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Copepod diversity in a subtropical bay based on a fragment of the mitochondrial COI gene

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Copepod communities in lagoons and embayments on subtropical islands in the Pacific Ocean are geographically isolated from other populations along continents and other islands. Nevertheless, taxonomic identifications suggest that many of these species are cosmopolitan. The genetic diversity of planktonic copepod species in a subtropical embayment, Kaneohe Bay, Hawaii, was investigated by pairing morphological identification of the species with sequencing a 710 bp fragment of the mitochondrial cytochrome oxidase *c* subunit I (mtCOI) gene. DNA sequences were obtained for six calanoid and three cyclopoid copepod species. The sequences of two oceanic species found in the bay, *Undinula vulgaris* and *Paracalanus parvus*, were ≤3.0% divergent from conspecifics in the coastal western Pacific Ocean. In contrast, sequences from the more estuarine, *Parvocalanus crassirostris* and *Bestiolina similis* specimens were ≥16.0% divergent from conspecifics of the western Pacific Ocean. The *Labidocera* sp. and *Acartia* sp. were ≥16.0% divergent from all congeners, while three *Oithona* species differed by ≥26.5% from congeners. These results suggest significant genetic isolation of the more estuarine species, although more sequence data for Hawaii and elsewhere will be needed to understand the population and genetic structure of coastal island copepod populations.

KEYWORDS: mtCOI; copepod; paracalanid; cyclopoid; Kaneohe Bay

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INTRODUCTION

Many subtropical embayments are highly productive environments that are economically important supporting fisheries and recreational activities. These habitats are often associated with coral reef communities, which are characterized by high standing stocks of invertebrate and vertebrate (fish) biomass, while having very low levels of nutrients in the surrounding water (Birkeland, 1997). Many reef habitats are under threat from global warming, pollution, invasive species and over-fishing (e.g. Smith *et al.*, 1981, 2010). The planktonic communities in the reef lagoons and surrounding coastal areas are an integral part of these habitats and they include the larval stages of most invertebrate and vertebrate reef inhabitants, as well as the holoplankton. The latter are typically dominated by copepods (McKinnon *et al.*, 2005), which are an important food source for other zooplankton, including ichthyoplankton, planktivorous reef fishes and reef invertebrates (Kimmerer, 1984; Hamner *et al.*, 1988).

Copepod communities associated with coral reef areas include one or more cyclopoids, mostly in the genus *Oithona*, and several calanoids, many in the family Paracalanidae (McKinnon and Thorrold, 1993; McKinnon and Ayukai, 1996; Hopcroft *et al.*, 1998; McKinnon and Klumpp, 1998; Hoover *et al.*, 2006; McKinnon *et al.*, 2008). Based on morphological identification, many species found in these communities have worldwide distributions. For example, *Parvocalanus crassirostris* has been reported from Long Island estuary, USA (Turner, 1982), Cananeia Lagoon, Brazil (Ara, 2004), Ariake Sea, Japan (Islam *et al.*, 2006), Shark Bay, Exmouth Gulf, Alligator Creek and Haughton River, Australia (Kimmerer and McKinnon, 1985; Robertson *et al.*, 1988; McKinnon and Ayukai, 1996; McKinnon and Klumpp, 1998), Danshuei and Tapong Bays, Taiwan (Lo *et al.*, 2004; Hwang *et al.*, 2006; Liu *et al.*, 2007), Kingston Harbour, Jamaica (Moore and Sander, 1979; Hopcroft *et al.*, 1998) and Kaneohe Bay, Hawaiian Islands, USA (Hoover *et al.*, 2006). However, these populations are not only geographically distant from each other, but their habitats are discontinuous, raising questions about species identification as well as species boundaries among populations.

The Hawaiian Islands are one of the most remote locations on this planet. The nearest landmass is more than 3000 km away (North America) and the nearest island, Johnston Atoll (area: 2.7 km^2), is over 1000 km away. Kaneohe Bay is a semi-enclosed embayment on the windward side of the island of Oahu. The bay is separated from the open ocean by a barrier reef (Fig. 1) characterized by healthy coral populations (Jokiel and Brown, 2004). Kaneohe Bay has been the focus of many studies of phytoplankton and zooplankton dynamics. Prior studies on the copepod community have focused on species diversity and community structure (Edmonson, 1937; Peterson, 1969; Hirota and Szyper, 1976; Scheinberg, 2004), grazing impact (Calbet *et al.*, 2000), predation risk (Kimmerer, 1984) and responses to

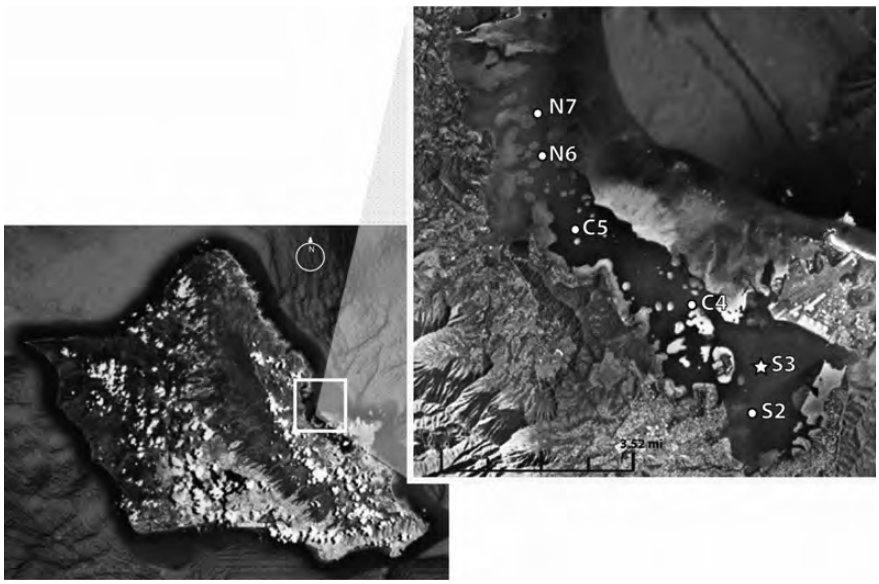

Fig. 1. Map of the island of Oahu (Hawaii), with an inset showing Kaneohe Bay and the sampling locations used to assess copepod genetic diversity. S, C and N indicate the southern, central or northern regions of the bay. Station S3 was the main sampling station for monthly sampling other stations were sampled to assess copepod genetic diversity within the bay. Images from Google Earth™.

