# Genetic diversity of hyperiid amphipods along a meridional transect in the Atlantic Ocean

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## Abstract

Detecting plankton community responses to a changing ocean environment requires knowledge of the diversity and distribution of species. Hyperiid amphipods are an important component of pelagic food webs as prey and as commensals and parasitoids of gelatinous zooplankton, but their species and genetic diversity remains incompletely known. Using mitochondrial cytochrome *c* oxidase subunit 1 (CO1) gene sequences, we assessed the genetic diversity of hyperiids collected along a meridional transect from 39 °N to 45 °S in the Atlantic Ocean in order to evaluate the congruence of genetic clades with morphologically-identified species, examine phylogeographic patterns within broadly-distributed species, and to contribute vouchered DNA barcodes into the public domain. CO1 sequences were obtained from 273 specimens representing 63 morphologically-identified hyperiid species belonging to 34 genera and 16 families, including sequences from 26 species with no prior reports in publicly-available databases. We recovered substantial amounts of genetic variation within nine nominal species, which were composed of two to four clades with 11–36% average sequence divergence between clades (range 10–45%). These are probably distinct or unrecognized biological species, however, additional sampling of morphological and/or genomic diversity is necessary to confirm this. Additionally, we examined the geographic distribution of genetic variation within *Lycaea pulex*, *Phronimopsis spinifera*, *Phrosina semilunata*, *Tryphana malmii*, and *Vibilia armata*, all species with broad or disjunct distributions across the Atlantic Ocean. We found that these species are composed of lineages with apparent geographic specializations to tropical, subtropical or temperate waters, suggesting more restricted biogeographic distributions than described for the inclusive morphospecies. This new collection of CO1 barcodes furthers our understanding of the diversity and distribution of hyperiids in the Atlantic Ocean and will support future research on this ecologically significant plankton group.

## Introduction

Zooplankton are important trophic intermediaries in the world's oceans that influence ocean biogeochemistry and carbon export to midwaters, as well as providing the forage base for higher trophic level predators. A number of recent international policy initiatives recognise the importance of zooplankton biodiversity to ecosystem function and aim to include plankton bioindicators to assess and monitor pelagic ecosystem health (Chiba et al., 2018; Giering et al., 2022). Zooplankton are rapid responders to climate change, due in part to small body size and short generation times, and they can provide a valuable and unadulterated record of biological response to long-term environmental change (Hays et al., 2005; Richardson

2008). However, achieving accurate assessments of zooplankton community change in response to climate change requires data at the species level, necessitating deep knowledge of the diversity and distribution of pelagic species. While the species and genetic diversity of some zooplankton groups are quite well characterized (Bucklin et al., 2021), other taxa have seen limited research effort. Here, we focus on hyperiid amphipods, a subdominant zooplankton group that are widely distributed and common in the global ocean ranging from tropical to polar ecosystems.

Hyperiid amphipods are abundant midwater zooplankton that serve as carbon-rich prey for higher trophic level predators (Shreeve et al., 2009; Choy et al., 2013), including commercially important fish species (Padovani et al., 2012). An ecologically significant group, the hyperiids typically live in association with a variety of gelatinous plankton, including salps, urochordates, ctenophores, and siphonophores (Harbison et al., 1977; Madin & Harbison 1977), and form commensal or parasitoid symbioses with their hosts for part or all of their life cycle (Fleming et al., 2014; Mazda et al., 2019). There is considerable diversity in the extent to which hyperiids are generalists or specialists to particular host taxa (Laval 1980). Hyperiids are often deleterious to their hosts, feeding on host tissues during later developmental stages, and may channel carbon and energy from their gelatinous host back to fishes that are feeding on hyperiids at the next trophic step (Riascos et al., 2012). While the majority of hyperiids have a commensal or parasitoid life history, several predatory free-living species are biomass dominants in high latitude pelagic

ecosystems (e.g., *Themisto* spp.), where they occupy a central trophic position within continental shelf and subantarctic/subarctic food webs (Bocher et al., 2001; Pinchuk et al., 2013; Havermans et al., 2019a). The approximately 284 described species (World Register of Marine Species, https://www.marinespecies.org/) of the suborder Hyperiidea are primarily epipelagic and mesopelagic in distribution.

