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DNA barcoding of Arctic Ocean holozooplankton for species identification and recognition

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ABSTRACT

Zooplankton species diversity and distribution are important measures of environmental change in the Arctic Ocean, and may serve as 'rapid-responders' of climate-induced changes in this fragile ecosystem. The scarcity of taxonomists hampers detailed and up-to-date monitoring of these patterns for the rarer and more problematic species. DNA barcodes (short DNA sequences for species recognition and discovery) provide an alternative approach to accurate identification of known species, and can speed routine analysis of zooplankton samples. During 2004–2008, zooplankton samples were collected during cruises to the central Arctic Ocean and Chukchi Sea. A ~700 base-pair region of the mitochondrial cytochrome oxidase I (mtCOI) gene was amplified and sequenced for 82 identified specimens of 41 species, including cnidarians (six hydrozoans, one scyphozoan), arthropod crustaceans (five amphipods, 24 copepods, one decapod, and one euphausiid); two chaetognaths; and one nemertean. Phylogenetic analysis used the Neighbor-Joining algorithm with Kimura-2-Parameter (K-2-P) distances, with 1000-fold bootstrapping. K-2-P genetic distances between individuals of the same species ranged from 0.0 to 0.2; genetic distances between species ranged widely from 0.1 to 0.7. The mtCOI gene tree showed monophyly (at 100% bootstrap value) for each of the 26 species for which more than one individual was analyzed. Of seven genera for which more than one species was analyzed, four were shown to be monophyletic; three genera were not resolved. At higher taxonomic levels, only the crustacean order Copepoda was resolved, with bootstrap value of 83%. The mtCOI barcodes accurately discriminated and identified known species of 10 taxonomic groups of Arctic Ocean holozooplankton. A comprehensive DNA barcode database for the estimated 300 described species of Arctic holozooplankton will allow rapid assessment of species diversity and distribution in this climate-vulnerable ocean ecosystem.

1. Introduction

1.1. Species diversity of holozooplankton

The marine zooplankton assemblage is both systematically diverse and taxonomically challenging. With more than 7000 species of 15 different phyla (Boltovskoy et al., 2002), routine characterization of the diversity, distribution, and abundance in oceanographic collections will require considerable time and effort. In addition, the holozooplankton are comprised of many groups, including the calanoid copepods, for which species identification is further complicated by the presence of numerous sibling species groups that can be difficult or impossible to distinguish using morphological characters (e.g., Frost and Fleminger, 1968; Frost, 1974, 1989), especially for larval or juvenile stages.

Approximately 300 species of holoplanktonic zooplankton have been recorded for the Arctic (Sirenko, 2001), excluding the wider variety of meroplanktonic larvae occurring particularly within the shallow waters of the marginal seas. The greatest diversity of Arctic holozooplankton occurs within the copepods (~150 species), which dominate the zooplankton community in both abundance and biomass (Kosobokova and Hopcroft, this issue); consequently this is the best-known group. Cnidarians are represented by ~50 species with prominent planktonic life

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phases. Mysids contribute ~30 species, most of which are epibenthic. Other groups are each represented by fewer than a dozen described species.

1.2. The Arctic Ocean environment

The Arctic Ocean is unique not only due to its permanent and seasonal ice cover, but with respect to its limited connection to other oceans. The exchange of deep-water biota between the Arctic deep-sea and the world's deep oceans is restricted, with little dispersal via the Bering Strait for the Pacific basin and limited dispersal via the Fram Strait for the Atlantic basin (e.g. Carmack and Wassmann, 2006). The Canada Basin remains further isolated from the Arctic's European basins by the Lomonosov and Alpha-Mendeleyev Ridges. Extreme environmental conditions and geographic barriers to exchange with the adjacent ocean regions have resulted in a zooplankton assemblage of species that is endemic to the Arctic Ocean and uniquely adapted to cold temperatures (Smith and Schnack-Schiel, 1990; Kosobokova and Hirche, 2000; Deibel and Daly, 2007), yet closely related to species found in the Atlantic and Pacific Oceans (e.g. Frost, 1974, 1989). Seasonally, planktonic fauna of the Barents and Chukchi Seas can become dominated by such related expatriates from the adjoining ocean regions (Olli et al., 2007; Hopcroft and Kosobokova, this issue).

Recent and projected changes in the extent and timing of the ice cover in the Arctic are expected to have profound impact on arctic marine ecosystems (ACIA, 2004). These effects will be pronounced within the zooplankton communities where seasonal life cycles are intricately coupled to the timing of ice-breakup and phytoplankton blooms (Smith and Schnack-Schiel, 1990; Deibel and Daly, 2007). Changes in the phenology of species, as well as overall productivity, can be anticipated with effects cascading to even the highest trophic levels (Edwards and Richardson, 2004; Richardson and Schoeman, 2004). We may also anticipate shifts of the biogeographic boundaries of individual species or entire zooplankton communities—whether subtle and progressive or as distinct regime shifts (e.g., Beaugrand, 2004; Hooff and Peterson, 2006). Although some of these changes in community composition are obvious, some may involve only the substitution of one species by another closely related one (Beaugrand, 2004; Hooff and Peterson, 2006).

Obtaining a comprehensive estimate of holozooplankton species diversity of the Arctic Ocean is made more difficult by the rather usual complications of taxonomic analysis by many experts working quite independently. Species names have changed over the past half century, resulting from taxonomic revisions that, in many cases, have split species (e.g., Jaschnov, 1955; Frost, 1974, 1989; Damkaer, 1975; Miller, 1988). Furthermore, both coastal and deep-water species have been frequently grouped or misidentified (see discussion in Hopcroft and Kosobokova, this issue). Despite these difficulties, it is imperative that taxonomic identification occurs reliably, and to the species level, to resolve long-term community changes likely to be associated with long-term changes in the climate of Arctic regions.

