MOLECULAR ECOLOGY
RESOURCES
Molecular Ecology Resources (2011) **11**, 19–31 doi: 10.1111/j.1755-0998.2010.02875.x

DNA BARCODING

Limitations of mitochondrial gene barcoding in Octocorallia

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Abstract

The widespread assumption that *COI* and other mitochondrial genes will be ineffective DNA barcodes for anthozoan cnidarians has not been well tested for most anthozoans other than scleractinian corals. Here we examine the limitations of mitochondrial gene barcoding in the sub-class Octocorallia, a large, diverse, and ecologically important group of anthozoans. Pairwise genetic distance values (uncorrected *p*) were compared for three candidate barcoding regions: the Folmer region of *COI*; a fragment of the octocoral-specific mitochondrial protein-coding gene, *msh1*; and an extended barcode of *msh1* plus *COI* with a short, adjacent intergenic region (*igr1*). Intraspecific variation was <0.5%, with most species exhibiting no variation in any of the three gene regions. Interspecific divergence was also low: 18.5% of congeneric morphospecies shared identical *COI* barcodes, and there was no discernible barcoding gap between intra- and interspecific *p* values. In a case study to assess regional octocoral biodiversity, *COI* and *msh1* barcodes each identified 70% of morphospecies. In a second case study, a nucleotide character-based analysis correctly identified 70% of species in the temperate genus *Alcyonium*. Although interspecific genetic distances were 2× greater for *msh1* than *COI*, each marker identified similar numbers of species in the two case studies, and the extended *COI* + *igr1* + *msh1* barcode more effectively discriminated sister taxa in *Alcyonium*. Although far from perfect for species identification, a *COI* + *igr1* + *msh1* barcode nonetheless represents a valuable addition to the depauperate set of characters available for octocoral taxonomy.

Keywords: cnidarians, DNA barcoding, invertebrates, systematics

Received 30 December 2009; revision received 15 April 2010; accepted 23 April 2010

Introduction

DNA barcoding has been envisioned and widely promoted as a reliable system by which unidentified specimens can be identified to known species by comparison to a reference database of molecular exemplars (e.g. Hebert *et al.* 2003a,b). Barcodes based on a fragment of the mitochondrial cytochrome oxidase I gene (*COI*) have been demonstrated to work well for species identification in many groups of animals, particularly vertebrates (Hebert *et al.* 2004; Ward *et al.* 2005; Smith *et al.* 2008; Tavares & Baker 2008; Baker *et al.* 2009) and arthropods (Smith *et al.* 2005; Hajibabaei *et al.* 2006). Their effectiveness and appropriateness as tools for species identification in many taxa, however, remain controversial on both philosophical (DeSalle *et al.* 2005; Will *et al.* 2005) and practical grounds (Meyer & Paulay 2005; Meier *et al.* 2006; Rubinoff *et al.* 2006; Roe & Sperling 2007). In particular, current distance-based analytical methods rely on the existence of a barcoding gap (i.e., a clear distinction between ranges of genetic distance values typical of intraspecific variation vs. interspecific divergence) and *a priori* specification of a threshold genetic distance value above which specimens can reliably be assigned to different species. The lack of a well-defined barcoding gap in many taxa, and the error rates associated with the use of threshold values and distance measures for species detection, dominate discussion in the recent literature (e.g., DeSalle *et al.* 2005; Meyer & Paulay 2005; Hickerson *et al.* 2006; Meier *et al.* 2006, 2008; Rach *et al.* 2008). In addition, it has been recognized from the outset that a lack of mitochondrial gene variation in some invertebrate groups, in particular the anthozoan cnidarians, could limit the utility of *COI* as a barcode for species identification (Hebert *et al.* 2003b).

Anthozoan cnidarians (e.g. scleractinian corals, sea anemones, and octocorals) are unusual among animals in having mitochondrial genomes that evolve relatively slowly (Shearer *et al.* 2002). Recent work suggests that the

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mitochondrial genome of some scleractinians evolves 5× more slowly than the nuclear genome (Chen *et al.* 2009), and 50–100× slower than the mt genomes of most other animals (Hellberg 2006). No regions of the anthozoan mt genome have sufficient variation to distinguish populations of conspecifics, and few gene regions exhibit enough variability to separate congeneric species (France & Hoover 2002; Shearer *et al.* 2002; Fukami & Knowlton 2005; Concepcion *et al.* 2006). The low substitution rates observed throughout the anthozoan mt genome have consequently led to predictions that *COI* will be unsuitable as a species-specific barcode in this group (Hebert *et al.* 2003b). Several recent tests of *COI* sequence data derived primarily from scleractinian corals show that low levels of interspecific divergence result in up to 40% of congeners (and occasionally different genera) sharing identical *COI* sequences (Huang *et al.* 2008; Shearer & Coffroth 2008). Tests of *COI* and *16S* sequences as species-specific markers for Zoanthinaria (colonial anemones) have, however, been more promising, distinguishing species groups and most morphospecies (Sinniger *et al.* 2008).

Based on scant data, it has been assumed that members of the diverse anthozoan sub-class Octocorallia share similarly slow rates of mt gene divergence with the other anthozoans (Shearer *et al.* 2002), but no studies have yet systematically examined variation of *COI* in this group. An early study that predated the *COI* barcoding initiative reported low rates of genetic divergence in octocoral *COI*, but at the 3′ region of the gene rather than the 5′ 'Folmer region' that was subsequently adopted as a universal barcode (France & Hoover 2002). The only study to focus specifically on the 'Folmer region' of *COI* found no intraspecific variation, but compared only four species representing three different octocoral families (Calderón *et al.* 2006). Following these initial reports of lack of variation there have been no further published attempts to explore the limitations of *COI* barcoding in octocorals.

In the present study we sequenced the 'Folmer region' of *COI* across a broad spectrum of octocoral species, genera, and families, allowing us to estimate average divergence values across a range of taxonomic levels, and to compare these with values for *msh1*, an octocoral-specific mitochondrial protein-coding gene that has been shown to evolve more rapidly than other coding regions of the octocoral mt genome (France & Hoover 2001; van der Ham *et al.* 2009). Although previous studies have found that some morphospecies of anthozoans cannot be discriminated using *COI* alone (Calderón *et al.* 2006; Shearer & Coffroth 2008), the percentage of correct species identifications that might nonetheless be expected in a regional survey of biodiversity has never been estimated. We used two case studies to evaluate the ability of (i) the *COI* 'Folmer region' alone (800 nt), and (ii) an extended barcode (~1845 nt) consisting of the 'Folmer region' plus an adjacent intergenic region (*igr1*, Brugler & France 2008) and a 5′ fragment of *msh1* to discriminate octocoral species. The first case study was a comprehensive survey of octocoral biodiversity conducted in Eilat (Gulf of Aqaba, northern Red Sea), Israel, a geographical region with a moderate diversity of species that are well known taxonomically relative to other regions of the Indo-Pacific (e.g., Grasshoff 2000; Benayahu *et al.* 2002). The second case study focused on a group of 10 Mediterranean and North Atlantic species belonging to the soft coral genus *Alcyonium*. This genus has been the focus of numerous past genetic studies, and species boundaries and clade relationships have been confirmed using a variety of nuclear molecular markers, including allozymes (McFadden 1999) and ribosomal ITS sequences (McFadden *et al.* 2001; McFadden & Hutchinson 2004). In both of these case studies we tested the ability of a mitochondrial barcode (*COI* alone, *msh1*, or the extended *COI+igr1+msh1*) to distinguish known octocoral species using both distance-based and character-based analyses.

