

## Introduction

### New insights into biodiversity, biogeography, ecology, and evolution of marine zooplankton based on molecular approaches

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Note: This article is the Introduction to the ICES JMS Themed Set: Patterns of biodiversity of marine zooplankton based on molecular analysis

Bucklin, A., Peijnenburg, K. T.C.A., Kosobokova, K., and Machida, R. J. New insights into biodiversity, biogeography, ecology, and evolution of marine zooplankton based on molecular approaches. – ICES Journal of Marine Science, 78: 3281–3287.

Received 26 September 2021; revised 26 September 2021; accepted 27 September 2021; advance access publication 19 October 2021.

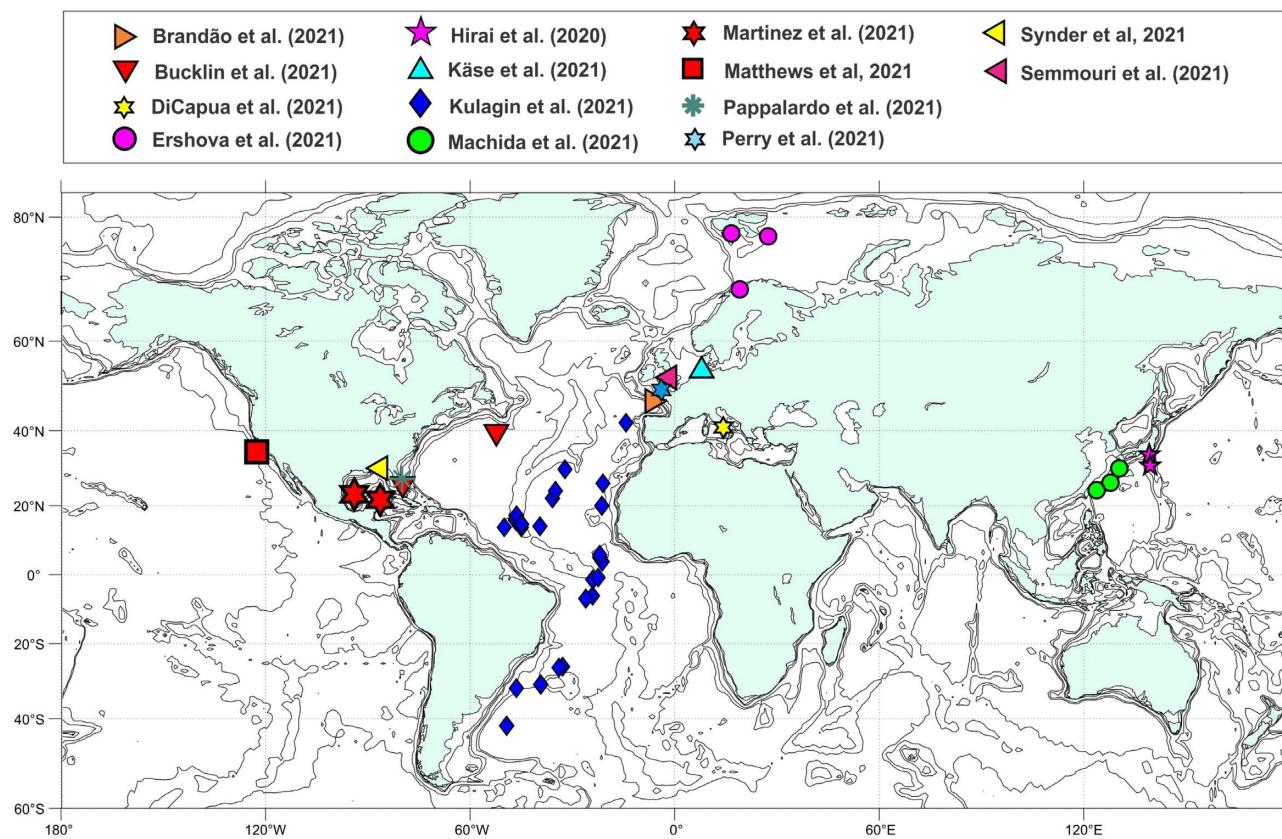
Marine zooplankton are key players in pelagic food webs, central links in ecosystem function, useful indicators of water masses, and rapid responders to environmental variation and climate change. Characterization of biodiversity of the marine zooplankton assemblage is complicated by many factors, including systematic complexity of the assemblage, with numerous rare and cryptic species, and high local-to-global ratios of species diversity. The papers in this themed article set document important advances in molecular protocols and procedures, integration with morphological taxonomic identifications, and quantitative analyses (abundance and biomass). The studies highlight several overarching conclusions and recommendations. A primary issue is the continuing need for morphological taxonomic experts, who can identify species and provide voucher specimens for reference sequence databases, which are essential for biodiversity analyses based on molecular approaches. The power of metabarcoding using multi-gene markers, including both DNA (Deoxyribonucleic Acid) and RNA (Ribonucleic Acid) templates, is demonstrated. An essential goal is the accurate identification of species across all taxonomic groups of marine zooplankton, with particular concern for detection of rare, cryptic, and invasive species. Applications of molecular approaches include analysis of trophic relationships by metabarcoding of gut contents, as well as investigation of the underlying ecological and evolutionary forces driving zooplankton diversity and structure.