nutrient inputs due to sewage or rainstorms (Smith *et al.*, 1981; Ringuet and Mackenzie, 2005; Cox *et al.*, 2006; Hoover *et al.*, 2006). Eight copepod species have been recently reported to occur in the bay, but taxonomic identification has been inconsistent over the years (Table I). Until now, morphological criteria have not been paired with molecular work to verify the identification of local species.

Although genetic markers have been used to confirm species identification (e.g. Bucklin *et al.*, 1995, 1998, 2010b), phylogenetic relationships (Machida *et al.*, 2004, 2006; Blanco-Bercial *et al.*, 2011a; Cepeda *et al.*, 2012) and population structure and connectivity (Caudill and Bucklin, 2004; Goetze 2005, 2011; Unal and Bucklin, 2010; Blanco-Bercial *et al.*, 2011b) in marine calanoid copepods, there is limited information available for cyclopoids and calanoids from subtropical estuaries. The mitochondrial cytochrome oxidase *c* subunit I (mtCOI) gene has been informative in a number of DNA barcoding studies to confirm species identification (e.g. Bucklin *et al.*, 1999, 2010a, b; Costa *et al.*, 2007; Ortman *et al.*, 2010). In calanoid copepods, the mtCOI gene is characterized by low intraspecific genetic divergence (up to 4% within species), while differentiation between species ranges from ~9 to >25% (Bucklin *et al.*, 1999, 2003, 2010a). A search for the mtCOI sequences for the copepod species identified in Kaneohe Bay in previous studies yielded a total of 31 sequences [National Center for Biotechnology Information (NCBI) GenBank database, search date: 3 December 2012]. In the current study, we surveyed the mtCOI diversity of calanoid and cyclopoid populations in Kaneohe Bay and compared these sequences with conspecifics and congeners from different regions of the world in the GenBank database.

METHOD

Sample collection and copepod cultures

Plankton samples were collected on 8 February 2010 from six locations spanning the northern, central and southern regions of Kaneohe Bay (Fig. 1). Monthly samples were collected at Station S3 between February 2010 and November 2011 for additional specimens to confirm species composition and diversity. Vertical net tows from 10 m to the surface were taken with a 0.5 m diameter, 64 μm mesh plankton net. Samples were concentrated and immediately preserved in 95% EtOH, put on ice in the field and then stored at −20°C. The alcohol was changed once within 24 h of collection, to maintain the integrity of sample DNA (Bucklin, 2000). Two species, *P. crassirostris* and *Bestiolina similis*, had been previously isolated from the bay and maintained continuously in the laboratory on a diet of *Isochrysis galbana* (McKinnon *et al.*, 2003; VanderLugt and Lenz, 2008). Individuals from these cultures were compared with field-caught individuals from Kaneohe Bay.

Species identification

Adult females were removed from preserved samples or cultures for identification, DNA extraction and

Table I: Planktonic copepod species listed in prior literature from Kaneohe Bay, Hawaii.

Order	Family	Species	Location(s)	Reference
Calanoida	Acartiidae	*Acartia* sp.	South	Bartholomew (1973), Hirota and Szyper (1976), Scheinberg (2004)
		Acartia hamata	South	Peterson (1969)
	Calanidae	*Undinula darwinii*[a]	South	Scheinberg (2004)
		Undinula vulgaris	North	Peterson (1969)
	Paracalanidae	*Parvocalanus crassirostris*	South	Calbet *et al.* (2000), Scheinberg (2004)
		Bestiolina similis	South	Scheinberg (2004)
		Paracalanus sp.	South	Bartholomew (1973), Newbury and Bartholomew (1976)
		Acrocalanus inermis[b]	South	Kimmerer (1984), Calbet *et al.* (2000)
		Acrocalanus sp.	South	Hirota and Szyper (1976)
	Pontellidae	*Labidocera* sp.	North, south	Peterson (1969), Hirota and Szyper (1976)
	Pseudodiaptomidae	*Pseudodiaptomus* sp.	North, south	Peterson (1969), Hirota and Szyper (1976)
Cyclopoida	Cyclopidae	*Cyclops* sp.	South	Peterson (1969)
	Oithonidae	*Oithona simplex*	South	Bartholomew (1973), Newbury and Bartholomew (1976), Hirota and Szyper (1976), Kimmerer, (1984), Calbet *et al.* (2000), Scheinberg (2004)
		Oithona nana	South	Bartholomew (1973), Newbury and Bartholomew (1976), Hirota and Szyper (1976), Kimmerer (1984), Calbet *et al.* (2000), Scheinberg (2004)

Location(s) = regions where the species was reported to occur within Kaneohe Bay.
[a]*Undinula darwinii* synonym of *C. darwinii* (Grice and Hulsemann, 1967; Walter and Boxshall, 2008).
[b]*Acrocalanus inermis* synonym of *Bestiolina inermis* (Walter and Boxshall, 2008).

sequencing. The paracalanid species in the bay had been previously examined by taxonomic experts F. Ferrari (Smithsonian Institution) and W. Lee (Hanyang University, Seoul, South Korea) and determined to be *P. crassirostris* and *B. similis*. These identifications were supported by descriptions in McKinnon *et al.* (McKinnon *et al.*, 2003) and Lawson and Grice (Lawson and Grice, 1973). The *Oithona* species previously described from the bay, *Oithona nana* and *Oithona simplex* (Table I), seemed to match poorly with the morphological variation observed in the samples. As a result, *Oithona* material from all three regions (North, central and South) of the bay was sent to taxonomic expert S. Nishida (ORI, U Tokyo) for identification. He identified three cyclopoid species in the material: *O. simplex*, *Oithona oculata* and *Oithona attenuata*, the latter two of which are first reported here for Kaneohe Bay. Identification of these *Oithona* species was supported using Nishida (Nishida, 1985). *Undinula vulgaris* has been previously reported, and identification of Kaneohe Bay specimens was supported using Björnberg (Björnberg, 1963, 1966). A *Labidocera* species was also identified in the bay. The *Labidocera* in Hawaiian waters had been tentatively identified as *Labidocera madurae* by Leis; however, there was some doubt about its proper identification (Leis 1978, 1982; Hassett and Boehlert, 1999). Thus, we refer to it as *Labidocera* sp.