1.3. DNA barcoding of marine holozooplankton

DNA barcodes (i.e., short DNA sequences for species recognition and discovery) can provide an alternative or ancillary means of identifying known species. To date, the barcode region of choice for animals is a 690 base-pair region of mitochondrial cytochrome oxidase I (mtCOI; Hebert et al., 2003; Stoeckle and Hebert, 2008). This gene region has been shown to discriminate species throughout the animal kingdom, including birds (Hebert et al., 2004), fish (Ward et al., 2005), and invertebrates (e.g., Schander and Willassen, 2005).

The marine holozooplankton assemblage provides an excellent opportunity to further examine the validity and usefulness of mtCOI as a universal DNA barcode for animal taxa. The taxonomic diversity of the Arctic holozooplankton assemblage—with species of as many as 15 phyla occurring in a single sample—allows demonstration of the feasibility of using DNA barcodes to accurately identify species of animals ranging from cnidarians to urochordates. The taxonomic complexity of many holozooplankton orders, e.g., sibling species of the copepod genus *Calanus* and other copepods, makes such integrated morphological and molecular systematic analysis particularly useful.

Use of DNA barcodes will allow accelerated analysis of species diversity in the Arctic pelagic realm, once a barcode library has been established, and can help ensure timely recognition of shifts in species composition, richness, and biogeographical distributions associated with environmental variability and climate change. Population genetic and phylogeographic analysis using mtCOI can reveal ecologically and taxonomically significant geographic variation within widespread marine species (Knowlton, 2000). Numerous published studies have used the mtCOI gene region most frequently identified as the "barcode region" to characterize holozooplankton species diversity, phylogeography, and phylogeny (e.g., Bucklin et al., 1997, 1998, 2003, 2007; Holland et al., 2004; Peijnenburg et al., 2004; Ünal et al., 2006; Bucklin and Frost, 2009).

This study represents initial progress toward a comprehensive DNA barcode database for Arctic Ocean holozooplankton. We report DNA barcode data for 82 individuals of 41 species representing ~15% of the known diversity for these groups in the region. The study was carried out through a partnership between two Census of Marine Life ocean realm field projects: the Census of Marine Zooplankton (CMarZ; www.CMarZ.org), which seeks to complete a taxonomically comprehensive, global-scale survey of holozooplankton species diversity entailing integrated morphological and molecular analysis, and the Census of Arctic Ocean Diversity (ArcOD; www.arcodiv.org), which is consolidating existing and new knowledge to understand patterns of biodiversity within the major realms of the Arctic Ocean and its marginal seas.

2. Methods

2.1. Collection of zooplankton for molecular analysis

Specimens were obtained from broadly distributed oceanographic research cruises in the Arctic region between 2003 and 2008. The primary expeditions sampled the Chukchi Sea (RUSALCA) during August 2004; and central Arctic Ocean during July 2005 (HLY 05-02) and August–September, 2007 (ARK-XXII/2). Station coordinates are given in Table 1 and shown in Fig. 1. In many cases, shallow water collections were made with 0.5 m diameter ring nets of 64 or 200 μm mesh. Deeper-water samples were collected from known strata using a Hydrobios Midi (0.25 m^2 mouth) Multinet, with hydrographic and geo-referencing data available for all stations. Zooplankton samples were examined under a dissecting microscope immediately following collection, and living representatives of a selection of the species present were removed for later barcoding. The bulk sample was then preserved in 5% formalin. In order to preserve the DNA, care was taken to avoid heating the sample and minimize the duration of light exposure. Specimens were identified to species using diagnostic morphological characters for each group. Identification was done for the Arctic Ocean (HLY 05-02), and

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Table 1
Species of Arctic holozooplankton analyzed for this study, with specimen voucher numbers, collection information, and GenBank accession numbers.