**Keywords:** diversity, DNA barcode, metabarcoding, zooplankton.

#### Background and motivation for this themed set

Molecular approaches are revolutionizing the analysis of biodiversity of marine zooplankton (Goodwin *et al.*, 2017; Rey *et al.*, 2020). These approaches have an acknowledged potential to detect the “hidden diversity” (Lindeque *et al.*, 2013; Bucklin *et al.*, 2011; Bucklin *et al.*, 2016) of the assemblage, which is characterized by numerous rare, sibling, and cryptic species. Despite the promise of molecular approaches in yielding new understanding and appreciation for ocean ecosystems, many challenges remain (Cordier *et al.*, 2021). Ensuring accurate and reliable application of molecular approaches for analysis of marine zooplankton bio-

diversity will require continued evaluation and comparison of results using different molecular markers (i.e. target gene regions, as well as different primers and protocols); development of methodologies that can ensure identification, discrimination, and detection of rare and cryptic species across the broad range of zooplankton taxonomic groups; and continued development of taxonomically comprehensive reference databases for all gene regions (Machida *et al.*, 2017; Bucklin *et al.*, 2021a). A particular need is to move molecular approaches from identification and detection of taxa to quantification in terms of (relative or absolute) abundance and/or biomass. It is also important to maintain expertise and capacity in morphological taxonomic identification



**Figure 1.** Collection locations of zooplankton analyzed in the articles included in this TS. Most of the symbols indicate regions where multiple samples were collected for each study; symbols for Kulagin *et al.* (2021) indicate station locations of individual samples.

of species to ensure the validation and ground truthing of new protocols (Pinheiro *et al.*, 2019). Integrative morphological and molecular taxonomic approaches will provide the necessary foundation for future research, monitoring, and management of the pelagic realm.

The objective of this themed article set (TS) is to bring together contributions on the broad theme of assessing the biodiversity of marine zooplankton based on novel molecular approaches. Contributions were encouraged on a variety of topics, including biodiversity, biogeography, and trophic dynamics of marine zooplankton. We sought papers highlighting best practices for barcoding and metabarcoding analysis; describing progress toward completion and use of DNA (Deoxyribonucleic Acid) sequence reference databases; and documenting the discovery, detection, and functional importance of rare and cryptic species. An important goal was to explore and interpret results from novel molecular approaches, including population genomics and metatranscriptomics. Recent progress in the many and various molecular approaches are providing powerful new resources for improved understanding and appreciation of the diversity of marine zooplankton, including progress toward answers for the important and overarching question of the actual number of species in the pelagic assemblage.

## An overview of the contributions in this themed set

The papers included in the TS describe results of research focused on key questions related to marine zooplankton biodiversity. The

studies range widely in taxonomic focus, from characterization of diversity across the pelagic assemblage to specific taxonomic groups or target species. These papers provide an excellent opportunity to understand variation in patterns of pelagic biodiversity across diverse ocean regions (Figure 1). Several studies report progress on continuing methodological developments, including ribonucleic acid (RNA) barcoding, metatranscriptomics, and population genomics. Established techniques, including DNA barcoding and metabarcoding, use a variety of target gene regions and protocols (Table 1).