Understanding evolution and diversification within the suborder Hyperiidea has long been challenging due to extensive homoplasious morphological characters that impede inference of accurate phylogenies. No single synapomorphic character unites the entire suborder (Vinogradov et al., 1996; Browne et al., 2007), and hyperiids are distinguished from other marine amphipods by a combination of traits that are correlated with their pelagic life history and symbioses with gelatinous plankton, for example, hypertrophied olfactory and visual systems, duplications of the eyes, and extraordinary shapes of the last segments of the pereopods (Hurt et al., 2013; Baldwin Fergus et al., 2015). These distinct hyperiid traits likely result from their ancestral benthic lifestyle and subsequent colonization of and evolution within the pelagic habitat (Zeidler 2016). Multi-gene molecular phylogenies have shed light on some aspects of evolutionary relationships within the Hyperiidea (Hurt et al., 2013; Copilaş-Ciocianu et al., 2019), in particular, by providing well-supported placements for several enigmatic or problematic taxa (*Cystisoma, Paraphronima, Tryphana*), and confirming two monophyletic radiations for the bathypelagic Physosomata and epi- and mesopelagic Physocephalata. Notably, taxon sampling within these

most recent molecular phylogenetic studies included 51 species, ~18% of the global diversity of the Hyperiidea. Few studies have examined genetic diversity within hyperiid species, with the exception of recent work on *Paraphronima* (Stenvers et al., 2021), *Hyperiella* (Havermans et al., 2019b), and *Themisto* (Tempestini et al., 2017; Tempestini et al., 2020; Murray et al., 2023).

Large-scale biodiversity and conservation studies of pelagic ecosystems often rely on molecular methods as one approach to quantifying biodiversity (Pierella Karlusich et al., 2022). Community amplicon sequencing, or metabarcoding surveys are increasingly being used due to their ability to detect a long tail of rare taxa, including cryptic or difficult-to-identify organisms (Sommer et al., 2017), their facility in providing research results for complex, high-diversity communities within a short timeframe (Hirai et al., 2020; Ershova et al., 2021), and their strengths in consistent identifications across marine regions, even in the absence of complete taxonomic descriptions of the fauna (Bucklin et al., 2016; Deagle et al., 2018). However, all community sequencing efforts fundamentally rely on well-curated and vouchered databases of reference sequences that link taxonomic names to DNA sequences for the target fauna (Leray et al., 2019), enabling classification of reads and assignment of taxonomy in metabarcoding studies. Gaps in taxon sampling for public sequence databases can therefore be a major limitation to operational use of metabarcoding as a survey tool (Hestetun et al., 2020). Although over 135,000 sequences are publicly available for metazoan plankton at the common DNA barcoding region

mitochondrial cytochrome *c* oxidase subunit I (CO1), these include DNA barcodes for only 83 species of hyperiids out of the ~284 species reported worldwide (29%; O'Brien, pers comm; Bucklin et al., 2021). Expanding representation of open ocean and deep pelagic crustaceans in DNA barcoding databases is urgent to support biodiversity assessments at large scale (Jazdzewska et al., 2021; Varela et al., 2021).

In this study, we examined genetic diversity and geographic patterns of genetic lineages within hyperiid species collected along a meridional transect in the Atlantic Ocean (39 °N to 45 °S; N = 276 individuals, N = 67 species). This work builds on our previous study (Burridge et al., 2017) that examined diversity and distribution patterns of oceanic hyperiids along this basin-scale transect and found intraspecific variation in morphological characters for a number of species. Our goals were to: (1) determine congruence of morphologically-identified species with mitochondrial CO1 barcodes, (2) examine phylogeographic patterns within species with broad latitudinal ranges or observed morphological variation, and (3) contribute specimen-vouchered DNA barcodes for this ecologically important plankton group into the public domain. The new genetic resources reported here support ongoing and future efforts to trace changing pelagic community composition and distributions in response to climate change.