Methods

Collection of material

Case study 1: regional biodiversity survey, Eilat (Gulf of Aqaba, northern Red Sea). Representatives of as many octocoral species as could be found during a 4-day survey of Eilat reefs down to 30 m were collected using SCUBA. For at least one specimen of each morphospecies, DNA was extracted immediately from live tissue using Qiagen's DNEasy Blood & Tissue Kit® following the manufacturer's protocol. Tissue from additional specimens was preserved in 95% EtOH for subsequent DNA extraction using the same method. Vouchers of all specimens were preserved in 70% EtOH and deposited in the collections of the Zoological Museum at Tel Aviv University (ZMTAU) (Table S1). Specimens were identified to morphospecies by YB based on traditional taxonomy, including microscopical examination of sclerites and other morphological characters. Identifications of members of the families Acanthogorgiidae, Melithaeidae, Nephtheidae, Plexauridae and Nidaliidae were similarly confirmed by L. van Ofwegen, Nationaal Natuurhistorisch Museum, Leiden.

Case study 2: North Atlantic and Mediterranean species of Alcyonium. Specimens of 10 *Alcyonium* species were collected at sites in the Mediterranean Sea (France, Spain) and North Atlantic coasts of Europe and North America from 1990 to 1994 (McFadden 1999; McFadden *et al.* 2001; McFadden & Hutchinson 2004) (Table S2). All specimens were preserved in liquid N_2 immediately following

collection, and stored frozen at −80°C. DNA was extracted from frozen tissue using a standard CTAB extraction protocol with the addition of Nucleon Phytopure® (McFadden *et al.* 2006a). *Alcyonium* species were identified by CSM using both traditional taxonomic methods and allozyme electrophoresis (e.g. McFadden 1999).

Deep-water samples. To estimate mean intra- and interspecific divergence values across a wide spectrum of octocoral taxa we also sequenced gorgonians that had been collected during a series of deep-sea coral expeditions to Hawaii (1993, 1996, 2006), Alaska (2003-2004), and the North Atlantic (2003–2005), with additional samples from museum collections or colleagues (Table S3). Whole colonies or portions thereof were sampled using remotely operated vehicles (ROV), human-occupied vehicles or trawls. Fragments for genetic analyses were preserved in 95–100% EtOH or frozen at −80°C. DNA was extracted using a modified CTAB protocol (France *et al.* 1996). Vouchers have been (or will be) deposited at the Yale Peabody Museum and USNM (Table S3). Specimens were identified to morphospecies by SCF, EP, JNT, L. Watling (U. Hawaii), and S. Cairns (USNM).

Amplification and sequencing

The 'universal' Folmer primers do not amplify *COI* in most octocorals (pers. obs.), necessitating the design of octocoral-specific primers for this region (e.g., Calderón *et al.* 2006). We amplified a fragment (~1.1 kB) that encompassed the entire 'Folmer region' of *COI* plus an adjacent intergenic region (*igr1*) from all specimens using primers COII8068F (McFadden *et al.* 2004) or COII8068xF (a degenerate version, Table S4) and COIOCTr (reverse complement of COIOCTf, France & Hoover 2002). Approximately 760 nt of *msh1* was amplified using forward primers ND42475F (Brugler & France 2008) or ND42599F (France & Hoover 2002) and mut3458R (Sánchez *et al.* 2003). Alternative primers for each gene region were used for some problematic deep-sea specimens (Table S4). Standard PCR protocols were used (e.g. Sánchez *et al.* 2003; McFadden *et al.* 2004; Brugler & France 2008). Amplified products were purified and sent to Cogenics (Houston, TX) for sequencing.

Analysis

Nucleotide sequences were aligned using MUSCLE v. 3.6 (Edgar 2004) or the LIN-S-I or GIN-S-I algorithm in MAFFT (Katoh *et al.* 2002), and translated protein-coding regions were adjusted by eye to conform to amino acid alignments. Pairwise distances (uncorrected *p*) were calculated, and PAUP* (Swofford 2002) was used to construct neighbour-joining phylograms with 1000 bootstrap replicates. Intraspecific variation was estimated as the maximum pairwise distance observed among conspecifics, i.e. the coalescent depth; the smallest interspecific distance observed between congeners was used as a measure of interspecific variation (Meier *et al.* 2008). For the *Alcyonium* data set, alignment files were viewed in MacClade 4.08 (Maddison & Maddison 2005) to identify nucleotide characters diagnostic for a species ('pure characteristic attributes', sensu Rach *et al.* 2008).

Results

Based on traditional taxonomy, we identified 49 morphospecies of octocorals from Eilat; these represented 23 genera and nine families, with the majority belonging to the fleshy octocoral families Alcyoniidae, Nephtheidae, and Xeniidae (Table S1). Sequences for *COI* and *msh1* were obtained for 1–6 specimens of each morphospecies, depending on availability of samples. Sequences for both genes were obtained from 7 to 11 individuals for each of 10 northern hemisphere species of *Alcyonium*, as well as two individuals of the South African *A. variabile*, and an outgroup, the South Atlantic *A. haddoni*. Among the deep-sea samples, we identified 16 morphospecies representing 10 genera from four families in the Holaxonia-Alcyoniina clade (McFadden *et al.* 2006b), and 28 morphospecies representing at least 12 genera from three families in the Calcaxonia clade (Table S3). Sequences for *COI* and *msh1* were obtained for 1–6 specimens of each morphospecies. A representative of each unique haplotype from each species was deposited in GenBank (Tables S1–S3).