## Metabarcoding analysis of marine zooplankton biodiversity and biogeography

The TS collection includes papers that use DNA metabarcoding to examine time/space patterns of biodiversity analyzed with varying degrees of taxonomic resolution, from phyla to functional groups to species. Several studies report increasing success in moving beyond presence-absence to reliable semi-quantitative estimations of abundance or biomass.

Brandão *et al.* (2021) reported the results of DNA metabarcoding using mitochondrial cytochrome oxidase I (COI) and the V1–V2 hypervariable regions of 18S ribosomal RNA (rRNA) to examine diversity and structure of meroplanktonic communities in the Iroise Sea (Northeast Atlantic Ocean). Metabarcoding allowed resolution of high heterogeneity in mesozooplankton over small spatial scales, significant regional variation across the continental shelf, and marked temporal variation between seasons, with

**Table 1.** Gene regions used for molecular analysis by the articles in this TS, with citations to articles that provide detailed information on primers and protocols.

TS publications	Gene regions	Primer names	Citations
Brandão <i>et al.</i> (2021)	COI	mlCOlIntF; jgHCO2198	Geller <i>et al.</i> (2013); LeRay <i>et al.</i> (2013)
	V1–V2 18S rRNA	SSU_F04; SSU_R22mod	Fonseca <i>et al.</i> (2010); Sinniger <i>et al.</i> (2016)
Bucklin <i>et al.</i> (2021a)	COI	LCO-1490; HCO-2198	Folmer <i>et al.</i> (1994)
	COI	COI-f; COI-609	Palumbi (1996); Bucklin <i>et al.</i> (2021a)
	CYB	CYB-L14841; CYB-H15149	Kocher <i>et al.</i> (1989)
Di Capua <i>et al.</i> (2021)	V4 18S rRNA	TAReuk454FWD1; TAReukREV3	Stoeck <i>et al.</i> (2010); Piredda <i>et al.</i> (2017)
Ershova <i>et al.</i> (2021)	COI	mlCOlIntF-XT; jgHCO2198	Wangensteen <i>et al.</i> (2018); Geller <i>et al.</i> (2013)
Hirai <i>et al.</i> (2020)	D2 28S rRNA	LSU Cop-D2F; LSU Cop-D2R	Hirai <i>et al.</i> (2017)
Käse <i>et al.</i> (2021)	V4 18S rRNA	528iF; 964iR	Fadeev <i>et al.</i> (2018)
Kulagin <i>et al.</i> (2021)	COI	LCO-1490; HCO-2198	Folmer <i>et al.</i> (1994)
	H3	H3AF; H3AR	Colgan <i>et al.</i> (1998)
Machida <i>et al.</i> (2021)	COI	mlCOlIntF; jgHCO2198	LeRay <i>et al.</i> (2013); Geller <i>et al.</i> (2013)
Martinez <i>et al.</i> (2021)	V9 18S rRNA	1389F; 1510R	Amaral-Zettler <i>et al.</i> (2009)
Matthews <i>et al.</i> (2021)	COI	mlCOlIntF; jgHCO2198	LeRay <i>et al.</i> (2013); Geller <i>et al.</i> (2013)
	V4 18S rRNA	Uni18S; Uni18SR	Zhan <i>et al.</i> (2013)
Pappalardo <i>et al.</i> (2021)	COI	jgLCO1490-F; jgHCO2198	Geller <i>et al.</i> (2013)
	V1–V2 18S rRNA	18s_SSU_F04; 18s_SSU_R22	Blaxter <i>et al.</i> (1998)
Parry <i>et al.</i> (2021)	V1–V2 18S rRNA	SSU_F04; SSU_R22	Fonseca <i>et al.</i> (2010)
Semmouri <i>et al.</i> (2021)	V4–V5 18S rRNA	F-566; R-1200	Hadziavdic <i>et al.</i> (2014)
Snyder <i>et al.</i> (2021)	V4 18S rRNA	384 F; 1147 R	Dopheide <i>et al.</i> (2008)

relative abundances of polychaete and decapod larvae peaking in spring and summer, while copepods predominated in offshore waters (Brandão *et al.*, 2021).

