown to be useful at two disparate levels of analysis: resolution of relationships among very closely related taxa and resolution of nodes between major groups of animals. Browne et al. (2007) sampled a wide range of amphipods and presented a preliminary molecular phylogeny based on COI; many lineage relationships between taxa within three hyperiid clades were highly supported. COI resolved relationships among giant clam species, which were shown to be monophyletic by Nuryanto et al. (2007). COI resolved deeper phylogenetic relationships among corals (Fukami et al. 2004, 2008).

It has been widely suggested that the inclusion of a large number of genomic regions in phylogenetic analysis—in a total evidence approach—will improve the quality of the inferences (Cummings et al. 1995). However, different markers—even different mitochondrial genes—differ markedly in amounts of nucleotide variation and rates of divergence, thus generating significant incongruence in the resultant tree topologies. The advantages of combining data partitions despite failed homogeneity tests are debatable (e.g., Giribet 2002); lack of congruence with other mitochondrial and nuclear makers has not prevented publication of innumerable studies that include COI in multigene phylogenetic analyses, frequently also including large- (28S) and small-subunit (18S) rRNA sequences.

There are examples of both congruence and lack of congruence of COI with other genes for molecular phylogenetic analysis. Compared with a reference topology for neogastropods based on whole mitochondrial genomes, Cunha et al. (2009) found that COI showed the lowest number of congruent internal branches and thus the lowest phylogenetic performance. Combined analysis of 28S rRNA and COI yielded highly congruent patterns of relationship at highly supported nodes within the sponge class Demospongia (Nichols 2005). Cárdenas et al. (2010) used COI to resolve phylogenetic relationships of sponges of the family Geodiidae and found congruent results with other gene regions, including 28S rRNA. COI provided a phylogenetic signal (identified in the combined analysis with 18S and 28S rRNA) for resolution of relationships within the isopod family Munnopsidae (Osborn 2009). Except for the resolution of basal taxa, Meland & Willassen (2004) found similar tree topologies for 18S rRNA and COI for species of the deep-sea mysid genus *Pseudomma*.

Trophic Relationships (Gut Contents)

Confirmation that short barcodes are diagnostic of species (Hajibabaei et al. 2006b) paved the way for their use in circumstances where full barcodes are impossible to obtain, as for degraded DNA in predator gut contents. Protocols are now available for barcoding in circumstances that necessitate dealing with fragmented DNA such as gut content and food web analysis (Carreon-Martinez & Heath 2010). Töbe et al. (2010) developed oligonucleotide probes based on barcodes for dominant copepods to detect the species in the guts of the larval krill *Euphausia superba* collected from the Lazarev Sea in the Southern Ocean; real-time, quantitative PCR results showed that the krill were feeding exclusively on *Oithona* sp. Krill was also the subject of an earlier study that employed short sequences of small-subunit (18S) rRNA to identify diatom prey from adult animals in the Indian Ocean (Passmore et al. 2006). In order to directly measure predation rates by older copepod stages upon copepod nauplii, Durbin et al. (2008) used species-specific primers and quantitative PCR to calculate ingestion rates based on DNA disappearance rates. The benthic marine worm *Xenoturbella* was found to be frequently contaminated with molluscan DNA; barcoding data (Bourlat et al. 2008) showed that the molluscan DNA originated from bivalves living in the same environment, indicating that the worm fed specifically on bivalve prey.

Dunn et al. (2010) conducted a diet study of deepwater sharks utilizing full length barcodes to identify prey species; they estimated that between 84 and 223 stomachs were needed to measure 90% of the extrapolated total prey richness. DNA identification was used to characterize prey species of large pelagic fishes by Smith et al. (2005). Especially for predators with diverse diets, group-specific primers, usually based on more conserved genes than COI, can expand the range of dietary items detected in gut contents. As an example, marine vertebrate diets were examined by Jarman et al. (2004) using probes based on 28S rRNA.

Forensics and Seafood Safety

DNA barcoding can be used for cooked or processed seafood. Smith et al. (2008a) obtained species-level identifications for smoked fish fillets of several genera; seafood sampled at commercial markets and restaurants was mislabeled at least in 25% of the cases (Barbuto et al. 2010, Cohen et al. 2009, Lowenstein et al. 2009, Marko et al. 2004, Wong & Hanner 2008). COI barcoding has been validated as a diagnostic marker for species identification (Dawnay et al. 2007) and is under consideration by the Food and Drug Administration in the United States as a replacement for the technique of protein isoelectric focusing for fish and fish product identification (Cohen et al. 2009, Yancy et al. 2007).

In one of the first applications of DNA barcoding in a human health context, barcodes were used to identify tuna sushi to the species level concomitant with mercury testing (Lowenstein et al. 2009). DNA barcodes enabled researchers to determine which species warranted inclusion in consumer advisories or trade restrictions, and whether the data used by health agencies accurately reflected mercury levels.