All edited and aligned sequences included the first (5') 800 nt of the *COI* coding region, encompassing the complete 'Folmer region' (nucleotide positions 29-736) (Folmer *et al.* 1994). There were no indels in this region, and nucleotide alignments were unambiguous. The *igr1* and *msh1* regions, however, both exhibited length variation (*msh1* typically exhibits amino acid length variation, maintaining the correct reading frame despite numerous indels; McFadden *et al.* 2006b; France 2007). Among *Alcyonium* species, *igr1* ranged from 107 to 112 nt, with a final alignment length of 113 nt; *msh1* ranged from 726 to 735 nt due to a 9-nt (3 aa) deletion in one clade. Among the other genera, *igr1* ranged from 94 nt (*Chrysogorgia* sp.) to 168 nt (*Titanideum frauenfeldii)* and *msh1* from 723 nt (*Radicipes* sp.) to 816 nt (*Lepidisis* sp. B1b) (241–272 aa). Among closely related taxa (e.g. congeners) nucleotide alignments were unambiguous, but as genetic distance increased the nucleotide alignment of *igr1* and amino acid alignment of certain regions within *msh1* became increasingly uncertain. We analysed separate alignments for the Eilat specimens, deep-sea Holaxonia-Alcyoniina, and deep-sea Calcaxonia; final alignment lengths ranged from 164–168 nt for *igr1* and 810-888 nt for *msh1*.

Mean divergence within taxonomic levels

Maximum intraspecific genetic distance values (coalescent depth) ranged from 0–1.25% (mean = 0.13%) for *COI*, 0–1.90% (mean = 0.17%) for *msh1*, and 0–1.82% (mean = 0.16%) for an extended barcode of *COI+igr1+msh1*, with a majority of species exhibiting no intraspecific variation in any of the three gene regions (Tables 1–3, Fig. 1). Minimum genetic distances among congeneric species pairs ranged from 0% to 4.75% (mean = 1.2%) for *COI*, 0% to 9.39% (mean = 3.1%) for *msh1*, and 0% to 7.12% (mean = 2.2%) for the extended barcode (Tables 1–3, Fig. 1). 18.5% of congeneric species pairs shared identical *COI* haplotypes, 15.1% shared *msh1* haplotypes, and 11.6% shared identical extended barcodes. Specimens identified to different genera shared the same extended barcode in only one case (*Xenia hicksoni* and *Heteroxenia ghardaqensis)*; another pair (*Paramuricea placomus* and *Placogorgia* sp.) shared identical *msh1* and *igr1* sequences, but differed at *COI*. In both of these cases, genus distinctions are unclear (see Discussion).

A commonly suggested threshold for species detection in barcoding studies is 10× the mean pairwise intraspecific genetic distance (Hebert *et al.* 2004). For octocorals, these values would be 1.3%, 1.7% and 1.6% respectively for *COI*, *msh1* and the extended barcode. Each of these threshold values results in high rates of false-negative identifications (failure to differentiate species). Including those cases in which different species shared identical barcode sequences, 62% of pairwise genetic distances among congeners fell below the 10X threshold for *COI*, 36% for *msh1*, and 45% for the extended barcode. In only three instances, however, did we observe a maximum intraspecific genetic distance greater than 0.5% (Fig. 1), and in each of those cases further scrutiny suggested the presence of cryptic species (see Discussion). Lowering the species detection threshold to 0.5%, however, still results in a false-negative rate of 38% (*COI*), 23% (*msh1*) or 25% (extended barcode).

Table 2 Means (±SD) and ranges of genetic distances (uncorrected *p*, expressed as percentage) observed within and between morphospecies of octocorals collected in Eilat, Israel using different mtDNA barcodes. Within species = maximum intraspecific value (coalescent depth); within genus = minimum distance between congeners. nt = total length (nucleotides) of aligned sequence; *n* = number of pairwise comparisons

Gene Region(s)	nt	Within species *n* = 18	Within genus *n* = 75
COI coding	800	0.12 (±0.30) 0–1.0	1.11 (±1.05) 0–4.75
COI + *igr1*	1044	0.16 (±0.44) 0–1.75	1.54 (±1.29) 0–5.28
msh1 coding	819	0.16 (±0.46) 0–1.90	3.36 (±2.49) 0–9.39
COI + *igr1* + *msh1*	1844	0.16 (±0.45) 0–1.82	2.36 (±1.79) 0–7.12

Case study 1: regional biodiversity survey, Eilat

Among the 49 morphospecies identified from Eilat, we detected 45 different *COI* coding sequences. Thirty-four taxa (69%) had unique *COI* haplotypes, but six pairs and one trio of morphospecies shared identical sequences, including one pair in which specimens were identified to different genera (*Xenia*, *Heteroxenia*) (Fig. 2). We detected intraspecific sequence variation in *COI* in only five species (Fig. 2). Addition of *igr1* and *msh1* sequences to the barcode did not further discriminate any of the species that had identical *COI* coding regions, but did reveal intraspecific variation in two additional species (data not shown). A 10X species detection threshold of 1.3% for *COI* would result in 71% false negatives, greatly

Table 1 Means (±SD) and ranges of genetic distances (uncorrected *p*, expressed as percentage) observed within and between 10 North Atlantic and Mediterranean species of *Alcyonium* assessed using different mtDNA barcodes. Within species = maximum intraspecific value (coalescent depth); within and between clades = minimum interspecific value. nt = total length (nucleotides) of aligned sequence; # unique = number of species identifiable using pure characteristic attributes (parentheses: number of identifiable Atlantic species). *n* = number of pairwise comparisons

Gene Region(s)	nt	Within species *n* = 10	Within clades *n* = 12	Between clades *n* = 34	# unique
COI coding	800	0.09 (±0.13) 0–0.38	0.08 (±0.14) 0–0.38	2.26 (±0.66) 1.38–2.88	1/10 (3/7)
COI + *igr1*	913	0.08 (±0.12) 0–0.33	0.15 (±0.27) 0–0.77	2.55 (±0.68) 1.65–3.31	3/10 (5/7)
msh1 coding	735	0.11 (±0.17) 0–0.41	0.31 (±0.38) 0–0.82	4.82 (±1.61) 2.45–6.62	2/10 (3/7)
COI + *igr1* + *msh1*	1648	0.09 (±0.10) 0–0.30	0.22 (±0.28) 0–0.74	3.57 (±1.09) 2.00–4.78	4/10 (7/7)

Table 3 Genetic distances (uncorrected $p \pm$ SD, expressed as percentage) within and between morphospecies of gorgonian octocorals collected on deep-sea cruises using different mtDNA 'barcodes'. For each 'barcode,' the first line shows in boldface means and ranges for all taxa combined, followed by means and ranges for Calcaxonia vs. Holaxonia/Alcyoniina clades. nt = total length (nucleotides) of alignment of barcode sequence; Within species = maximum intraspecific value (coalescent depth); within genus = minimum distance between congeners; n = number of pairwise comparisons for combined data/Calcaxonia/Holaxonia-Alcyoniina, respectively