Polymerase chain reaction and DNA sequencing

DNA was extracted from individual adult females using the lysis buffer method described in Lee and Frost (Lee and Frost, 2002). For each species, a 710 bp fragment of the mtCOI gene was targeted for amplification with polymerase chain reaction (PCR) using previously published primers L1384 (GGTCATGTAATCATAAAGA TATTG) (Machida *et al.*, 2004), and HCO2198 (TAAACTTCAGGGTGACCAAAAAATCA) (Folmer *et al.*, 1994). PCR amplifications were performed in 25 μL reaction volumes using 2.5 μL 10× PCR Buffer minus Mg^{2+}, 1.5 mM $MgCl_2$, 0.3 μM of each primer, 0.2 mM of each dNTP, Invitrogen Taq polymerase (recombinant) at 0.05 units/μL, and 3 μL of DNA extract. Reaction conditions included denaturing at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 42°C for 30 s, extension at 72°C for 1 min then the final extension step at 72°C for 4 min. PCR products were visualized on 1–1.5% agarose gels with ethidium bromide staining. Acceptable amplifications were purified using shrimp alkaline phosphatase and exonuclease I (USB Corp.; 30 min at 37°C followed by 95°C for 15 min), and sequenced on an ABI 3730XL machine (BigDye terminator chemistry). mtCOI sequences were obtained from a total of 159 specimens of eight species identified morphologically, and sampled at three regions within Kaneohe Bay.

Sequence analysis

DNA sequences were evaluated for data quality using Geneious (v5.5.7; Kearse *et al.*, 2012). Forward and reverse strands were aligned using ClustalW (v2.0) within Geneious (Larkin *et al.*, 2007). Ambiguous bases in one strand were corrected if the base was sequenced more clearly on the second strand, otherwise the base was left as ambiguous ("N" rather than A, G, C or T). A final consensus sequence was created from this alignment for each specimen, containing only bases that were called with high confidence. All sequences for specimens morphologically identified as the same species were aligned (ClustalW), and all unique mtCOI haplotypes were translated to amino acids to assess for the presence of stop codons or indels (insertions–deletions) within the open reading frame (ORF). Observation of either of these within the mtCOI ORF would indicate that a pseudogene may have been amplified, rather than the intended mtCOI functional gene copy (Buhay, 2009). No stop codons or interruptions in the reading frame were observed in any Kaneohe Bay sequences. All sequences were then compared with the GenBank database for closest matches, in order to verify amplification of copepod mtDNA.

In order to evaluate the systematic identity of Kaneohe Bay copepods, phylogenetic trees were inferred for calanoids and cyclopoids (separately) that included both the new Kaneohe Bay mtCOI material and all mtCOI sequences present in the GenBank database from species within the same genera, and the same family for the Pontellidae. Separate alignments were created for subsets of the calanoids (Paracalanidae, *Acartia* sp., *U. vulgaris*, Pontellidae) and all the cyclopoids using ClustalW. Each alignment was trimmed to equal length (calanoids 499 bp, cyclopoids 584 bp), and any sequences that were shorter than the final 499 or 584 bp length were removed from further analysis. Unique haplotypes were identified using Mothur (v1.25.1) (Schloss *et al.*, 2009), which clusters sequences into groups based on their uncorrected pairwise genetic distance. Haplotypes were clustered in Mothur by assigning sequences into operational taxonomic units (OTUs), where all sequences within each unit were <1 or 2% genetically divergent from the average sequence in the OTU (2% for calanoids, 1% for cyclopoids). For calanoids, all GenBank haplotypes determined to be >2% genetically divergent (>10 substitutions, 499 bp alignment) were included in final alignments, along

with all unique (>1 bp difference) Kaneohe Bay calanoid sequences. Within the genus *Acartia*, there were a few cases where the <2% divergent OTUs contained multiple species. In these cases, a representative of each species within the OTU was selected for final phylogenetic analyses. For cyclopoids, all GenBank haplotypes determined by Mothur to be >1% genetically divergent (>6 substitutions, 584 bp alignment) were included in final alignments, along with all unique (>1 bp difference) Kaneohe Bay cyclopoid sequences. Accession numbers for all GenBank haplotypes used in analyses can be found in Supplementary data, Tables SI and SII.

These final alignments were used to infer maximum-likelihood (ML) trees as well as calculate genetic distances between all unique OTUs. The best nucleotide substitution model for both alignments was determined using jModelTest (v0.1.1) (Posada, 2008) by computing their likelihood scores and identifying the best substitution model using the Akiake information criterion. From this analysis, it was determined that the general time reversible (GTR) substitution model with a proportion of invariable sites (+I) and rate variation among sites (+G) (GTR + I + G) was the best model for both sequence alignments. The best ML tree for each alignment was inferred using PhyML (v3.0) (Guindon and Gascuel, 2003; Guindon *et al.*, 2010), with the GTR + I + G substitution model and 1000 bootstrap replicates. Uncorrected pairwise genetic distances were calculated from each final alignment using Mothur.