No.	*Group* *Genus and species*	Voucher no.	Cruise	Date	Station	Latitude (N)	Longitude	GenBank Acc. no.
	Amphipoda							
1	*Cyclocaris guilelmi*	Am37.1.1	Healy 05-02	9-Jul-05	7	74.428	151.733°W	FJ602460
	Cyclocaris guilelmi	Am37.1.2	Healy 05-02	9-Jul-05	7	74.428	151.733°W	FJ602461
2	*Cyphocaris bouvieri*	Am44.1.1	Healy 05-02	30-Jun-05	3	72.368	155.224°W	FJ602469
	Cyphocaris bouvieri	Am44.1.2	Healy 05-02	30-Jun-05	3	72.368	155.224°W	FJ602470
3	*Eusirus holmi*	Am38.1.2	Healy 05-02	14-Jul-05	9	75.252	155.907°W	FJ602462
	Eusirus holmi	Am38.2.1	ARK-XXII/2	26-Aug-07	307	86.289	94.441°E	FJ602463
	Eusirus holmi	Am38.3.2	ARK-XXII/2	22-Sep-07	400	77.366	123.422°E	FJ602464
	Eusirus holmi	Am42.1.1	Healy 05-02	14-Jul-05	9	75.252	155.907°W	FJ602468
4	*Hyperoche medusarum*	Am76.1.1	BLF0802	2-Aug-08	KF19	71.022	164.874°W	FJ602471
5	*Themisto libellula*	Am40.3.1	ARK-XXII/2	21-Aug-07	290	82.568	86.462°E	FJ602465
	Themisto libellula	Am40.2.1	Healy 05-02	10-Jul-05	8	74.578	151.935°W	FJ602466
	Themisto libellula	Am40.2.2	Healy 05-02	10-Jul-05	8	74.578	151.935°W	FJ602467
	Copepoda							
1	*Aetideopsis minor*	Co149.2.1	ARK-XXII/2	8-Aug-07	260	84.497	36.079°E	FJ602480
	Aetideopsis minor	Co149.2.2	ARK-XXII/2	8-Aug-07	260	84.497	36.079°E	FJ602481
	Aetideopsis minor	Co149.2.3	ARK-XXII/2	8-Aug-07	260	84.497	36.079°E	FJ602482
2	*Aetideopsis rostrata*	Co154.2.1	ARK-XXII/2	14-Aug-07	268	82.809	61.022°E	FJ602483
	Aetideopsis rostrata	Co154.2.2	ARK-XXII/2	14-Aug-07	268	82.809	61.022°E	FJ602484
	Aetideopsis rostrata	Co154.2.3	ARK-XXII/2	14-Aug-07	268	82.809	61.022°E	FJ602485
3	*Augaptilus glacialis*	Co282.1.1	ARK-XXII/2	8-Aug-07	260	84.497	36.079°E	FJ602513
4	*Calanus glacialis*	Co147.4.1	ARK-XXII/2	21-Aug-07	290	82.568	86.462°E	FJ602479
5	*Calanus hyperboreus*	Co220.2.1	ARK-XXII/2	21-Sep-07	389	78.356	124.545°E	FJ602504
6	*Centropages abdominalis*	Co394.3.1	BLF0802	2-Aug-08	KF15	70.897	164.494°W	FJ602518
7	*Gaetanus brevispinus*	Co158.2.1	ARK-XXII/2	14-Aug-07	268	82.809	61.022°E	FJ602492
	Gaetanus brevispinus	Co158.2.2	ARK-XXII/2	14-Aug-07	268	82.809	61.022°E	FJ602493
	Gaetanus brevispinus	Co158.2.3	ARK-XXII/2	14-Aug-07	268	82.809	61.022°E	FJ602494
8	*Gaetanus tenuispinus*	Co144.4.1	ARK-XXII/2	25-Aug-07	301	84.536	90.097°E	FJ602477
9	*Heterorhabdus compactus*	Co157.2.1	ARK-XXII/2	14-Aug-07	268	82.809	61.022°E	FJ602490
	Heterorhabdus compactus	Co157.2.3	ARK-XXII/2	14-Aug-07	268	82.809	61.022°E	FJ602491
10	*Heterorhabdus norvegicus*	Co182.2.1	ARK-XXII/2	14-Aug-07	268	82.809	61.022°E	FJ602500
11	*Hyalopontius typicus*	Co278.1.1	ARK-XXII/2	25-Aug-07	301	84.536	90.097°E	FJ602509
12	*Lucicutia anomala*	Co065.2.1	ARK-XXII/2	26-Aug-07	307	86.289	94.441°E	FJ602476
13	*Lucicutia polaris*	Co160.1.1	Healy 05-02	3-Jul-05	5	73.367	153.683°W	FJ602496
	Lucicutia polaris	Co160.1.3	Healy 05-02	3-Jul-05	5	73.367	153.683°W	FJ602497
	Lucicutia polaris	Co160.2.1	ARK-XXII/2	8-Aug-07	260	84.497	36.079°E	FJ602498
	Lucicutia polaris	Co160.2.3	ARK-XXII/2	8-Aug-07	260	84.497	36.079°E	FJ602499
14	*Lucicutia pseudopolaris*	Co159.2.1	ARK-XXII/2	25-Aug-07	301	84.536	90.097°E	FJ602495
15	*Metridia longa*	Co188.4.2	ARK-XXII/2	29-Aug-07	312	88.141	119.978°E	FJ602501
16	*Mimocalanus damkaeri*	Co277.1.1	ARK-XXII/2	26-Aug-07	307	86.289	94.441°E	FJ602508
17	*Neocalanus cristatus*	Co227.2.1	BLF0802	17-Aug-08	KF01	70.646	166.003°W	FJ602505
	Neocalanus cristatus	Co227.2.2	BLF0802	17-Aug-08	KF01	70.646	166.003°W	FJ602506
	Neocalanus cristatus	Co227.2.3	BLF0802	17-Aug-08	KF01	70.646	166.003°W	FJ602507
18	*Paraeuchaeta glacialis*	Co146.2.1	ARK-XXII/2	21-Aug-07	285	82.133	86.412°E	FJ602478
19	*Paraeuchaeta norvegica*	Co196.2.1	ARK-XXII/2	14-Aug-07	268	82.809	61.022°E	FJ602502
	Paraeuchaeta norvegica	Co196.2.3	ARK-XXII/2	14-Aug-07	268	82.809	61.022°E	FJ602503
20	*Scaphocalanus acrocephalus*	Co155.1.2	Healy 05-02	28-Jun-05	2	72.317	155.783°W	FJ602486
	Scaphocalanus acrocephalus	Co155.2.2	ARK-XXII/2	12-Aug-07	268	82.809	61.022°E	FJ602487
	Scaphocalanus acrocephalus	Co155.2.4	ARK-XXII/2	12-Aug-07	268	82.809	61.022°E	FJ602488
21	*Scaphocalanus brevicornis*	Co284.1.1	ARK-XXII/2	2-Sep-07	397	77.631	123.621°E	FJ602514
	Scaphocalanus brevicornis	Co284.1.4	ARK-XXII/2	2-Sep-07	397	77.631	123.621°E	FJ602515
22	*Scaphocalanus polari*	Co156.2.1	ARK-XXII/2	25-Aug-07	301	84.536	90.097°E	FJ602489
23	*Scolecithricella minor*	Co290.1.3	ARK-XXII/2	22-Sep-07	397	77.631	123.621°E	FJ602516
	Scolecithricella minor	Co290.1.4	ARK-XXII/2	22-Sep-07	397	77.631	123.621°E	FJ602517
24	*Temorites brevis*	Co281.1.1	ARK-XXII/2	14-Aug-07	268	82.809	61.022°E	FJ602510
	Temorites brevis	Co281.1.2	ARK-XXII/2	14-Aug-07	268	82.809	61.022°E	FJ602511
	Temorites brevis	Co281.1.3	ARK-XXII/2	14-Aug-07	268	82.809	61.022°E	FJ602512
	Decapoda							
1	*Hymenodora glacialis*	Cr14.3.3	ARK-XXII/2	21-Aug-07	290	82.568	86.462°E	FJ602519
	Hymenodora glacialis	Cr14.3.4	ARK-XXII/2	21-Aug-07	290	82.568	86.462°E	FJ602520
	Hymenodora glacialis	Cr14.2.3	Healy 05-02	30-Jun-05	5	73.367	153.683°W	FJ602521
	Hymenodora glacialis	Cr14.3.1	ARK-XXII/2	21-Aug-07	290	82.568	86.462°E	FJ602522
	Hymenodora glacialis	Cr14.2.1	Healy 05-02	30-Jun-05	5	73.367	153.683°W	FJ602523
	Hymenodora glacialis	Cr14.2.2	Healy 05-02	30-Jun-05	5	73.367	153.683°W	FJ602524
	Hymenodora glacialis	Cr14.3.2	ARK-XXII/2	21-Aug-07	290	82.568	86.462°E	FJ602525
	Euphausiacea							
1	*Thysanoessa* sp.	Eu55.1.3	ARK-XXII/2	21-Aug-07	290	82.568	86.462°E	FJ602526
	Thysanoessa sp.	Eu55.1.4	ARK-XXII/2	21-Aug-07	290	82.568	86.462°E	FJ602527
	Chaetognatha							
1	*Eukrohnia hamata*	Ch19.1.1	Healy 05-02	29-Jun-05	3	72.368	155.224°W	FJ602472
	Eukrohnia hamata	Ch19.4.1	Healy 05-02	29-Jun-05	3	72.368	155.224°W	FJ602473