Gene Region(s)	nt	Within species n = 6/5/1	Within genus n = 23/18/5
COI coding		**0.21 (±0.51)**	**0.41 (±0.89)**
		0–1.25	**0–4.25**
Calcaxonia	800	0 (±0)	0.17 (±0.28)
		0–0	0–0.88
Holax/Alcyon	800	1.25	1.28 (±1.68)
		1.25	0.13–4.25
COI + *igr1*		**0.29 (±0.72)**	**0.52 (±0.94)**
		0–1.75	**0–4.39**
Calcaxonia	968	0 (±0)	0.30 (±0.43)
		0–0	0–1.19
Holax/Alcyon	968	1.75	1.29 (±1.76)
		1.75	0.11–4.39
msh1 coding		**0.29 (±0.72)**	**1.25 (±1.58)**
		0–1.77	**0–7.07**
Calcaxonia	888	0 (±0)	0.92 (±0.80)
		0–0	0–1.96
Holax/Alcyon	810	1.77	2.42 (±2.96)
		1.77	0.14–7.07
COI + *igr1* + *msh1*		**0.29 (±0.72)**	**0.85 (±1.17)**
		0–1.76	**0–5.59**
Calcaxonia	1856	0 (±0)	0.58 (±0.47)
		0–0	0–1.38
Holax/Alcyon	1778	1.76	1.80 (±2.26)
		1.76	0.12–5.59

underestimating total biodiversity; a 0.5% threshold would still result in 19% false negatives, but would flag three potential cryptic species as distinct (see Discussion).

Case study 2: North Atlantic and Mediterranean Alcyonium species

We detected 11 distinct *COI* haplotypes among the 10 species of northern hemisphere *Alcyonium*. Five species exhibited no intraspecific variation (Fig. 3). *Alcyonium glomeratum* and *A. palmatum* each had two haplotypes, the most common of which they shared with one another and with *A. acaule*. The clade comprising *A. coralloides*, *A. hibernicum*, *A. bocagei* and *Alcyonium* sp. M2 included 7 *COI* haplotypes: *A. coralloides* and *A.* sp. M2 each possessed two unique haplotypes, and the remaining three haplotypes were shared by more than one species. *A. digitatum* was the only northern hemisphere *Alcyonium* species that could be identified unequivocally based on a unique *COI* haplotype. The other nine species all shared at least one haplotype with another species.

Addition of the *igr1* and *msh1* regions to the barcode sequence resulted in better discrimination of *Alcyonium* species, separating *A. siderium* from *A.* sp. A, and *A. coralloides* from the other three species in that clade (Fig. 3). Not all species were distinguishable, however, even with this extended barcode. The most frequent haplotype found in *A. acaule*, *A. glomeratum* and *A. palmatum* was shared by all three species, and *A.* sp. M2 shared haplotypes with *A. bocagei* and *A. hibernicum*. Mean minimum genetic distances among sister taxa belonging to the same clade ranged from 0.08% (*COI*) to 0.31% (*msh1*) (Table 1), comparable to the mean maximum intraspecific genetic distance values estimated across octocoral taxa. In addition, not all sister species were reciprocally monophyletic, and bootstrap support for species-level clades was generally weak (Fig. 3).

Although the '10X rule' and reciprocal monophyly both would fail to distinguish sister taxa, a majority of *Alcyonium* species nonetheless possessed diagnostic nucleotide substitutions that allowed character-based species assignment (Fig. 4). When only the seven species that occur in the North Atlantic were included in the analysis, the ability to correctly assign species identity to a specimen based on diagnostic nucleotide characters was 100%. Species pairs such as *A. siderium* – *A.* sp. A and *A. bocagei* – *A. hibernicum* that were not reciprocally monophyletic and differed by <0.06% (extended barcode) had at least one 'pure characteristic attribute' (i.e. a nucleotide shared by all members of that species but not by its sister taxon; Rach *et al.* 2008) that allowed unequivocal species assignment. Among the four Mediterranean species, *A. coralloides* and *A.* sp. M2 could be distinguished from one another and from the other two species using a character-based approach, but *A. acaule* and *A. palmatum* remained inseparable.

Discussion

As anticipated from past studies of other anthozoans (Shearer *et al.* 2002; Hellberg 2006; Huang *et al.* 2008; Shearer & Coffroth 2008) and from published estimates of variability in octocoral mitochondrial genes (France & Hoover 2002; McFadden *et al.* 2004; Calderón *et al.* 2006; van der Ham *et al.* 2009), *COI* and *msh1* both exhibited little intraspecific variation in octocorals (Fig. 1), a potential advantage for use of either or both gene regions as species-specific barcodes. However, both genes also exhibited relatively little divergence among congeneric species, with the result that maximum intraspecific and