RESULTS

Species diversity based on morphology

Species diversity increased from South to North based on the survey of the copepods in Kaneohe Bay (Fig. 1, Table II). Two paracalanid, *B. similis* and *P. crassirostris*, and two cyclopoid, *O. simplex* and *O. attenuata*, species were common throughout the bay. A third *Oithona* species, *O. oculata*, was found primarily in the North Bay. Three other calanoid species, *U. vulgaris, Labidocera* sp. and *Acartia* sp., were found in the central and northern bay. The four species that characterized the southern bay were abundant year-round based on 1.5 years of monthly sampling.

mtCOI sequence analysis

Based on the sequence data, we identified six calanoids and three cyclopoid species, only two of which matched to known species with published sequence data (*U. vulgaris* and *Paracalanus parvus*). mtCOI sequences from all other species were at least 14% different from published sequences. Few mtCOI sequences were available for cyclopoids in the GenBank database, and the sequences obtained for the Kaneohe Bay cyclopoids were at least 25% different from each other and from published sequences.

Paracalanidae (Calanoida)

Five unique haplotypes were sequenced for specimens identified as *P. crassirostris* morphologically (Table II). All five haplotypes translated into identical amino acid sequences. Of the 32 individuals identified as *P. crassirostris* and sequenced, 29 sequences (94%) clustered into a single clade with four haplotypes (KH1–KH4, Fig. 2), which were obtained from individuals that were collected throughout the bay and from the laboratory cultures. Divergence among these four haplotypes ranged from 0.2 to 1.2%, which is in the range of expected

Table II: Number of individuals sequenced from each region of Kaneohe Bay, the resulting number of unique haplotypes for each, and GenBank accession numbers

Species	South	Central	North	Lab	# Seq.	Haplotypes	Accession Numbers
Parvocalanus crassirostris	8	8	8	5	29	4: KH1-KH4	KC594153-6
P. crassirostris (O)	—	1	1		2	1: KH5	KC594157
Bestiolina similis	9	7	6	2	25	7: KH3-KH9	KC594122-8
B. similis (O)	—	2	2		4	2: KH1-KH2	KC594120-1
Paracalanus parvus	—	—	1		1	1: KH1	KC594152
Acartia sp.	—	5	8		13	9: KH1-KH9	KC594111-9
Labidocera sp.	—	2	13		15	10: KH1-KH10	KC594129-38
Undinula vulgaris	—	2	9		11	9: KH1-KH9	KC594158-66
Oithona simplex	11	8	5		24	7: KH1-KH7	KC594145-51
Oithona attenuata	11	7	8		26	2: KH1-KH2	KC594139-40
Oithona oculata	—	—	9		9	4: KH1-KH4	KC594141-4

Individuals were identified using morphological criteria and are separated by source (region of the Bay or laboratory culture).
For each species, the total number of individuals sequenced (# Seq.), number of and label for unique haplotypes (KH), and GenBank accession numbers (respectively) for each unique haplotype are given. (O), outlier sequences, potentially cryptic species. —, species was not found in the sample in that region.

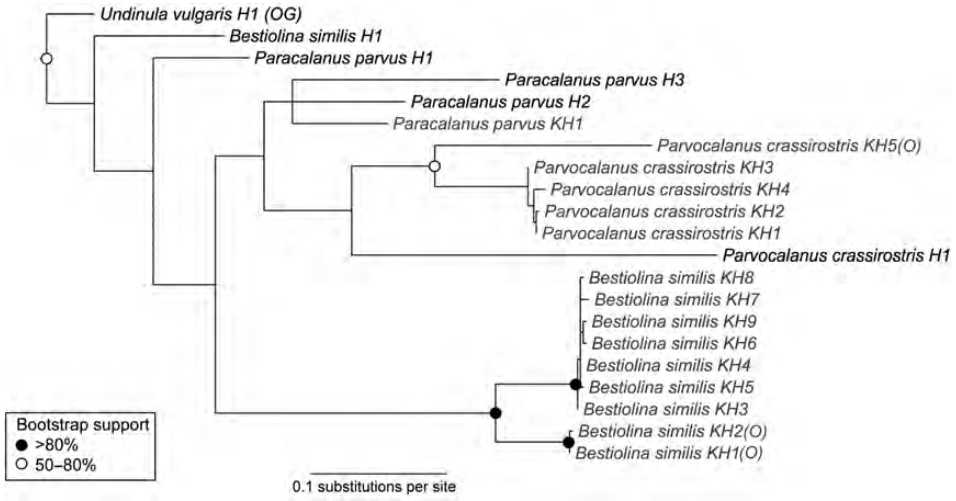

Fig. 2. ML phylogenetic tree including only the Paracalanidae, with *U. vulgaris* H1 as the outgroup (OG). H#: is the haplotype ID for each species which corresponds to Supplementary data, Table SI sequence accession numbers. KH# (and grey font): indicates species is from Kaneohe Bay and corresponds to Table II. Closed circles indicate >80% bootstrap support for a given node and open circles indicate 50–80% bootstrap support from 1000 bootstrap replicates. Scale bar: 0.1 bp substitutions per nucleotide site in the alignment.

within-species genetic variability for this gene. A fifth unique haplotype was obtained for two individuals collected from the northern region of Kaneohe Bay and was 14.3% divergent from the primary clade. This outlier, which was named *P. crassirostris* KH5 (O), may be a "cryptic", genetically divergent lineage from the rest of the local *P. crassirostris*, showing a level of divergence (>9%) which is more often observed among species.

Ten unique haplotypes were found for individuals identified as *B. similis* (Table II). One specimen appeared to have been misidentified. Alignment of its sequence with other Paracalanids identified it as *P. parvus* (KH1, Fig. 2), with 99% sequence identity to a *P. parvus* mtCOI in the GenBank database (HQ150069; collected in the Spermonde archipelago, Indonesia). Of the remaining nine unique haplotypes for *B. similis*, seven belonged to a single clade with genetic divergence ranging from 0.2 to 1.0% (KH3–KH9). Eighty-four percent of the sequences belonged to this group, and one of these haplotypes (KH6) included a single amino acid substitution. A secondary clade with two unique haplotypes was identified for thirteen percent of sequences. These two haplotypes, identified as *B. similis* KH1 and KH2 (O), were 0.2% divergent from each other and 8.6–9.2% divergent from the primary clade. Translations of these two haplotypes indicated that their amino acid sequences were identical to each other and all other Kaneohe Bay sequences except for KH6.