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Table 1 (*continued*)

No.	*Group* *Genus and species*	Voucher no.	Cruise	Date	Station	Latitude (N)	Longitude	GenBank Acc. no.
2	*Heterokrohnia* sp.	Ch26.1.1	ARK-XXII/2	8-Aug-07	260	84.497	36.079°E	FJ602474
	Heterokrohnia sp.	Ch26.1.2	ARK-XXII/2	8-Aug-07	260	84.497	36.079°E	FJ602475
	Hydrozoa							
1	*Aeginopsis laurentii*	Hy109.1	Healy 05-02	15-Jul-05	10	75.777	158.527°W	FJ602536
2	*Aglantha digitale*	Hy106.1.4	ARK-XXII/2	21-Aug-07	290	82.568	86.462°E	FJ602534
	Aglantha digitale	Hy106.2.11	RUSALCA	24-Aug-04	107	70.889	187.324°W	FJ602535
3	*Botrynema brucei*	Hy07.2.3	ARK-XXII/2	3-Sep-07	328	87.816	169.714°E	FJ602530
4	*Euphysa flammea*	Hy111.1.1	RUSALCA	15-Aug-04	23	68.520	188.540°W	FJ602537
	Euphysa flammea	Hy111.1.3	RUSALCA	15-Aug-04	23	68.520	188.540°W	FJ602538
	Euphysa flammea	Hy111.2.1	BLF0802	4-Aug-08	BF03	71.113	163.035°W	FJ602539
5	*Paragotoea bathybia*	Hy104.1.1	ARK-XXII/2	26-Aug-07	307	86.289	94.441°E	FJ602531
	Paragotoea bathybia	Hy104.1.2	ARK-XXII/2	26-Aug-07	307	86.289	94.441°E	FJ602532
	Paragotoea bathybia	Hy104.1.3	ARK-XXII/2	26-Aug-07	307	86.289	94.441°E	FJ602533
6	*Rathkea octopunctata*	Hy112.1.1	RUSALCA	20-Aug-04	89	72.284	183.284°W	FJ602540
	Rathkea octopunctata	Hy112.1.3	RUSALCA	20-Aug-04	89	72.284	183.284°W	FJ602541
	Scyphozoa							
1	*Chrysaora melanaster*	Sc10.1	RUSALCA	20-Aug-04	79	72.047	185.241°W	FJ602545
	Nemertea							
1	*Dinonemertes arctica*	Ne03.1.1	Healy 05-02	24-Jul-05	15	72.863	156.973°W	FJ602542

Cruise names are explained in the text. Latitude is given as decimal degrees North; longitude is decimal degrees East or West.