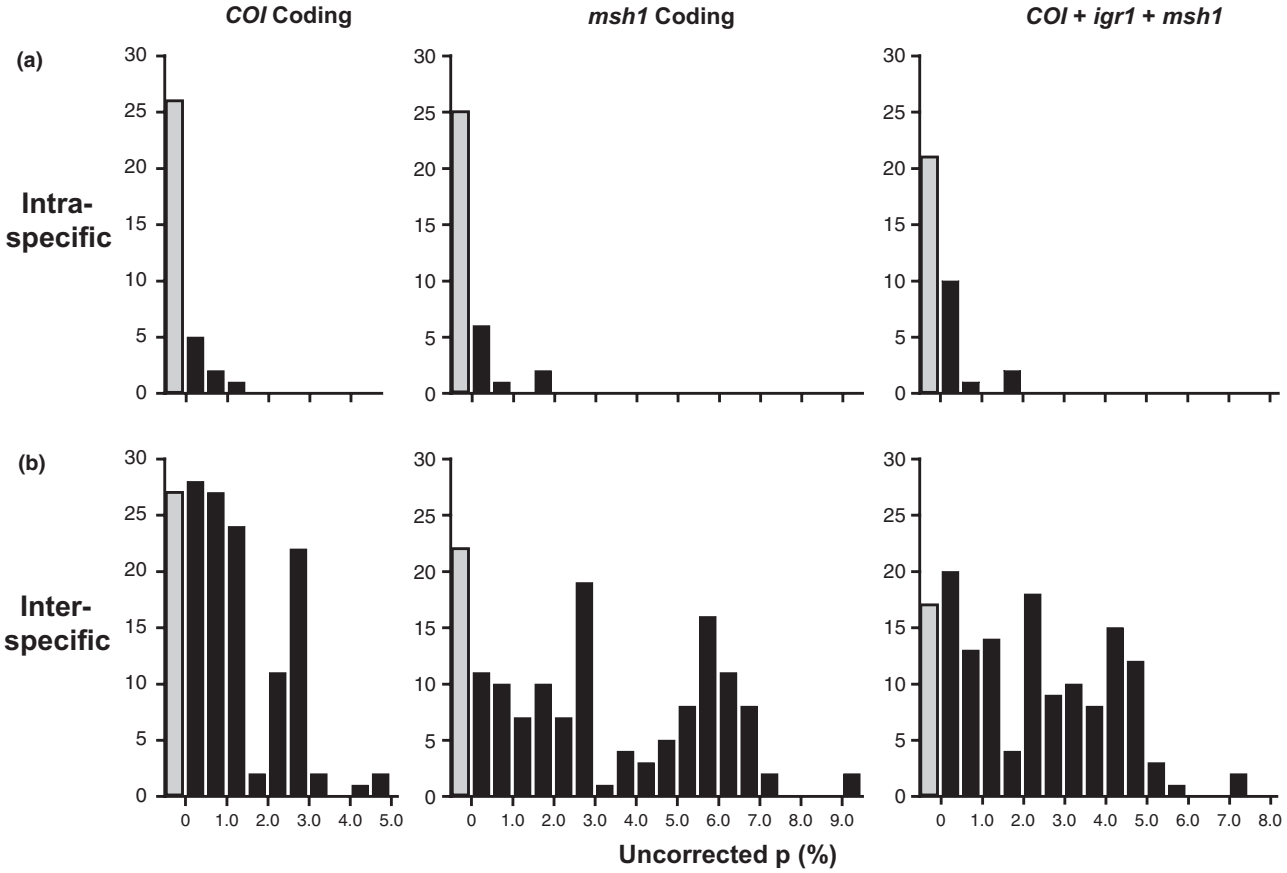

Fig. 1 Frequency histograms of pairwise genetic distance values (uncorrected *p*) across all octocoral taxa sequenced in this study. Separate histograms are shown for *COI*, *msh1*, and an extended barcode consisting of both of those coding regions plus *igr1*, an intergenic region adjacent to *COI*. Pairwise comparisons were made (a) among conspecific individuals (maximum intraspecific distances) and (b) between congeneric species (minimum interspecific distances). Grey bars represent genetic distances of 0, i.e. (a) no intraspecific variation observed or (b) congeners with identical barcode sequences.

minimum interspecific genetic distances overlap. In particular, 18.5% of congeneric morphospecies shared identical sequences and were therefore inseparable using the standard (*COI*) molecular barcode. In at least one case, specimens identified to different genera by traditional taxonomy shared identical *COI* sequences (but see below). The percentage of octocoral congeners that share *COI* barcodes is, however, less than the 40% reported for scleractinian corals (Shearer & Coffroth 2008).

The lack of a gap between intraspecific and interspecific distance values makes the use of a mitochondrial gene barcode problematic for distance-based species identification in octocorals, as specimens whose sequences differ by <1.0% might or might not be conspecific. Our results do suggest, however, that genetic distances >0.5% are likely to be indicative of species-level differences. In at least two of the three cases in which specimens identified to the same morphospecies differed by >0.5%, subsequent phylogenetic and morphological studies have suggested that they are indeed different species. The two distinct haplotypes of *Sinularia polydactyla* collected in Eilat belong to very different clades within that speciose genus, and morphological characters that distinguish them at the clade level have subsequently been identified (McFadden *et al.* 2009). Likewise, when the two divergent *msh1* haplotypes of *Sarcophyton gemmatum* found at Eilat are included in a well-sampled phylogeny of that genus (McFadden *et al.* 2006a) they fall into different clades, suggesting that they, too, represent different species. These examples illustrate cases in which a molecular barcode has proven effective for taxon discovery in octocorals, alerting taxonomists to previously unrecognized cryptic species or misidentifications of highly variable, closely related taxa. Our results also highlight the need for thorough verification, and in some cases revision of certain octocoral taxa. As discussed in the literature (e.g., DeSalle *et al.* 2005; Rubinoff *et al.* 2006), integrated taxonomic approaches utilizing traditional morphological, molecular, and geographical data will always be necessary to confirm species boundaries,

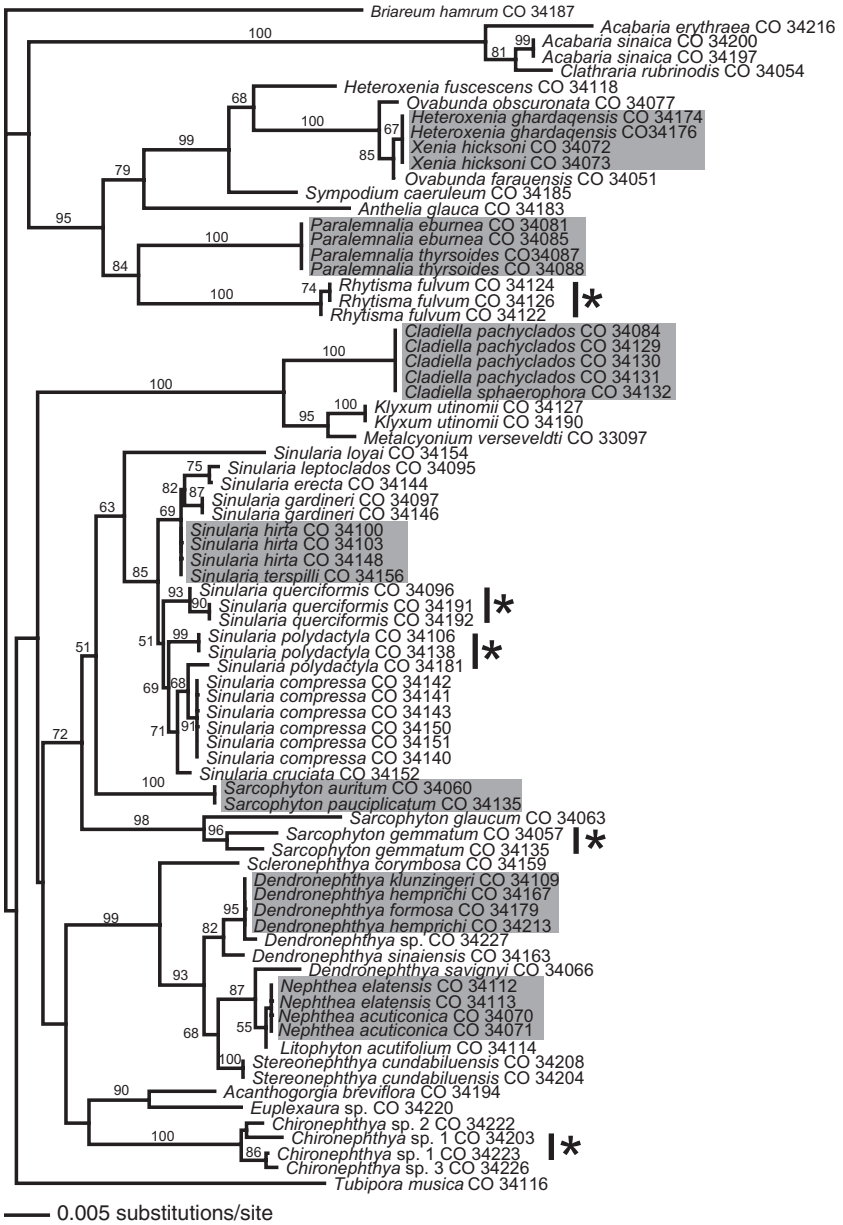

Fig. 2 Neighbour-joining tree (uncorrected *p*) based on *COI* coding region (800 nt) sequenced for octocoral taxa collected in a 4-day biodiversity survey at Eilat, Israel. Grey boxes enclose specimens identified to two or more different morphospecies that shared the same *COI* haplotype. Asterisks indicate specimens identified to the same morphospecies that had different *COI* haplotypes. Numbers on nodes are bootstrap values (% of 1000 replicates).

but our results suggest that *COI* sequence differences of >0.5% effectively flag cases worthy of further scrutiny in Octocorallia.