The paracalanid sequences obtained from Kaneohe Bay were compared with published mtCOI sequences (Fig. 2). The *B. similis* sequences (KH1–KH9) were 16.0–16.6% divergent from the previously published *B. similis* haplotype (H1; AB679188) which represented all 17 *Bestiolina* mtCOI sequences in the GenBank database, all from Palau, Micronesia. The two clades from Kaneohe Bay clustered into two adjacent branches of the ML tree shown in Fig. 2. For *P. parvus*, the sequence from Kaneohe Bay (KH1) was most similar to a sequence obtained from Indonesia (H2; Fig. 2), while the other two published sequences (*P. parvus* H1, H3) were more divergent. The three *P. crassirostris* mtCOI sequences (a single haplotype) in GenBank had been obtained from specimens collected off the Coast of China (H1; HM045396), and were the only representatives of the genus *Parvocalanus* with mtCOI sequences in the GenBank database. The two *P. crassirostris* clades from Kaneohe Bay were most similar to each other (Fig. 2), and these sequences [KH1–KH5(O)] were 17.8–20.0% divergent from the published *P. crassirostris* haplotype (H1). Interestingly, a broader comparison that excluded local conspecifics suggests that *P. crassirostris* KH1–KH4 were most similar to *P. parvus* H1 (15.0%, coast of China) rather than to *P. crassirostris* H1. Similarly, *P. crassirostris* KH5(O) was most similar to *P. parvus* KH1 (16.2%; Table III).

Acartia sp. (Acartiidae: Calanoida)

Nine unique haplotypes were found among the *Acartia* sp. (Table II). These were very similar to each other; they translated into identical amino acid sequences with 0.2–1.4% divergence among haplotypes and formed a single clade (KH1–KH9; Fig. 3). A comparison of these sequences to those from 13 congeners published

Table III: Uncorrected pairwise genetic distances within and between the closest species for each clade in Kaneohe Bay

		Kaneohe Bay conspecifics (%)		Closest haplotype (%)		
Kaneohe Bay haplotype(s)	# Seq.	Mean	SD	Species, location	Mean	SD
With published conspecifics						
Parvocalanus crassirostris KH1–KH4	29	0.8	0.4	*Paracalanus parvus* H1, Coast of China	15.0	0.4
Parvocalanus crassirostris KH5(O)	2	—	—	*Paracalanus parvus* KH1, Kaneohe Bay	16.2	—
Bestiolina similis KH3–KH9	25	0.6	0.2	*Paracalanus parvus* H1, Coast of China	15.0	0.2
Bestiolina similis KH1–KH2(O)	4	0.2	—	*Paracalanus parvus* H1, Coast of China	15.4	0
Paracalanus parvus KH1	1	—	—	*Paracalanus parvus* H2, Spermonde Archipelago, Indonesia	1.2	—
Undinula vulgaris KH1–KH9	11	0.9	0.3	*Undinula vulgaris* H1, Coast of China	2.7	0.3
Without published conspecifics						
Labidocera sp. KH1–KH10	15	1.1	0.6	*Labidocera rotunda* H2, Japan	16.7	0.3
Acartia sp. KH1–KH9	13	0.8	0.4	*Acartia tonsa* H4, E. Coast USA	17.1	0.4
Oithona simplex KH1–KH7	24	1.1	0.8	*Oithona similis*, South Korea	27.6	0.2
Oithona attenuata KH1–KH2	26	0.2	—	*Oithona dissimilis* H3, Japan	29.6	0.1
Oithona oculata KH1–KH4	9	0.6	0.3	*Oithona similis*, South Korea	29.1	0.3

The mean and standard deviation (SD) were calculated for all relevant pairwise comparisons and are shown in percentages. The 'Closest Haplotype' includes the haplotype with the minimum genetic distance from the listed species from Kaneohe Bay when compared with all others, excluding that of local conspecifics. See Figs. 2–6 for additional information on relationships between and across species. # Seq. = number of individuals sequenced that are of the haplotype(s).