DiCapua *et al.* (2021) used the V4 region of 18S rRNA to analyze marine metazoan diversity in eDNA samples collected from the Gulf of Naples, where time-series collections have been made for over 30 years at a Mediterranean long-term ecological research site. Copepoda predominated in the resulting dataset, with resolution of genera possible based on the marker used and the available reference database. The next most frequent group was Annelida, primarily the invasive benthic polychaete, *Hydroides elegans*. Holozooplankton groups included Siphonophora, Rotifera, and Appendicularia, with occasional mass occurrences of Scyphozoa. Meroplankton showed high diversity, including representatives of 11 phyla, from Porifera to Craniata.

Martinez *et al.* (2021) described epipelagic zooplankton diversity based on metabarcoding with the V9 region of 18S rRNA for samples collected in deep water regions of the Gulf of Mexico during Summer 2015. Fourteen phyla were detected and classified: Arthropoda were dominant, with Calanidae, Metridiinidae, and Euphausiidae the most conspicuous families. Other abundant phyla were Cnidaria (Hydrozoa), Chordata (Tunicata), and Chaetognatha. Metabarcoding suggested the presence of a core community with high local diversity throughout the Gulf of Mexico (GoM). The authors conclude that metabarcoding can provide a solid foundation for broad-scale biodiversity assessments of marine zooplankton, which will allow evaluation of the effects of environmental changes in the zooplankton communities of the GoM.

Parry *et al.* (2021) analyzed V1–V2 regions of 18S rRNA to compare taxonomic richness and composition of samples based on metabarcoding. Vertical net tows were done in the English Channel in the top 50 m of the water column; samples collected by hyperbenthic sledge in the benthic boundary layer (BBL). Major contrasts in plankton composition were related to seasonal cycles, rather than to pelagic-BBL differences. Metabarcoding clearly augmented tra-

ditional methods to examine community dynamics of both the water column and BBL.

### Detection of rare and cryptic species

An important focus of the TS was exploration of novel approaches and applications of DNA metabarcoding of marine zooplankton diversity, including detection of rare and cryptic species and improved taxonomic resolution using custom reference databases. Traditional morphological/microscopic taxonomic analysis of marine zooplankton inevitably underestimates species diversity, due to the prevalence of rare and cryptic species in the pelagic assemblage (Snelgrove *et al.*, 2017). An overarching question that remains is the actual number of species in the marine zooplankton assemblage.

Ershova *et al.* (2021) found 3–4 times higher species richness of zooplankton using COI metabarcoding compared to morphological taxonomic estimates. They concluded that metabarcoding with COI can provide improved taxonomic resolution, universal taxonomic coverage of metazoans, reduced primer bias, and—with the availability of a comprehensive reference database—rapid and relatively inexpensive processing of hundreds of samples at higher taxonomic resolution than morphological taxonomic analysis. They also reported that relative numbers of sequence counts were highly and significantly correlated with relative biomass estimates from morphological analysis (i.e. length-weight regressions) for a wide range of metazoan taxa, with the highest correlations for Crustacea.

Snyder *et al.* (2021) examined the power of metabarcoding to provide accurate estimates of species-level diversity of planktonic Ciliata from the Northeastern Gulf of Mexico, based on specially-designed V4 18S rRNA primers (384F and 1147R). The Ciliata provide an excellent—and challenging—opportunity to discover, detect, and understand the functional importance of rare and cryptic species among microzooplankton; ciliates are extremely challenging to discriminate using traditional methods.

## Trophic dynamics

Molecular approaches, including metabarcoding, are widely used to examine food web dynamics in a wide variety of ocean ecosystems. DNA metabarcoding can detect tiny, rare, and soft-bodied prey in the gut contents of marine zooplankton, that would otherwise be overlooked by morphological (microscopic) analysis.

Käse *et al.* (2021) used an integrative approach, including analysis of water samples and consumer gut contents, to examine trophic relationships of the planktonic community at Helgoland Roads in the North Sea. Using network analysis, they sought to trace predator–prey dynamics and look for evidence of prey selectivity. DNA metabarcoding analysis based on the V4 region of 18S rRNA suggested that flexible food web interactions in the eukaryotic plankton community are more common than specific predator–prey relationships, and that there are strong seasonal and occasionally rapid fluctuations, in some cases driven by blooms of autotrophs. A key finding is that zooplankton predators select specific prey during some time periods, but are opportunistic in terms of prey choice throughout the year.