Evaluation of the effectiveness of molecular barcodes in octocorals is hindered greatly by the poor state of our knowledge of species boundaries in these organisms. Cases in which specimens identified to different morphospecies share a barcode can and should motivate additional taxonomic work to test species boundaries and quantify intraspecific morphological variation. Many octocoral species were described in the late 19th and early 20th centuries based on collections made using trawls and dredges, which typically recover damaged colonies or colony fragments. The descriptions of the vast majority of these species lack images of the living animal, *in situ* or otherwise, and also lack sufficient detail to encompass the morphological variation observed in material collected using modern methods such as SCUBA or ROVs. Identification of species is therefore difficult to impossible without a thorough re-examination of type material, and in some cases revision of the relevant genus. In addition, octocoral taxonomy is still based on an antiquated typological species concept, whereby specimens that differ from one another in a small set of subjectively assessed morphological character states are deemed different species (McFadden *et al.* 2006a). Descriptions of new species are still frequently based on single specimens (e.g. Watling 2007; van Ofwegen

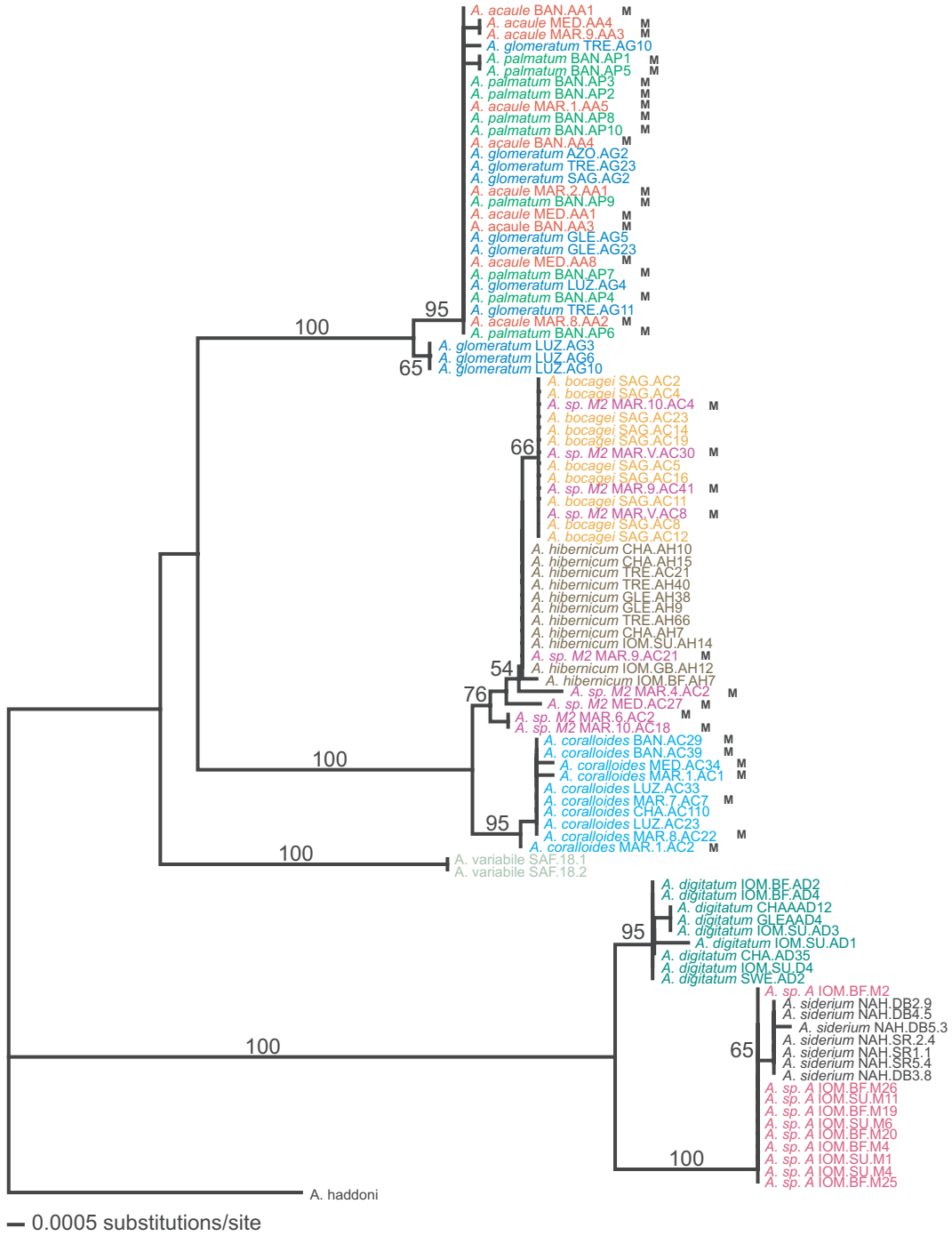

Fig. 3 Neighbour-joining tree based on an extended barcode of *COI+igr1+msh1* for 10 northern hemisphere species of *Alcyonium*. Specimens belonging to the same species share the same colour. M: Specimens collected in the Mediterranean Sea. Bootstrap values on nodes are % of 1000 replicates. Tree is rooted using the southern hemisphere *A. haddoni* as an outgroup.

2008a,b), and therefore intraspecific variation is often not depicted. Furthermore, integrated taxonomic approaches are rarely applied to confirm that morphologically distinguishable forms indeed represent different species rather than intraspecific or environmentally induced variants. In this study, for example, sequences from the holotype of *Iridogorgia fontinalis*, described by Watling (2007) from a single specimen, were identical to those from the holotype of *I. magnispiralis*, calling into question the distinctiveness of the new species. In such cases in which