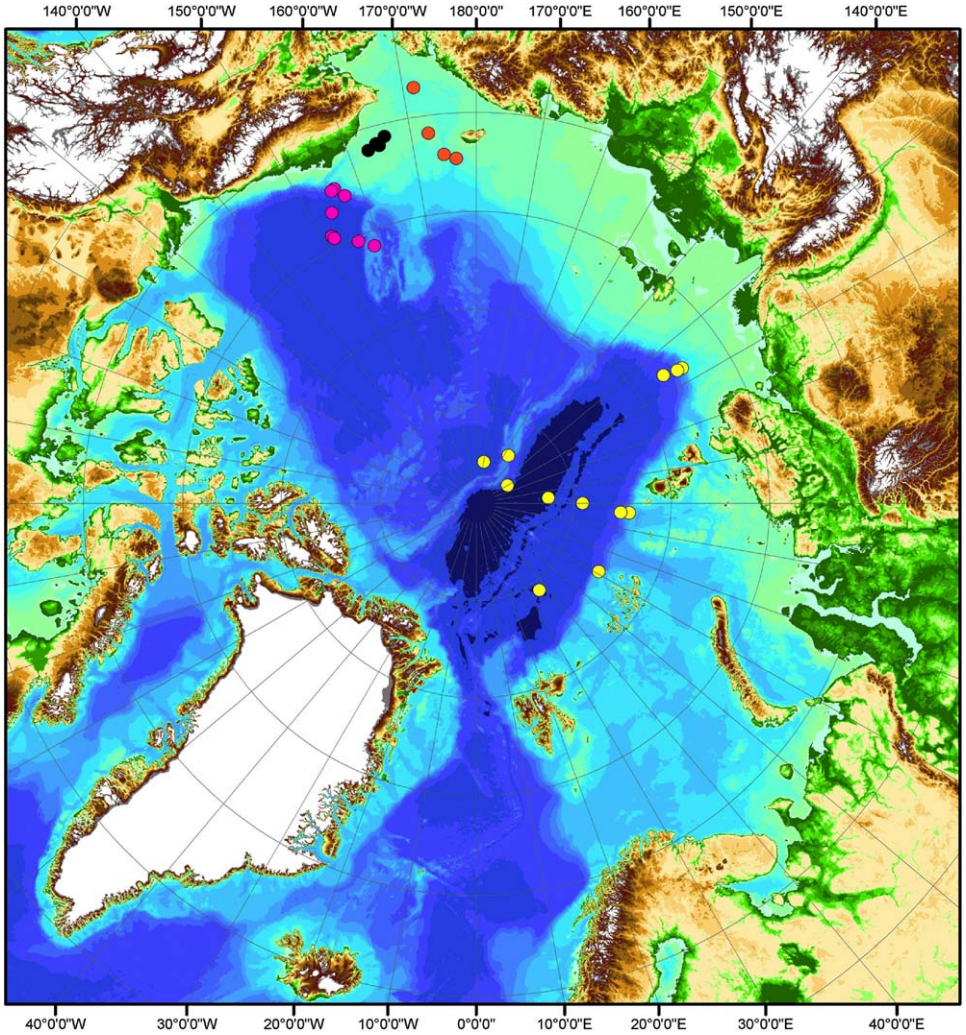

Fig. 1. Regions sampled and collection locations for samples analyzed for this study. The map shows the activities of several different expeditions: Chukchi Sea (RUSALCA) from August 2004 (orange-red dots) and Arctic basin cruises of the USCGC *Healy* (HLY 05-02) during July 2005 (pink dots) and FS *Polarstern* (ARK-XXII/2) during August–September, 2007 (yellow dots), *Blue Fin* August 2008 (black dots). Station coordinates are given in Table 1.

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Chukchi Sea (RUSCALA) jointly by KNK and RRH; for the 2007 *Polarstern* cruise (ARK-XXII/2) by KNK; and for minor cruises by RRH. Identified specimens for molecular analysis were placed in labeled vials, and preserved immediately in 95% ethyl alcohol; the alcohol was changed once 24 h after collection. Detailed preservation protocols are as previously published (Bucklin, 2000). Identified specimens were shipped to the University of Connecticut for molecular analysis and DNA barcoding.

Additional taxonomic and environmental information for the FS *Polarstern* cruise (ARK-XXII/2) is available from the Alfred Wegner Institute for Polar and Marine Science. Additional zooplankton species diversity, pelagic community composition, and environmental information for other sampled regions is available in related publications (Hopcroft and Kosobokova, this issue; Kosobokova and Hopcroft, this issue).

2.2. MtCOI sequence analysis

DNA was purified from individual specimens by phenol extraction and ethanol precipitation (Bucklin, 2000) or using DNA purification kits (DNeasy, Qiagen). A 708 base-pair region of mtCOI was amplified in a GeneAmp 9600 PCR machine (Applied Biosystems, Inc.). Various PCR primers were used, including consensus primers made according to published sequences (Folmer et al., 1994):

LCO-1490 5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′
HCO-2198 5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′

The PCR protocol was 94 °C for 1 min, 45 °C for 2 min, and 72 °C for 3 min, for 40 cycles.

DNA sequencing was done by direct sequencing of PCR amplification products, according to published protocols (Bucklin, 2000). For copepod species that did not work with LCO-1490 and HCO-2198, a different reverse primer was designed and used to obtain barcode sequences:

HCO-Co-2358 5′-CCH ACD GTA AAY ATR TGR TG-3′

The non-standard DNA base designations indicate mixtures of two or more nucleotide bases, to facilitate primer annealing to DNA templates with variable bases at those sites. The PCR reaction protocol was 95 °C for 3 min; then 95 °C for 45 s, 45 °C for 1 min, 72 °C for 1.5 min for 40 cycles; then a final extension of 72 °C for 3 min. For cnidarian species that did not work with LCO-1490 and HCO-2198, an additional reverse primer was designed and used to obtain barcode sequences (Ortman, 2008):

HCO-Med-2414 5′-GGA ACT GCT ATA ATC ATA GTT GC-3′

For species of diverse groups that did not amplify with LCO-1490 and HCO-2198, another alternative reverse consensus primer was designed and used to obtain the barcode sequence (Bucklin et al., unpublished):

HCO-2607 5′-ACA TAG TGG AAA TGT GCT ACA ACA TA-3′

The PCR protocol using these primers was 94 °C for 1 min, 45 °C for 2 min, and 72 °C for 3 min, for 40 cycles.

DNA sequencing was carried out on an American Biotechnology, Inc. (ABI) Prism 3100 4-capillary Genetic Analyzer. Sequences were obtained from the forward and/or reverse primer; bidirectional sequences were obtained when deemed necessary to confirm the sequence. One individual was sequenced for each of 17 species; 15 species were characterized using two individuals; the remaining species were sequenced for three or more individuals. Two or three different PCR and sequencing reactions were done when only one specimen of a given species was available for analysis. All sequences were manually checked for accurate machine reading.