## Best practices for integrative taxonomic analysis

The concept of integrative molecular–morphological analysis as a “best practice” for characterizing patterns of biodiversity of marine zooplankton is a primary result of several of the papers in this TS. These studies use various molecular approaches in tandem with morphological taxonomic (microscopic)—and other—analyses, resulting in new appreciation and insights into this fast-moving field.

Matthews *et al.* (2021) studied impacts of key protocol options for metabarcoding zooplankton samples collected during the California Cooperative Fisheries Investigations monitoring program in the California Current Ecosystem. Community composition was resolved by metabarcoding with COI and V4 18S rRNA, and compared with digital imaging of samples, providing evidence of significant relationships between relative sequence numbers (read abundances) and proportions of carbon biomass, with better agreement for the more dominant taxonomic groups. Findings for protocol optimization included the Polymerase Chain Reaction (PCR) replication and careful subsampling to avoid decreased sensitivity to rare taxa; pH neutralization had no effect. A primary conclusion was the best practice recommendation for paired metabarcoding and morphological analyses for characterization of zooplankton community structure and biomass.

Pappalardo *et al.* (2021) examined zooplankton diversity of the Florida Current in the Gulf Stream System of the North Atlantic Ocean using COI and V1–V2 18S rRNA metabarcoding. They also emphasized the continuing need for morphological taxonomic experts, who can identify specimens for DNA sequencing of the target gene regions for essential improvements to reference databases. Evaluation of morphological variation among geographic populations is essential for evaluating the taxonomic significance of genetic differentiation existence and the potential for cryptic species.

Semmouri *et al.* (2021) examined marine zooplankton communities of the North Sea using integrative molecular (metabarcoding) and morphological approaches. The metabarcoding gene region was the V4–V5 region of 18S rRNA, with long-read sequencing (Oxford Nanopore, Cambridge, UK) that allowed species-level resolution for some taxonomic groups. Comparative analysis revealed no significant correlation between diversity indices based

on molecular versus morphological approaches, most likely because not all taxonomic groups were successfully sequenced, and disparities existed between relative abundances based on counts and those estimated from sequence reads. The authors concluded by stating that it remains critically important to continue to maintain expertise in morphological taxonomic identification of zooplankton.

## Choice of genetic markers for molecular analysis of zooplankton diversity

An important topic is the impact of choice of the marker gene region used for metabarcoding on the results and interpretation of biodiversity (Table 1). Among the most common gene regions used are hypervariable regions of 18S rRNA. The mitochondrial COI barcode region remains a frequent choice, despite challenges and some limitations (Schroeder *et al.*, 2021; Bucklin *et al.*, 2021b). A novel and intriguing line of investigation is the use of RNA-based markers (Cristescu, 2019; Lenz *et al.*, 2021), which is explored here in compelling detail by Machida *et al.* (2021) and Hirai *et al.* (2020).

Machida *et al.* (2021) examined zooplankton samples from coastal waters near the Ryukyu Islands, Japan and compared several methods for assessing taxonomic composition and diversity: complementary DNA, genomic DNA, mitochondrial COI amplicons, metatranscriptome sequences, and morphological identifications. There were significant correlations among results for all methods based on operational taxonomic unit richness, Shannon's H index, and phylum-level community composition. After careful analysis, the results were interpreted to indicate probable mitochondrial pseudogene contamination from sequencing of genomic DNA and taxonomic bias from PCR-based methods, leading to the recommendation that PCR-free approaches be used whenever possible.

Hirai *et al.* (2020) developed a novel metabarcoding approach and used it in combination with microscopy to examine copepod community structure and diversity over the Izu Ridge, off the southern coast of Japan. The region exhibits rapid environmental changes due to high levels of nutrients and strong turbulence around the complex topography. The ratio of rRNA:rDNA for the 28S gene region showed positive correlation with chlorophyll a concentration, providing a useful indicator of physiological responses of epipelagic copepods to available food supply.