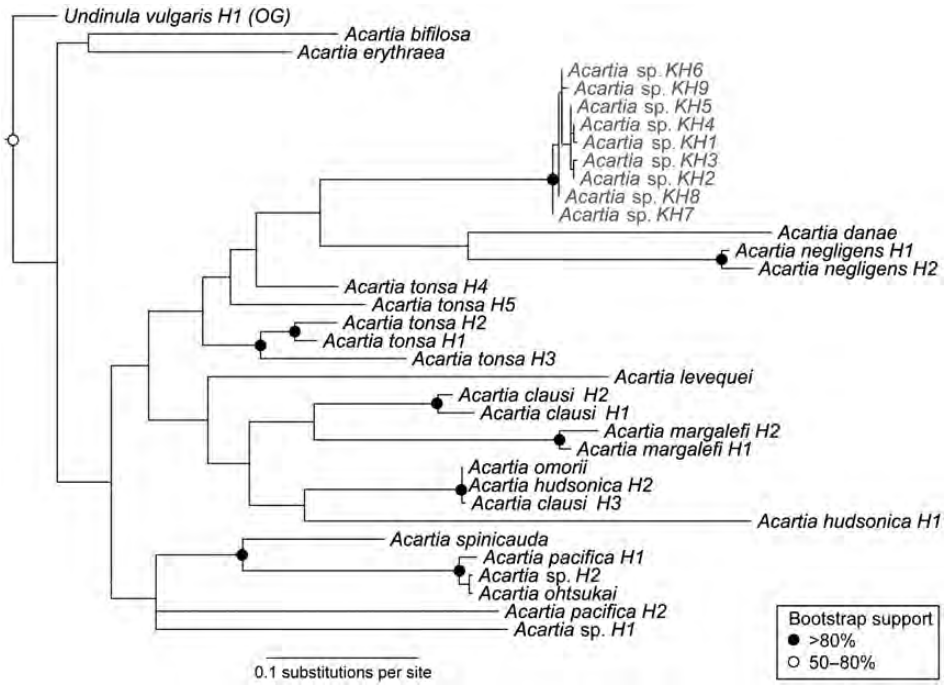

Fig. 3. ML phylogenetic tree including only the genus *Acartia*, with *U. vulgaris* H1 as the outgroup (OG). H#: is the haplotype ID for each species which corresponds to Supplementary data, Table SI sequence accession numbers. KH# (and grey font): indicates species is from Kaneohe Bay and corresponds to Table II. Closed circles indicate >80% bootstrap support for a given node and open circles indicate 50–80% bootstrap support from 1000 bootstrap replicates. Scale bar as in Fig. 2.

in GenBank showed 16.6–25.9% divergence from all other *Acartia* sequences (Fig. 3). The Kaneohe Bay sequences were most similar to an *A. tonsa* haplotype (H4; JF304090; East Coast, USA; Table III).

Undinula vulgaris (Calanidae: Calanoida)

Nine unique haplotypes were found among *U. vulgaris* (Table II). Divergences among these haplotypes were low, represented synonymous substitutions and ranged

from 0.2 to 1.4%. These haplotypes (KH1–KH9) were compared with the 11 published *Undinula* mtCOI sequences, all *U. vulgaris*, which clustered into two haplotypes; one haplotype represented the sequences from Chinese coastal waters (H1; EU599561) and the second haplotype represented the sequences from the Northwest Atlantic (H2; GU171333). The Kaneohe Bay sequences formed a single clade that was 2.2–3.0% divergent from the *U. vulgaris* from Chinese coastal waters (Table III, Fig. 4) and 8.2–9.2% divergent from the haplotype from the Northwest Atlantic (Fig. 4). Given that within-species divergence in the mtCOI gene is typically <4%, the high degree of similarity between the sequences from Kaneohe Bay and the haplotype from Chinese coastal waters would place them into the same species.

Labidocera sp. (Pontellidae: Calanoida)

Ten unique haplotypes with divergences ranging between 0.2 and 2.2% were found for the *Labidocera* sp. specimens (Table II). All haplotypes translated into identical amino acid sequences. They were compared with sequences from three other *Labidocera* species, and six other mtCOI sequences in the database from other copepods in the same family, Pontellidae. The Kaneohe Bay sequences (KH1–KH10) formed a single clade, and were 16.0–20.4% divergent from the other *Labidocera* species and 18.6–22.9% divergent from all other Pontellid haplotypes (Fig. 5). Out of all published *Labidocera* species, the Kaneohe Bay sequences were most similar to *L. rotunda* (H2; AB206442; Table III).

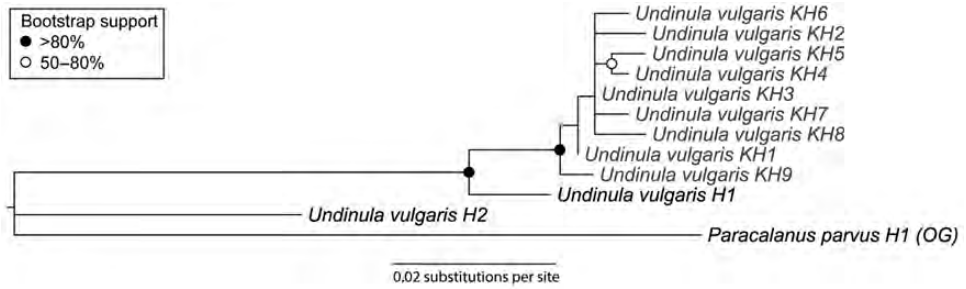

Fig. 4. ML phylogenetic tree including only the genus *Undinula*, with *P. parvus* H1 as the outgroup (OG). H#: is the haplotype ID for each species which corresponds to Supplementary data, Table SI sequence accession numbers. KH# (and grey font): indicates species is from Kaneohe Bay and corresponds to Table II. Closed circles indicate >80% bootstrap support for a given node and open circles indicate 50 to 80% bootstrap support from 1000 bootstrap replicates. Scale bar: 0.02 base pair substitutions per nucleotide site in the alignment.

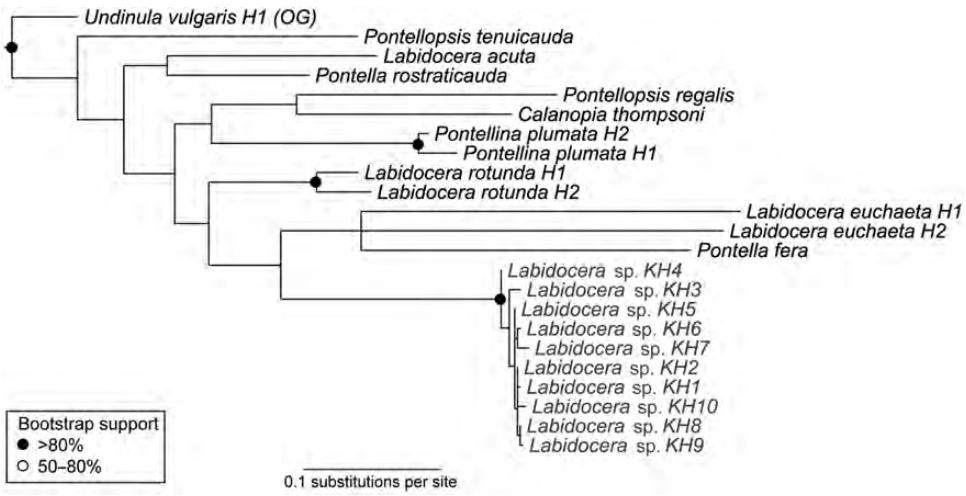

Fig. 5. ML phylogenetic tree including only the Family Pontellidae, with *U. vulgaris* H1 as the outgroup (OG). H#: is the haplotype ID for each species which corresponds to Supplementary data, Table SI sequence accession numbers. KH# (and grey font): indicates species is from Kaneohe Bay and corresponds to Table II. Closed circles indicate >80% bootstrap support for a given node and open circles indicate 50–80% bootstrap support from 1000 bootstrap replicates. Scale bar as in Fig. 2.