All mtCOI sequences were aligned using CLUSTAL X (Thompson et al., 1997). The complete alignment was trimmed to a length of 650 base-pairs for further analysis. Multiple alignments, calculation of percent pairwise differences, and GenBank searches were done to confirm the accuracy and validity of the sequences, detect artefactual sequences and pseudogenes, and recognize errors in species identification. The nucleotide sequences were further checked by aligning the translated protein (amino acid) sequences, and comparison with consensus protein sequences for this gene region. The mtCOI sequences were submitted to the National Center for Biotechnology Information (NCBI) GenBank database using the BARCODE submission portal. The GenBank record includes the nucleotide sequences in text format, conceptual translations to protein (amino acid) sequences, specimen voucher number, collection date, geospatial coordinates of the collection site, PCR primer names and sequences, and names of the persons collecting and identifying the specimens. GenBank Accession Numbers are provided in Table 1.

Specimens not consumed in the molecular analysis were assigned a voucher number. Voucher numbers for small organisms that were used entirely for analysis were assigned to specimens that were collected in the same sample and identified by the same person. Specimen vouchers are stored permanently in the lead author's laboratory in 95% ethyl alcohol at −20 °C. The person responsible for identifying and providing the specimen for barcoding also frequently retains additional specimens preserved in formalin. DNA vouchers are kept for all barcoded specimens, assigned the same voucher number, and stored in an ultra cold (−80 °C) freezer in the lead author's laboratory. All information associated with each barcode is maintained in an ACCESS specimen tracking database. Most information is also part of the GenBank record and can be obtained using GenBank accession numbers (Table 1).

The mtCOI sequences were analyzed and compared using the Neighbor-Joining (NJ) algorithm of the Molecular Evolutionary Genetics Analysis (MEGA, Version 4) software package (Kumar et al., 2001). Kimura-2-Parameter (K-2-P) distances were used for the tree reconstruction; the tree was bootstrapped using 1000 subreplicates. The cluster analysis was shown with a radial tree topology, with bootstrap results (shown as percentages of 1000 subreplicates) at nodes supported by greater than 80% values. K-2-P distances were calculated for pairwise comparisons between individuals of the same species, individuals of different species of copepods, and individuals of different taxonomic groups. Frequency distributions of K-2-P distances were statistically compared using two-sample *t*-tests for distributions with unequal variances.

3. Results

DNA sequences for the barcode region of mitochondrial cytochrome oxidase I (mtCOI) were obtained for 82 specimens of 41 species, including cnidarians (six hydrozoans, one scyphozoan), arthropod crustaceans (five amphipods, 24 copepods, one decapod, and one euphausiid); two chaetognaths; and one nemertean (Table 1).

K-2-P genetic distances for pairwise comparisons across the entire dataset ranged widely from 0.0 to 0.7 with a mean of 0.3867 (Table 2). Between individuals of the same species, K-2-P distances ranged from 0.0 to 0.2 with a mean of 0.0075; between

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different species of copepods distances ranged from 0.1 to 0.4 with a mean of 0.2705; and between species of different groups, distances ranged from 0.2 to 0.7 with a mean of 0.4441 (Table 2). The frequency distribution of within-species K-2-P distances was non-overlapping with differences between species (Fig. 2), and differed significantly from those between species of copepods by two-sample *t*-tests ($t = -82.4$, $p < 0.0001$) and between species of different groups ($t = -144.5$, $p < 0.0001$). Distances between species of copepods and species of different groups also differed significantly ($t = -65.5$, $p < 0.0001$).

Phylogenetic analysis of mtCOI barcode sequences by Neighbor-Joining yielded an unrooted tree, displayed in radial format (Fig. 3), confirmed the monophyly of individuals of the same species (100% bootstrap value) for all species for which multiple specimens were sequenced (Fig. 3). Sequence divergence was exceptionally large between barcodes for individuals of the hydrozoan *Aglantha digitale* (K-2-P = 0.046). Another pair of sequences tentatively identified as the euphausiid *Thysanoessa* sp. were exceptionally divergent for individuals of the same species (K-2-P = 0.146); neither barcode matched any *Thysanoessa* or euphausiid species currently in the GenBank database, and these sequences were not considered to be conspecific for the statistical analysis of sequence differences.

Of six genera for which more than one species was analyzed, two were shown to be monophyletic, including the copepods *Aetideopsis minor* and *A. rostrata*; and *Paraeuchaeta glacialis* and *P. norvegica*. Species of the other four genera were not resolved, including the copepods *Calanus glacialis* and *C. hyperboreus*; *Gaetanus brevispinus* and *G. tenuicornis*; *Lucicutia anomala*, *L. polaris* and *L. pseudopolaris*; and *Scaphocalanus acrocephalus*, *S. brevicornis*, and *S. polaris*.

At higher taxonomic levels, the crustacean Order Copepoda was resolved with a bootstrap value of 83% and the Phylum Cnidaria was resolved with a bootstrap value of 90% (Fig. 3).

Table 2
Kimura-2-Parameter genetic distances for pairwise comparisons between mtCOI sequences for species of Arctic Ocean holozooplankton.

	All **pairs**	Within **species**	Between **copepods**	Between **groups**
Mean	**0.3867**	**0.0075**	**0.2705**	**0.4441**
S.D.	**0.1206**	**0.0188**	**0.0528**	**0.0781**
Median	**0.3940**	**0.0020**	**0.2710**	**0.4340**

Mean, standard deviation (S.D.) and median values are given for all pairwise comparisons (all pairs), comparisons between individuals of the same species (within species), different species of copepods (between copepods), and between species of different taxonomic groups (between groups). See text for explanation.