## Materials and methods

### *Sampling and identification*
Zooplankton was collected at 26 stations on Atlantic Meridional Transect Cruise

22 (AMT22) between October 16 and November 19, 2012 (fig. 1; table S1). Oblique tows were conducted at night with paired bongo nets (CalBOBL, 200 μm, 333 μm mesh) and a rectangular midwater trawl net (RMT1, 333 μm mesh) from epipelagic and upper mesopelagic depths to the sea surface (average maximum depth of tow was 242.5 m). Bulk plankton was preserved immediately in 100% ethyl alcohol and the alcohol was replaced after 24 hours. Details regarding sample collection for AMT22 and identification of hyperiids were reported in (Burridge et al., 2017) and this study used the specimens as in this previous study. Identifications were made using a range of taxonomic keys (Bowman 1973; Bowman and Gruner 1973; Bowman 1985; Sheader 1986; Zeidler 1990; Shih 1991; Vinogradov et al., 1996; Zeidler 1999; Zeidler 2003b, Zeidler 2003a, 2004b, a, 2009; 2012; 2015; 2016). Representatives of all species were imaged using a Zeiss automated stacking light microscope and ZEN Microscopy Software. Specimens used for DNA extraction were selected based on size, preservation stage and morphological variation observed within morphologically-identified species. For most specimens, pereopod #6 was removed and used for DNA extraction. Members of the family Platyscelidae have pereopods that are tucked underneath the abdomen by a hard integument and are difficult to access. We therefore used tissue of uropods or the entire urosome (depending on size) for some members of this family. DNA extracts as well as specimens used for DNA extraction were deposited as vouchers in the collection of Naturalis Biodiversity Center, Leiden, The Netherlands (museum codes RMNH.CRUS.A.5401-5673). DNA

sequence data as well as specimen images were deposited in the Barcode of Life Data Systems (BOLD) under IDs NBCHyAmp-001 – NBCHyAmp-273 (table 1 and available through this link: dx.doi.org /10.5883/DS-HAMA2) and are part of the quality controlled MetaZooGene Atlas and Database (https://metazoogene.org /mzgdb).

### DNA barcoding

DNA was extracted from tissues using NucleoMag B-Beads and binding buffer MB2 using the magnetic separator NucleoMag SEP. A ~685 base pair fragment of the mitochondrial cytochrome oxidase subunit 1 (CO1) gene was amplified from each specimen. Polymerase Chain Reaction (PCR) amplifications included PCR buffer CL 2.5 μL (Qiagen), $MgCl_2$ (25 mM) 0.5 μL, BSA (100mM) 0.5 μL, dNTP (2.5 mM) 0.5 μL, Qiagen Tag (5 U/ μL) 0.25 μL, primers M13–jgLCO1490F (10 pMol/ μL) 1 μL and M13F-jgHCO2198R (10 pMol/ μL) 1 μL, and 2.0 μL of template DNA. PCR was performed with primers jgLCO1490F and jgHCO2198R (Geller et al., 2013), which were tailed with M13F and M13R for sequencing (Messing 1983). PCR conditions were 94 °C for 3 min of denaturation, followed by 40 cycles of 94 °C for 15 sec, 50 °C for 30 sec, 72 °C for 40 sec, and a final extension of 72 °C for 5 minutes. Sequencing of forward and reverse strands was carried out by Baseclear B.V. (Leiden, the Netherlands).