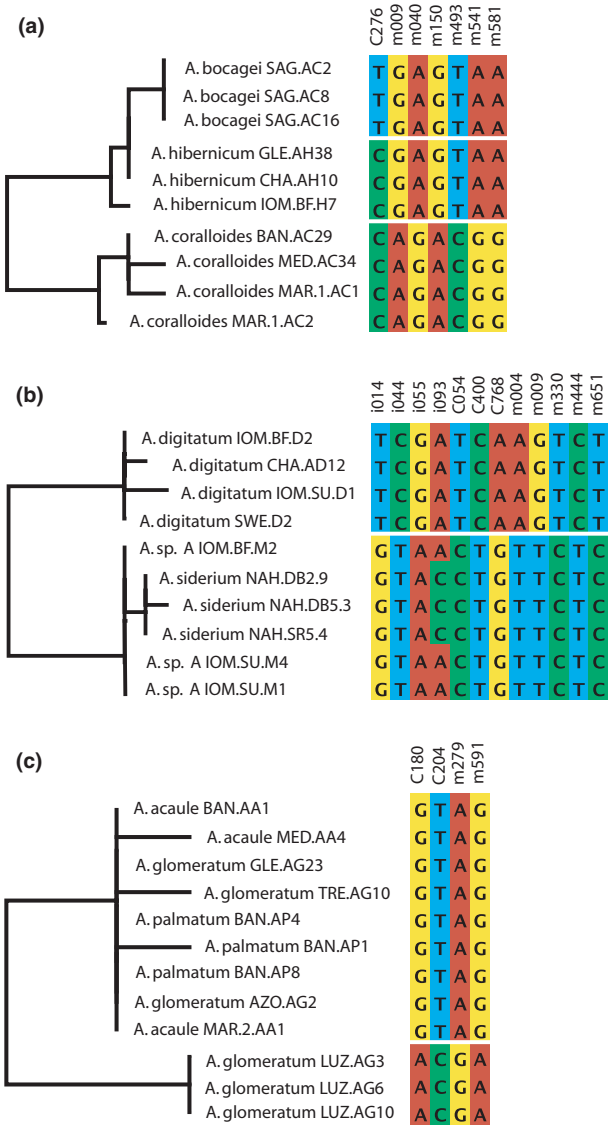

Fig. 4 Individual nucleotide characters distinguishing species of *Alcyonium* within each of three distinct clades. For simplicity, only 3–4 specimens of each species are shown (including a representative of each distinct *COI* + *igr1* + *msh1* haplotype detected for a species). Species-diagnostic nucleotide positions are labelled by gene (C = *COI*; m = *msh1*; i = *igr1*). (a) *Alcyonium coralloides* clade, excluding *A.* sp. M2. (b) *A. digitatum* clade. (c) *A. acaule* clade.

recognized morphospecies share a barcode sequence(s), species boundaries should be confirmed using independent criteria. Until such time, a wholesale rejection of mitochondrial markers as inadequate for species identification in octocorals is premature.

Genetic distances within and among genera

The distributions of pairwise genetic distance values among congeneric species (Fig. 1) reflect additional uncertainty surrounding the definitions of higher taxa in Octocorallia. For instance, the one case in which specimens identified to two different genera (*Xenia*, *Heteroxenia*) shared identical barcode sequences is more likely because of the lack of a clear distinction between these two genera (Fabricius & Alderslade 2001) than the inability of mtDNA markers to separate well-defined genera. Similarly, among the deep-sea taxa an observed lack of genetic distinction between *Paramuricea placomus* and *Placogorgia* sp. is not surprising, as the sclerites of these two genera have long been recognized to represent a continuum of morphological variation (Bayer 1959; Grasshoff 1977).

Large pairwise distances among congeners also reflect taxonomic uncertainty. For instance, two species of *Acanthogorgia* included here differed from one another by 4–7%, not surprising considering that this genus has previously been shown to be polyphyletic and to comprise two divergent clades (McFadden *et al.* 2006b). Among the bamboo corals (Isididae), pairwise genetic distances between congeners in the nominal genera *Isidella*, *Keratoisis*, and *Lepidisis* range from 0% to 2% for *msh1* (all are identical at *COI* and *igr1*). Earlier genetic analyses of *msh1* and *igr4*, however, revealed highly divergent clades that do not correspond to currently accepted bamboo coral taxonomy (France 2007; van der Ham *et al.* 2009), and a reassessment of morphological characters within the subfamily Keratoisidinae has revealed synapomorphies sufficiently significant to warrant erecting new genera (France & Watling, unpub. data). In the present study, if pairwise genetic distances had been restricted to comparisons within clades (i.e. hypothetical new genera) rather than nominal genera, the maximum interspecific distance observed among isidids would be only 0.3%.

Alcyonium as a test case for mtDNA barcoding

Circularities of the type illustrated above are common in groups such as Octocorallia that are poorly known and poorly sampled; a lack of sequence divergence among morphospecies can be interpreted as either inadequate molecular variation or faulty taxonomy, depending on personal bias. For that reason, we used the genus *Alcyonium* as a test of mtDNA barcodes specifically because species boundaries in this genus have been confirmed previously using a variety of independent criteria. A molecular phylogenetic study based on the rapidly evolving nuclear ribosomal internal transcribed spacer (*ITS*) regions separated the northern hemisphere representatives of this genus into three distinct clades among which *ITS* is too divergent (>25%) to align with any certainty (McFadden *et al.* 2001). Within each clade, however, species exhibit only 1-8% divergence at *ITS* and are often difficult to distinguish morphologically (Verseveldt

1964, 1973; Feldman 1973; Weinberg 1977; Manuel 1981; Groot & Weinberg 1982). *Alcyonium* therefore provides a rigorous test of the applicability of mitochondrial barcodes to octocorals, because it includes sister species known to be extremely similar both genetically and morphologically, but demonstrated via allozymes or reproductive differences to be reproductively isolated in sympatry (McFadden 1999; McFadden *et al.* 2001).

In this taxonomically challenging genus, the extended *COI+igr1+msh1* barcode correctly identified 7 of 10 species when a character-based analysis was applied. Within the *A. coralloides* clade (Fig. 3), the extended barcode failed to distinguish the Mediterranean species *A.* sp. M2 from two Atlantic species, *A. bocagei* and *A. hibernicum*. Based on a study of shared *ITS* polymorphisms, however, it has been proposed that *A. bocagei* and *A. hibernicum* both arose from hybrid speciation events, and that *A.* sp. M2 may have been one of the progenitors (McFadden & Hutchinson 2004), a scenario that could explain the sharing of mitochondrial haplotypes. The failure of mitochondrial barcodes to distinguish taxa with histories of hybridization or introgression is well known (Rubinoff *et al.* 2006). The other cases in which a character-based analysis of the extended barcode failed to distinguish species occurred among the three species in the *A. acaule* clade. This is the one clade of *Alcyonium* for which we lack confirmation of species boundaries using allozyme data.

COI or msh1 as an octocoral barcode?

Screening of variation across the octocoral mitochondrial genome suggests that the *msh1* gene, unique to this cnidarian subclass, is the most rapidly evolving protein-coding region in an otherwise slowly evolving genome (van der Ham *et al.* 2009), and therefore the most promising candidate for a mitochondrial barcode. For pairwise comparisons among congeners, genetic distances at *msh1* were typically 2–3× greater than those at *COI* (Tables 1–3). Overlap between intra- and interspecific genetic distances were, however, comparable for the two gene regions (Fig. 1), and in character-based analyses *msh1* was often no more effective than *COI* at distinguishing species. All of the species pairs that shared identical *COI* sequences in the Eilat survey were also identical at *msh1*; among the deep-sea calcaxonian taxa, however, some morphospecies that shared *COI* haplotypes did differ at *msh1*. Among the North Atlantic *Alcyonium* species, each gene alone distinguished just three of seven species, whereas combining the two coding regions plus *igr1* revealed enough pure characteristic attributes to distinguish all seven species reliably.