Marine Invasive Species

Barcodes can provide accurate identification of marine invasive species. In some instances, COI variation can be analyzed to reveal the geographic source region and pathway of invasion. Genetic approaches to the study of marine invasions were reviewed recently by Geller et al. (2009), who summarize barcoding studies as well. Two studies that showcase the usefulness of COI in this regard include a study of invasive pathways of a bryozoan (Mackie et al. 2006) and comparative analysis of COI diversity of native and introduced populations of an amphipod (Ashton et al. 2008).

METHODOLOGY—PRESENT AND FUTURE

High-Throughput Sequencing and Environmental Barcoding

Barcodeing is moving to massively parallel sequencing platforms to speed analysis of COI from mixed genomes, as well as whole genomes for the target species. Barcodes for larval fishes have been successfully determined using high-throughput sequencing (Richardson et al. 2006). High-throughput sequencing is also useful for determining COI barcodes from bulk environmental samples. Sometimes called environmental barcodeing, this methodology offers an accurate, cost-effective, and rapid estimation of species diversity of marine communities based on barcodes. DNA or mRNA is extracted from bulk environmental samples and analyzed for COI sequence diversity. High-throughput sequencing for exhaustive analysis of cDNA libraries can help reduce artifacts due to mispriming and pseudogenes. Also, short (<100 bp) sequences are sufficient to identify species in most cases by matching to a comprehensive taxon- or region-specific database of DNA barcodes of known species. As increasingly complete DNA barcode databases become available, increasingly accurate estimation of new or undescribed species will be possible. Machida et al. (2009) demonstrated the power of environmental barcodeing by analyzing a zooplankton sample collected in the Equatorial Pacific Ocean; they detected 189 species among 828 COI sequences, using threshold differences of 9% between species and distance-based clustering. Creer et al. (2010) analyzed the highly diverse but generally cryptic meiofaunal assemblage using environmental barcodeing.

Real-Time Quantitative PCR

A natural extension of DNA barcodeing is the use of the barcode sequence to design probes for real-time, or quantitative, PCR (QPCR), which can be used to estimate absolute and relative abundances of species in unsorted bulk samples. Pan et al. (2008) used real-time PCR assays based on COI for detection and relative quantification of decapod crustacean larvae.

DNA Microarrays

DNA microarrays are matrices with a detector DNA sequence (probe) affixed in each cell, which will bind (hybridize) to the labeled target DNA in the test sample. DNA barcodes can be used to design probe sequences for up to ~7,000 species, although new analytical software may be

required. The main limitation of the array-based approach is that it requires advance knowledge of sequences in target species. Because of a lack of exact matches, undiscovered haplotypes or geographic variants could fail to anneal properly to the probes on the array. This problem could be avoided by providing a set of several different probes per species, which limits the use of microarrays for large-scale biodiversity projects. However, DNA microarrays made for regional assemblages have the potential to accelerate routine at-sea or lab-based taxonomic analysis of marine samples for known species.

The *in silico* study on the effectiveness of mammalian COI and cytochrome *b* (cyt *b*) sequences for probe design suggested that both genes yield a high number of probes (Hajibabaei et al. 2007a). However, as the behavior of oligonucleotide probes in hybridisation experiments cannot be predicted, *in silico* results should be viewed with caution. Another study suggests that COI and cyt *b* are not well suited for probe design in fish species (Kochzius et al. 2010), and similar findings were reported for COI in fungi (Chen et al. 2009). Sequences of mitochondrial 12S and 16S rRNA may be more suitable for the design of functional probes in fish species (Kochzius et al. 2008). This result is particularly interesting as COI is markedly superior for sequence-based species identification of fishes; 16S rRNA frequently fails to discriminate closely related species. It is likely that microarray-based identification of marine metazoan species will require probes designed not solely from COI but from a combination of genes.

Sources of Error

Heteroplasmy. Strict maternal inheritance of mtDNA should result in homoplasmcy (i.e., only one mtDNA type within a cell or individual). Heteroplasmy (i.e., the presence of a mixture of more than one type of mtDNA within a cell or individual) can be caused by the differential amplification of nuclear pseudogenes (Bensasson et al. 2001), paternal leakage (Kvist et al. 2003), somatic mutation (Moum & Bakke 2001), and cross-species transfer (Barbara et al. 2007). Mussels of the *Mytilus edulis* species group appear to be exceptions to usual mtDNA inheritance patterns; homoplasmic individuals with mtDNA similar to the heteroplasmic mtDNA types have been found (Hoeh et al. 1991). These observations are best explained by the hypothesis that biparental inheritance of mitochondrial DNA can occur in *Mytilus* (e.g., Ort & Pogson 2007, Šmietanka et al. 2009). Further complexity in interpreting *Mytilus* barcodes and other mtDNA patterns is introduced by recombination after hybridization between species (see Riginos & Henzler 2008).

Pseudogenes. Nuclear mitochondrial pseudogenes, or numts (i.e., nonfunctional copies of mtDNA in the nucleus), have been found in major clades of eukaryotic organisms. Studies have revealed high frequencies in some taxa (e.g., the snapping shrimp *Alpheus*; Williams & Knowlton 2001). They can be easily coamplified with orthologous mtDNA when using conserved universal primers. This is problematical for DNA barcoding, particularly as nucleotide substitutions are not expected to occur as rapidly in the nucleus as in the mitochondrial genome (Brown et al. 1979). However, recent studies (Smith et al. 2007, Song et al. 2008) have presented strategies to reduce the risk of misinterpreting barcoding results.