Oithona (Cyclopoida)

Three species of *Oithona* were identified morphologically: *O. simplex*, *O. attenuata* and *O. oculata*. We found single genetic lineages with two to seven unique haplotypes per species (Table II). All haplotypes within a species represented synonymous substitutions. *O. attenuata*, with two unique haplotypes (KH1–KH2), had a within-species genetic distance of 0.2%. Four unique haplotypes were found for *O. oculata* (KH1–KH4) and their within-species divergence ranged of 0.2–1.0%. Seven unique haplotypes were found among *O. simplex* specimens (KH1–KH7). The sequences had a mean genetic distance of 1.1%, ranging from 0.2–2.4%.

Few mtCOI sequences for *Oithona* are currently available through GenBank, none of which corresponded to the species found in Kaneohe Bay (*O. simplex*, *O. attenuata*, *O. oculata*). Thus, the present sequences are the first submitted for these three species to this public database. The Kaneohe Bay sequences were compared with published sequences from *O. dissimilis* (AB604158-164) and *O. similis* (JN230860-871; Fig. 6). In contrast to the calanoid sequences, the *Oithona* sequences were characterized by 3 bp insertions (1 amino acid) or 3 bp deletions (1 amino acid) among the species. The *O. similis* sequences aligned with no gaps with the Kaneohe Bay sequences for *O. simplex* and *O. attenuata*, while *O. dissimilis* sequences showed a single amino acid deletion. In contrast, the Kaneohe Bay *O. oculata* sequences had a single amino acid insertion in their alignment relative to the *O. similis* sequences. All *Oithona* species were highly divergent from one another (Table III; Fig. 6), with uncorrected pairwise genetic distances ranging from 26.5 to 33.4% between species, with *O. similis* and *O. dissimilis* showing the greatest divergence (Fig. 6).

DISCUSSION

Kaneohe Bay is characterized by shallow fringing reefs along much of the shoreline, 60 patch reefs scattered throughout the bay and a protective barrier reef that encloses much of the northern region (Smith *et al.*, 1973; Hunter and Evans, 1995; Jokiel and Brown, 2004). High standing stocks of copepods are found in the open waters adjacent to the reefs (Bartholomew, 1973; Hirota and Szyper, 1976; Hoover *et al.*, 2006). The copepods are an important food source for both pelagic and reef predators. The reefs shelter a diverse array of zooplanktivorous predators, many of them fishes, which are capable of removing large quantities of copepods from the water column as water flows over or around them (Hamner *et al.*, 1988, 2007; Yahel *et al.*, 2005). Thus, the copepods are an integral part of the ecology of both pelagic and benthic communities in the Bay.

Nutrient loading due to eutrophication and changes in land use has led to concerns about its effect on pelagic communities, leading to a number of scientific investigations in Kaneohe Bay (Bathen, 1968; Szyper, 1978; Smith *et al.*, 1981; Calbet and Landry, 1999; Hoover *et al.*, 2006; De Carlo *et al.*, 2007; Ostrander *et al.*, 2008; Drupp *et al.*, 2011), including studies on the copepod populations (Table I). In spite of this interest, a comprehensive investigation on the copepod biodiversity combining morphological and molecular criteria has not been carried out. DNA barcoding using the mtCOI gene has been an effective tool in species identification in eukaryotes, including copepods (e.g. Stoeckle, 2003; Bucklin *et al.*, 2010b, 2011). Furthermore, although adult copepods can be identified using taxonomic keys, the earlier developmental stages, which typically

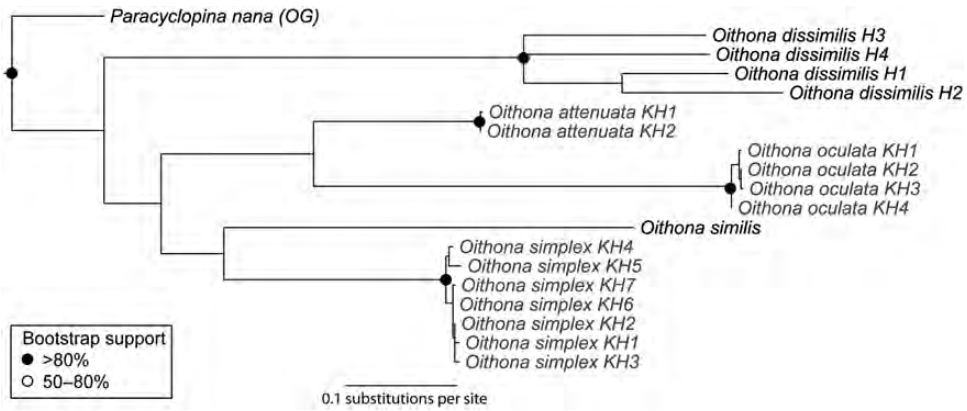

Fig. 6. ML phylogenetic tree including only the genus *Oithona*, with *Paracyclopina nana* as the outgroup (OG). H#: is the haplotype ID for each species which corresponds to Supplementary data, Table SII sequence accession numbers. KH# (and grey font): indicates species is from Kaneohe Bay and corresponds to Table II. Closed circles indicate >80% bootstrap support for a given node and open circles indicate 50 to 80% bootstrap support from 1000 bootstrap replicates. Scale bar as in Fig. 2.

outnumber adults in the water column (Hoover *et al.*, 2006), are difficult to identify. Thus, the mtCOI sequence data presented here can be used for rapid identification of all stages, including eggs and nauplii.