Alternative molecular markers that have been explored to date do not appear to be any more promising as barcodes for Octocorallia than our extended mitochondrial barcode. Other mitochondrial protein-coding and rDNA regions typically exhibit levels of interspecific variation similar to or less than that of *COI*, and the utility of mt intergenic regions is limited by extreme length variation among taxa (van der Ham *et al.* 2009). The internal transcribed spacers (*ITS-1*, *ITS-2*) frequently lack sufficient variation to discriminate sister taxa (McFadden *et al.* 2001; Aguilar & Sánchez 2007) and, in addition, often exhibit intraindividual sequence polymorphisms (McFadden *et al.* 2001; McFadden & Hutchinson 2004; Dueñas & Sánchez 2009). Few nuclear markers have yet been identified that exhibit adequate levels of variation to distinguish species of octocorals, and those that have (e.g. SRP54; Concepcion *et al.* 2008) have proven difficult to amplify reliably across diverse taxa (pers. obs.). For now, we suggest continued use of an extended mitochondrial barcode for octocorals: *msh1 + igr1* encompasses the most variable regions of most octocoral mt genomes, while *COI* allows comparison to other taxa for which an extensive database already exists.

In addition to an apparent two-fold difference in rates of evolution between the *COI* and *msh1* coding regions, our data also suggest some rate heterogeneity among the different taxa included in our study. In particular, deep-sea Calcaxonia exhibited significantly less genetic variation at both *COI* and *msh1* than other taxa (Table 3); we observed no intraspecific variation in any of the five calcaxonian species for which multiple individuals were sampled, and genetic distances among congeners of Calcaxonia were an order of magnitude lower for *COI* and 2–4× lower for *msh1*. Whether these observed differences reflect actual rate differences, or, alternatively, are artefacts of the smaller sample size for intraspecific comparisons or more narrowly defined genera in this group remain to be tested.

Conclusions

As has been reported previously for other anthozoans (Huang *et al.* 2008; Shearer & Coffroth 2008), the absence of a significant gap between intra- and interspecific genetic distance values imposes a limit on the use of mitochondrial barcodes (*COI, msh1,* or extended *COI + igr1 + msh1*) for species identification in Octocorallia. Overlap between intra- vs. interspecific genetic distances will give rise to false negatives, i.e. an underestimate of biodiversity as a result of different species being lumped together because of lack of sequence divergence. Our data suggest, however, that intraspecific variation (coalescent depth) rarely if ever exceeds 0.5% at either *COI* or *msh1*, making it generally safe to conclude

that specimens differing by >1% are indeed different species. Given the poor state of octocoral taxonomy and our lack of understanding of morphological characters in many genera, the potential for identification of cryptic species and genera using mtDNA barcodes is still high, as evidenced by the outcome of our Eilat biodiversity study as well as some other recent studies (McFadden *et al.* 2006a, 2009; van der Ham *et al.* 2009). For specimens that differ by <1% at *COI* or *msh1*, however, it will not be possible to conclude without additional biological, morphological or molecular study whether or not species boundaries are present.

Interspecific divergence values <1% do not, however, preclude use of a mitochondrial barcode to assign species identity in cases in which clades have been well sampled both intra- and interspecifically (e.g., Meyer & Paulay 2005). Nucleotide character-based analysis successfully identified specimens of *Alcyonium* in cases where sister species differed by <0.1%; specimens could reliably be assigned to species that differed from one another by only a single nucleotide when that difference represented a pure characteristic attribute (sensu Rach *et al.* 2008). In the regional biodiversity survey at Eilat, our ability to detect cryptic species of *Sinularia* and *Sarcophyton* was further enhanced by the existence of well-sampled *msh1* phylogenies for those genera (McFadden *et al.* 2006a, 2009). As other octocoral genera are sampled exhaustively and a database of exemplar sequences grows, our ability to assign species identifications confidently using character-based approaches will improve, despite the relative lack of interspecific divergence observed in Octocorallia.

Neither *COI* nor an extended barcode encompassing additional, more variable regions of the octocoral mitochondrial genome (*igr1* + *msh1*) meets all of the criteria required of an ideal, species-specific genetic marker. Given the poor state of octocoral taxonomy, however, and our current lack of understanding of morphological variation in this group, these imperfect molecular markers can and will nonetheless provide valuable guidance and assistance, as we search for a stable genus- and species-level taxonomy in this diverse, neglected and ecologically important group of organisms. Studies that combine molecular character-state data (i.e. barcodes) with traditional morphological taxonomic approaches have already greatly increased our understanding of species boundaries and intraclade relationships and have identified new, diagnostic morphological character states useful for field identification in several speciose and ecologically important genera of octocorals (McFadden *et al.* 2006a, 2009; France 2007). We urge traditional taxonomists and molecular systematists to continue to work together to better understand species boundaries and to construct well-sampled phylogenies. Only through such collaborative, integrated approaches will our ability to confidently identify species of octocorals using molecular barcodes continue to improve.

Acknowledgements

We thank Vanessa Brisson for assistance in the laboratory, Leen van Ofwegen for identification of specimens, the Interuniversity Institute at Eilat for assistance and use of facilities, and Alex Shlagman for professional curatorial skills. We also thank the Israel Nature and Parks Authority for providing collection permits, and the Eilat-Ashkelon Pipeline Company (EAPC) for allowing research on their premises. This research was supported by the Assembling the Cnidarian Tree of Life project (NSF grants EF-0531570 to C.S. McFadden and EF-0531779 to P. Cartwright), and by grants #NA05OAR4601061 from NOAA's Office of Ocean Exploration and OCE-0624601 from NSF's Ocean Sciences Division–Biological Oceanography Program to S.C. France. P.A. Nevarez was supported by Howard Hughes Medical Institute award 52005123 to Harvey Mudd College. Grants from - Sigma Xi (GIAR G20061021830514629) and the American Museum of Natural History (Lerner Gray Fund) allowed E. Pante to sample specimens from the MNHN collection (Paris, France).

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Morphospecies of octocorals identified from collections made during a 4-day biodiversity survey at Eilat, Israel

Table S2 Species of *Alcyonium* included in the comparison of barcode sequences. Each unique haplotype sequenced for a species was deposited in GenBank

Table S3 Deep-sea octocorals analyzed, including location and depth of collection

Table S4 Primers used to amplify the regions of *COI*, *igr1*, and *msh1* used as molecular 'barcodes'

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