Selection. Although the barcode region is frequently interpreted as a selectively neutral marker of species identity, numerous studies have confirmed the influence of selection on patterns of diversity and rates of divergence (e.g., Meiklejohn et al. 2007). A marine barcoding study by Foltz et al. (2004) found less COI diversity among species of the star fish *Leptasterias* that brood their young, indicating the influence of life history factors on patterns of COI nucleotide substitutions. Galtier et al. (2009) reviewed evidence for mitochondrial selective sweeps (i.e., reduction or elimination of

sequence variation resulting from recent and strong natural selection) and hypothesized that levels of intraspecific mtDNA divergence may primarily reflect the time elapsed since the last selective sweep. Selective sweeps would thus interfere with interpretations of demographic processes based on mtDNA but could aid species identification using barcodes by reducing intraspecific variation and making the barcoding gap more pronounced.

The formalin problem. Barcoding of marine organisms has been slowed by preservation and storage of samples in formalin solutions, which are not always adequately buffered for pH. Both the aqueous matrix and the rapid acidification of unbuffered formalin have exacerbated the difficulty of recovering DNA from archived samples (Tang 2006). Long-term storage in buffered formalin does not preclude some types of molecular genetic analysis, assuming caution is paid to possible sources of artifact (Zimmermann et al. 2008). Numerous protocols exist to recover DNA from formalin-preserved tissue; barcodes obtained from formalin-preserved marine specimens have been reported for zooplankton (Bucklin & Allen 2004, Kirby & Lindley 2005) and fish (Zhang 2010). Even if new protocols can be developed for DNA recovery from formalin-preserved tissue, marine specimens intended for molecular analysis should be preserved and maintained in ethanol or be deep frozen (Tang 2006).

Buyer beware. The rapid expansion of COI sequences in the NCBI GenBank database has provided an increasingly useful and comprehensive library for species identification of diverse marine metazoans. However, closer examination can reveal numerous errors and artifacts with various causes, including taxonomic misidentification of species, DNA sequencing quality control concerns (e.g., sequencing or manual editing errors, stop codons, and insertions and deletions in reading frames), unrecognized pseudogenes, and microbial contaminants (e.g., Siddall et al. 2009), among others. Errors are becoming more evident as barcode databases are more densely populated. Erroneous sequences can be identified by comparative examination within a particular taxonomic group; for example, Buhay (2009) reported numerous errors after examination of crustacean COI sequences on GenBank.

FUTURE ISSUES FOR DNA BARCODING

1. The new taxonomy. Concern that only a tiny cadre of expert taxonomists can identify and describe marine species has become the basis for renewed public interest and reaffirmed understanding of the scientific importance of species-level biodiversity throughout the global ocean. We can expect DNA barcoding to continue to rebuild the field of taxonomy and retain—or recover—the body of knowledge of marine biodiversity.
2. The Rosetta Stone for marine biodiversity. The growing database of gold standard DNA barcodes serves as a Rosetta Stone for marine biodiversity, providing the keys to link species names, morphology, and DNA sequence variation. The COI sequence library will serve to translate or decipher the complex array of morphological characters that are used to describe and discriminate species by traditional taxonomists (Hajibabaei et al. 2007b, Stoeckle & Hebert 2008).
3. Environmental barcoding. Analysis of marine biodiversity will proceed through barcoding of specimens that have not been—or cannot be—identified by recognized taxonomic authorities. Some specimens may be new or undescribed species,

whereas others may be simply damaged or poorly preserved. In many cases, high-throughput sequencing will profoundly outstrip the speed with which traditional sample analysis can be performed. Barcodes from unidentified specimens are being used to analyze species-level diversity and estimate numbers of novel species. New analytical approaches may eventually allow accurate classification of unknown barcodes.

4. Whole-genome barcoding. We may expect that, in the not-too-distant-future, it may become both possible and cost-effective to characterize whole genomes (or whole mitochondrial genomes) for the same purposes now served by the COI barcode region for species identification, discrimination, and discovery.
5. Conservation of biodiversity. Until recently, the prospects for rapidly cataloging tens of thousands species associated with hot spot habitats were very dim. Amid genuine fears that species extinction will outpace discovery, DNA barcoding offers the promise of greatly accelerating the rate of biodiversity analysis for threatened or endangered marine habitats with exceptionally high species diversity, such as coral reefs (Neigel et al. 2007).
6. Ocean monitoring and observing. O'Dor et al. (2010) summarized a comprehensive analysis of new technologies for ocean observing, including the use of DNA barcoding for rapid and remote analysis of species diversity of ocean ecosystems. Operational approaches to marine biodiversity that use barcodes to monitor ecosystem health, inform fisheries management, and detect species diversity for near real-time ocean observing are on the horizon.

DISCLOSURE STATEMENT

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