Using both criteria, we identified three cyclopoid species in the genus *Oithona* and six calanoid species. Two paracalanids were particularly abundant and they were found in all bay samples. They were identified morphologically as *B. similis* and *P. crassirostris* (this study; Davis *et al.*, 1999; Scheinberg, 2004; Hoover *et al.*, 2006), but may have been identified as *Acrocalanus* sp. or *Paracalanus* sp. in the earlier literature (Table I). The third paracalanid, a single specimen of *P. parvus*, may have been advected into the bay (Hooff and Peterson, 2006). A single *Acartia* species, *U. vulgaris* and *Labidocera* sp. were identified using both morphological and molecular criteria. These three species were common in the central and northern bay, and rare in the southern section. *Undinula vulgaris* is common in the coastal waters surrounding Oahu, where they can occur in very high abundances (Hassett and Boehlert, 1999). We did not find *Undinula darwinii* (= *Cosmocalanus darwinii*, Grice and Hulsemann, 1967 cited in Walter and Boxshall, 2008) or *Pseudodiaptomus* sp. in our samples; however, these have been reported as rare in previous studies. Two of the cyclopoid species, *O. simplex* and *O. attenuata*, were abundant throughout the bay, and this agrees with earlier reports of two common cyclopoid species (Table I). The third species, *O. oculata*, was less abundant and it was collected at the northern stations. The presence of three species of cyclopoids was confirmed using the molecular marker, mtCOI.

mtCOI sequence data identified two additional clades among the paracalanids. Both *B. similis* and *P. crassirostris* included one common clade each and a second less common one. The second clades, identified by the letter "O" differed by more than 8% from the primary clades. In Kaneohe Bay, the "O" clades were found in the northern and central regions of the bay, where water exchange with surrounding coastal waters is higher than in the South (Bathen, 1968). The level of genetic divergence between the clades is large enough that they may represent second cryptic species for both *B. similis* and *P. crassirostris*. Within these two genera, there are multiple species, and the "cryptic" clades described here may belong to one of these other congeners. However, given the paucity of genetic information available for subtropical paracalanids, the Kaneohe Bay sequences could not be compared with sequences from these congeners.

The sequences obtained for the copepods from Kaneohe Bay were all unique haplotypes, when compared with mtCOI sequences found in the GenBank database. Only *U. vulgaris* and *P. parvus* sequences were similar enough (<4%) to be considered the same species based on mtCOI similarity. These two species are cosmopolitan and they are found in coastal and oceanic waters in the North Pacific central gyre (McGowan and Walker, 1979; Hannides, 2007). The sequences in GenBank that were most similar to those from Kaneohe Bay were from individuals collected from the western Pacific (*U. vulgaris*: Chinese coastal waters; *P. parvus*: Indonesia). The similarity of the Kaneohe Bay species to published sequences from other regions of the Pacific suggests that there may be connectivity between populations here around Oahu and the western Pacific Ocean; however, a more thorough phylogenetic assessment would be necessary to identify if there are haplotypes that overlap between the two regions.

The other calanoid species from Kaneohe Bay belong to genera that inhabit coastal regions and bays (Lenz, 2012), which are characterized by discontinuous habitats (e.g. Caudill and Bucklin, 2004). Thus, it is not surprising that the sequences for *B. similis* and *P. crassirostris* differed by more than 16.0% from their presumed conspecifics collected from other localities (Palau and China, respectively). The genus *Acartia* has been studied extensively and a large number of sequences from this genus have been deposited in the GenBank database. The mtCOI sequences from Kaneohe Bay differed substantially from all other sequences in this genus, supporting the conclusion that the *Acartia* from Hawaii belongs to a species that is different from those that are currently published (Fig. 3). The situation for *Labidocera* was similar: there were no matches between the sequences from Kaneohe Bay and those from other pontellids (Fig. 5). The *Labidocera* from Hawaii had been identified as being most similar to *L. madurae* (Leis, 1982; Hassett and Boehlert, 1999). However, the Kaneohe Bay sequences could not be compared with those of *L. madurae*, since there were no mtCOI sequences for this species in the GenBank database.

The genus *Oithona* includes many cosmopolitan species based on morphological identification (Nishida, 1985). Members of this genus are often among the most abundant copepods in pelagic habitats (Paffenhöfer, 1993; Nielsen and Sabatini, 1996; Gallienne and Robins, 2001). Many of these species are widespread and this has been confirmed using molecular markers for three Atlantic species (Cepeda *et al.*, 2012). The three *Oithona* species identified for Kaneohe Bay occur along the coasts and estuaries on a number of continents (Omori and Hamner, 1982; Nishida, 1985; Turner, 1986; Böttger-Schnack, 1988; Hopcroft and Roff, 1996; Hsieh and Chiu, 2002). Despite their apparent abundance and widespread distribution, the number of mtCOI sequences available for the genus

Oithona was very small. The mtCOI sequences for the five *Oithona* species (three from this study) were all highly divergent from each other (>20%; Fig. 6). Similar genetic distances were reported between *O. nana* and *Oithona atlantica*, and *O. nana* and *Oithona similis*, but not between *O. atlantica* and *O. similis* using the 28S ribosomal DNA gene as a molecular marker (Cepeda *et al.*, 2012).

The copepod community in Kaneohe Bay is similar to those of other shallow sub-subtropical bays and lagoons associated with coral reefs. It is dominated by small species, which are difficult to identify using taxonomic keys alone. The mtCOI sequence data presented here provide molecular markers that can be used to confirm taxonomic identifications for all life stages. The two common paracalanids, *B. similis* and *P. crassirostris*, were found to be highly divergent from conspecifics from other locations and may thus be different species. In addition, high variability in the mtCOI sequences suggests that there may be additional genetic population structure in these two species. In contrast, single clades were found in the other calanoid and cyclopoid species in the bay.

SUPPLEMENTARY DATA

Supplementary data can be found online at http://plankt.oxfordjournals.org.

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