4. Discussion

Knowledge of zooplankton species composition is fundamental to understanding ocean ecosystem dynamics and health. Holozooplankton occupy a pivotal position in ocean food webs, and patterns of zooplankton abundance and diversity may determine recruitment patterns and success of many commercially harvested fisheries (Cushing, 1995). Zooplankton may also be considered to be short-lived small-bodied 'rapid-responders' for climate change (Richardson, 2008), resulting in shifting patterns of diversity, distribution, and abundance that can be documented only with analysis of high-resolution sampling on both temporal and spatial scales.

Despite the importance of understanding patterns of species diversity of holozooplankton, accurate and timely access to this information may be difficult or impossible for Arctic ocean regions. Primary reasons for this difficulty are the increasing scarcity of expert taxonomists and the under-sampling of polar ocean domains.

New approaches to accelerate the taxonomic analysis of zooplankton samples are critically needed, and DNA barcoding provides a number of particular advantages and opportunities. The Arctic zooplankton assemblage represents an unusual opportunity to test a community barcoding approach, due to its relatively low species diversity (Sirenko, 2001), while including species that reflect the usual phylogenetic diversity of holozooplankton (Fig. 3). To date, most marine barcoding studies have been designed to

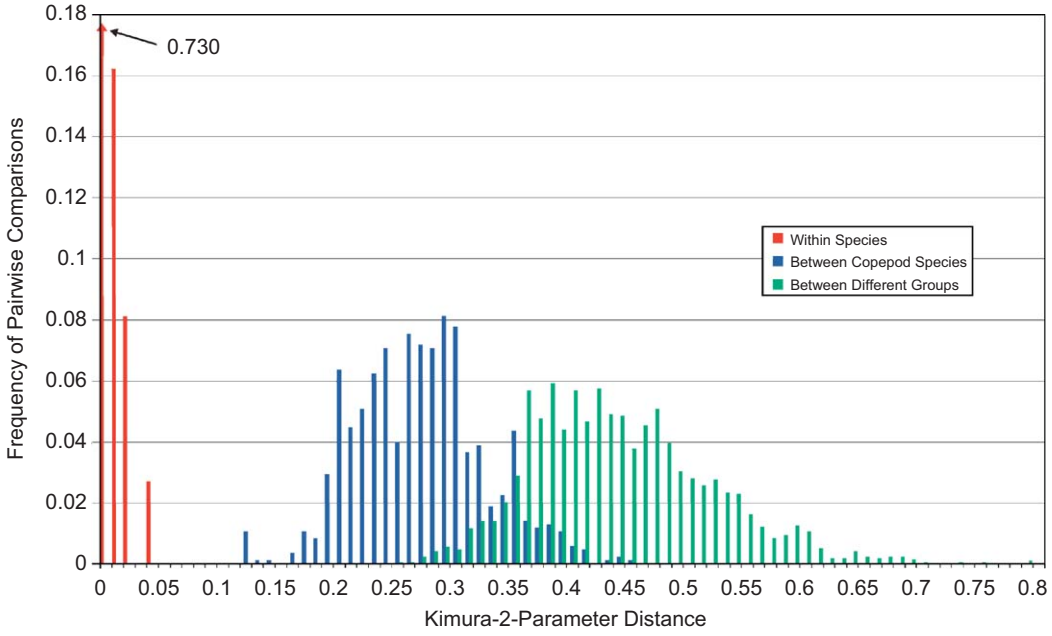

Fig. 2. Frequency distribution of Kimura-2-Parameter (K-2-P) genetic distances for pairwise comparisons within species, between species of copepods, and between species of different groups. Arrow indicates that the first histogram bar (K-2-P distance = 0) is off the scale, with a frequency of 0.730. The distributions differ significantly by *t*-tests of the means (see Table 2 for summary statistics and the text for statistical analysis and explanation).