## Ecological and evolutionary drivers of genetic diversity

Patterns of molecular diversity and population structure of marine zooplankton can provide novel insights into the underlying ecological and evolutionary processes. Peijnenburg and Goetze (2013) reviewed evidence that selection may be a dominant driver of the evolution of open-ocean zooplankton. Several studies have examined the genetic consequences of exceptionally large population sizes and extensive geographical ranges exhibited by many marine species (Plough, 2016; Marlétaz *et al.*, 2017; Filatov, 2019). Two papers in the TS provided new insights into these questions for regional- to global-scale patterns of population genetic diversity of selected species groups.

Kulagin *et al.* (2021) examined molecular variation of COI and histone 3 (H3) variation of species of the euphausiid genus, *Nematocarcinus*, collected at numerous stations throughout the North and South Atlantic Ocean (Figure 1). Parallel analyses were done

of diagnostic morphological traits, including chitin structures and photophores. The results revealed the underlying drivers of speciation in this genus, which included both restricted dispersal, due to the physical structure of the ocean, and limited inter-breeding, due to sexual selection based on morphological divergence.

Bucklin *et al.* (2021b) reported significant population genetic differentiation and structure of the deep-dwelling, non-migrating euphausiid, *Stylocheiron elongatum*, in the Gulf Stream System of the North Atlantic Ocean. Analysis of COI and mitochondrial cytochrome b (CYB) provided evidence of persistent population structure driven by natural selection. These findings were considered in the context of the lack of population genetic differentiation of globally distributed species of *Stylocheiron* based on the COI barcode region, which is consistent with purifying selection (Hughes, 2005), which eliminates even slightly-disadvantageous mutations and may be especially effective in extremely large populations.

## Summary and forward look

Molecular approaches have become essential tools for analysis of marine zooplankton biodiversity, including targeted gene regions for DNA barcoding (Bucklin *et al.*, 2021a) and metabarcoding (Bucklin *et al.*, 2016; Rey *et al.*, 2020), as well as population genomics (Bucklin *et al.*, 2018), transcriptomics, and metatranscriptomics (Cristescu, 2019; Lenz *et al.*, 2021). Considered together, the papers in this TS provide clear evidence of the promise and prospects of molecular approaches for new insights into the diversity, biogeography, ecology, and evolution of marine zooplankton, as well as new understanding and appreciation of their importance for the functioning of ocean ecosystems. There are a number of remaining challenges. Primary is the need for taxonomically-complete DNA reference databases for target gene regions, based on morphologically-identified species across the broad taxonomic range of marine zooplankton (Machida *et al.*, 2017; Bucklin *et al.*, 2021a). Also critically needed are reference genomes and transcriptomes for species representing all the taxonomic groups of the pelagic assemblage (GIGA Community of Scientists, 2014; Lewin *et al.*, 2018; Blasiak *et al.*, 2020). Continued development of transcriptomic resources will provide tools to reveal physiological responses and adaptive potential of species, and allow prediction of future changes in pelagic ecosystems (Lenz *et al.*, 2021). Widespread application of molecular approaches for monitoring of ocean ecosystems and management of fisheries will require protocols that yield accurate quantitative data, ideally including both abundance and biomass. There is every reason for confidence in the promise and prospects of molecular approaches to continue to yield new understanding of the underlying ecological and evolutionary drivers of biodiversity, structure, and function of the marine zooplankton assemblage.

## Data availability statement

No new data were generated or analysed in support of this research.

## Funding

This publication resulted in part from support provided by the Scientific Committee on Oceanic Research (SCOR). Funds were also

contributed by the U.S. National Science Foundation (Grant OCE-1840 868) and by national SCOR committees.

## Conflict of interest statements

The authors report no conflicts of interest

## Acknowledgements

The motivators of this Themed Set acknowledge with sincere appreciation the authors of the contributed papers. This publication is a contribution from the Scientific Committee on Oceanic Research (SCOR) Working Group 157, *MetaZooGene: Toward a new global view of marine zooplankton biodiversity based on DNA metabarcoding and reference DNA sequence databases* (<https://metazoogen.e.org/> and <https://scor-int.org/group/157/>). Additional contributions were made by members of the Working Group on Integrated Morphological and Molecular Taxonomy (WGIMT) of the International Council for the Exploration of the Sea (ICES).

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Handling Editor: Howard Brownman