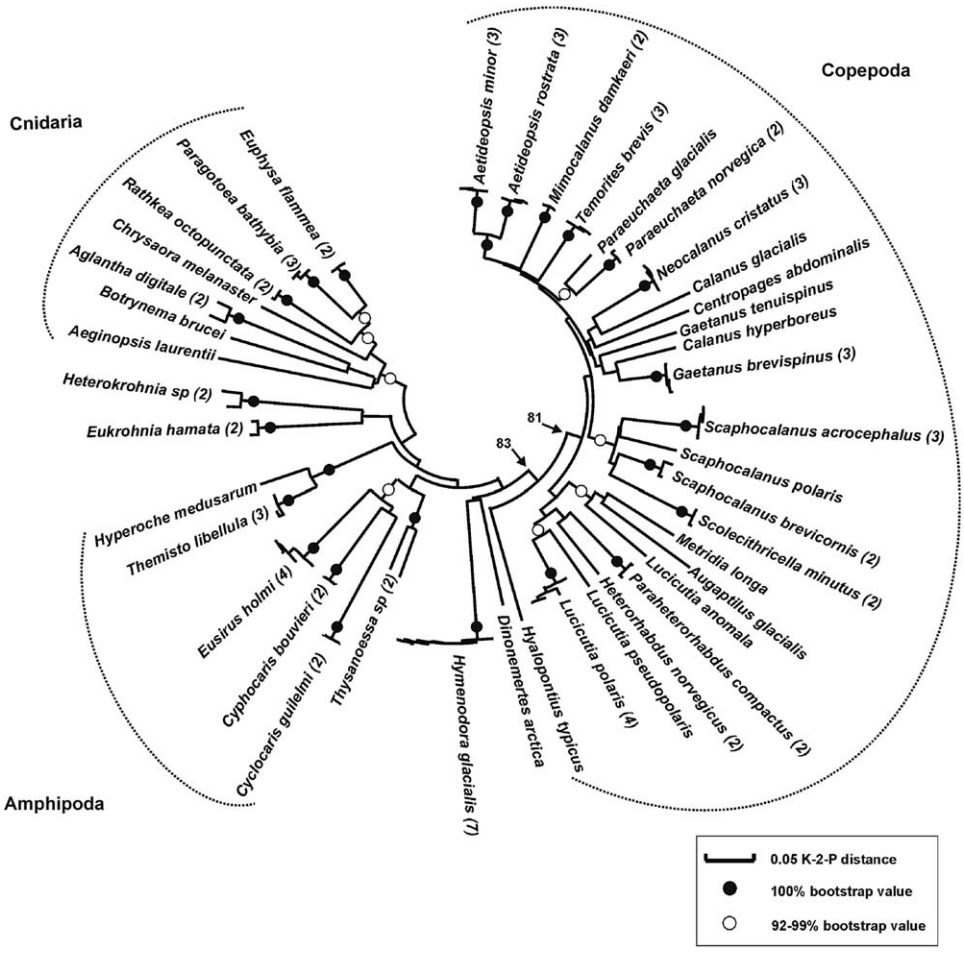

Fig. 3. Unrooted mtCOI gene tree for 82 identified specimens of 41 Arctic holozooplankton species reconstructed by Neighbor-Joining with Kimura-2-Parameter (K-2-P) distances; bootstrapping was done for 1000 sub-replicates. Multiple barcodes for the same species clustered with 100% bootstrap values in all cases; the 24 species of copepods were resolved with an 83% bootstrap value; the seven species of Cnidarians were resolved with a 90% bootstrap value. Number of individuals analyzed for each species is given in parentheses.

discriminate species of a particular taxonomic group or lineage. In contrast, this study uses DNA barcodes to characterize a taxonomically diverse assemblage of organisms inhabiting the pelagic environment of an entire oceanographic province (see Longhurst, 2001) and an entire ocean. For the Arctic Ocean, changes in species abundance, composition and distribution can be anticipated to occur in the near future as climate change alters water temperature, and both the timing and magnitude of productivity cycles (Grebmeier et al., 2006; Bluhm and Gradinger, 2008). The establishment of expatriate species from adjoining regions, and even the introduction of exotic species through increased commercial traffic are likely scenarios for the future Arctic Ocean (ACIA, 2004).

DNA barcoding studies of the diverse groups that comprise the holozooplankton assemblage have confirmed that partial mtCOI sequences allow accurate identification and discrimination of species of copepods (e.g., Bucklin et al., 2003; Bucklin and Frost, 2009); euphausiids (Bucklin et al., 2007); other crustaceans (Lefébure et al., 2006; Costa et al., 2007); gastropod mollusks (Remigio and Hebert, 2003); and cnidarians (Ortman, 2008); among others. The present study supports the use of partial mtCOI sequences for accurate species identification and discrimination. For the 27 species (out of 41) for which more than one individual was sequenced for this study, mtCOI barcodes of the same species were clustered together with a 100% bootstrap value in the gene tree reconstruction (Fig. 3). For six genera for which multiple species were sequenced, mtCOI variation resolved a unique monophyletic lineage for two genera (>90% bootstrap value); another four genera were not resolved (Fig. 3).

The monophyly of species in gene trees reconstructed by Neighbor-Joining is a useful indication of the reliability of the sequence for species identification, but is not the only approach to species identification using barcodes (e.g., Lefébure et al., 2006; Kelly et al., 2007; Rach et al., 2008; Ross et al., 2008; Zhang et al., 2008), and monophyly may not be a necessary condition for the use of DNA sequence for species identification (Knowles and Carstens, 2007). While this does not negate its usefulness for species identification, the mtCOI barcode region is generally considered to have limited usefulness as a character for phylogenetic reconstruction due to its rapidly evolving DNA sequence (Brown et al., 1979; Miyata et al., 1982). However, Machida et al. (2006) resolved phylogenetic relationships among congeneric species of the copepod genus *Neocalanus* using a longer region of mtCOI. And closer examination suggests that mtCOI barcode sequences have some resolving power for some groups at the genus level and above, with resolution of deeper branches dependent upon taxon and character sampling. For crustaceans, Costa et al. (2007) concluded that the mtCOI barcode region was 95% successful in classifying species to the correct order. Remigio and Hebert (2003) found evidence of resolution of

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the recognized subclasses of gastropod molluscs using mtCOI. In this study, all seven species of the Phylum Cnidaria were resolved with a 90% bootstrap value and all 24 species of the crustacean Order Copepoda were resolved with an 83% bootstrap value, although other crustacean orders represented by fewer species (amphipods, euphausiids, and decapods) were not resolved (Fig. 3).

5. Conclusions

Creation of a taxonomically comprehensive DNA barcode database for the known holozooplankton species diversity of the Arctic Ocean will require the coordinated efforts of expert morphological taxonomists and molecular biologists, who together can accurately assign DNA barcodes to identified specimens across the diverse groups found throughout the diverse marine habitats of the region. Once complete, a DNA barcode database for the Arctic Ocean will allow rapid and accurate identification of specimens and characterization of patterns of species distribution and diversity, and eventually near-real-time characterization of species diversity, distribution, and abundance. Comparisons with the growing global holozooplankton barcode database may eventually reveal geographic variation among oceans that is consistent with cryptic species—although there is no evidence for this yet. A caution is that species identifications using DNA barcode sequences will require a comprehensive dataset, including all those species for which identifications are being sought. Numerous studies have confirmed the pitfalls of attempting to use DNA sequences to identify or classify specimens of a species for which a barcode has not been determined. Accelerated reporting of the results from oceanographic research and monitoring cruises is particularly important to facilitate the tracking of climate effects in fragile environments such as the Arctic Ocean.

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