### Sequence analysis

Forward and reverse sequences were combined and checked for errors in Geneious (Kearse et al., 2012). Final sequences were aligned with ClustalW in MEGA6
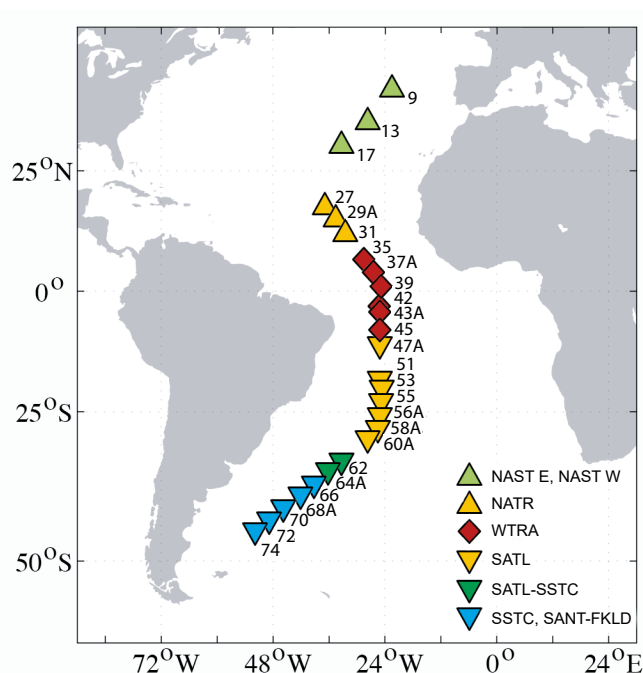
FIGURE 1    Sampling locations for hyperiid specimens during Atlantic Meridional Transect Cruise 22 (AMT22). Oceanic provinces defined by Longhurst (1998) are indicated by symbols and abbreviations: NAST E/W (Northeast/Northwest Atlantic subtropical gyral), NATR (North Atlantic tropical gyral), WTRA (Western tropical Atlantic), SATL (South Atlantic gyral), SATL-SSTC (transition zone to Southern subtropical convergence), SSTC (Southern subtropical convergence), SANT (Subantarctic water ring), FKLD (Southwest Atlantic shelves) (Longhurst 2007). Station numbers as in tables 1 and S1

(Tamura et al., 2013) resulting in a final CO1 alignment of 550-bp. Nucleotide sequences were translated to protein to confirm an absence of stop codons and frameshift mutations. BLAST searches were performed to compare our DNA barcodes to published hyperiid sequences. A total of 54 hyperiid CO1 sequences were retrieved from GenBank and used in phylogenetic analyses (Browne et al., 2007; Bucklin et al., 2010a, Bucklin et al., 2010b). Genetic distances between and within morphologically-identified hyperiid species were determined using Kimura-2-Parameter (K2P) models, calculated in MEGA6 (Tamura et al., 2013). The barcode gap between species was evaluated using the Automatic Barcode Gap Discovery (ABGD) method and used to delimit Operational Taxonomic Units (OTUs) (Puillandre et al., 2012) using both K2P & Jukes-Cantor models (Kimura 1980; Puillandre et al., 2012; Kumar et al., 2016). Phylogenetic trees

were inferred using maximum likelihood (ML) in RAxML-HPC2 on CIPRES using the best fitting nucleotide substitution models (Stamatakis 2006; 2014). Clade support was assessed by 1000 bootstrap replicates. For the overall dataset including a maximum of five sequences per species and published sequences, the mtREV protein model was selected and sequences were translated into protein. To obtain insights into phylogeographic patterns, ML phylogenies were also inferred for subsets including all specimens within *Phronimopsis spinifera* Claus, 1879 (N = 17), *Phrosina semilunata* Risso, 1822 (N = 13), *Tryphana malmii* Boeck, 1871 (N = 10), *Vibilia armata* Bovallius, 1887 (N = 19), and the genera *Lycaea* (N = 18) and *Primno* (N = 94) using the GTRCAT nucleotide model. All phylogenies were rooted at the midpoint except *Tryphana malmii* for which we had a suitable outgroup.

### Results

#### DNA *reference barcodes*

Mitochondrial CO1 sequences were obtained from 273 hyperiid specimens, representing 63 identified species in 34 genera and 16 families (table 1, table S3). Family names are reported here as designated by Zeidler (2016). Amplification using universal CO1 primers failed for *Hyperietta stebbingi* Bowman, 1973; *Iulopis loveni* Bovallius, 1887 and *Thamneus rostratus* Bovallius, 1887, and no sequence data were obtained for these species.

Results of genetic analyses and re-examination of morphological characters clarified original identifications for ten species reported in Burridge

et al., (2017) for which species names were revised (table 1). Specimens initially identified as *Lanceola* sp. 1 and *Lestrigonus* sp. 1 matched GenBank sequences for *Lanceola sayana* Bovallius, 1885 EF989696.1 (100%) and *Lestrigonus schizogeneios* Stebbing, 1888 EF989684.1 (98%), respectively, and were revised in this study. Specimens provisionally identified as *Lycaea* sp. 1 and *Lycaea* sp. 2 in Burridge et al., (2017) were genetically identical and failed to match any existing species description. Hence, they are here referred to as *Lycaea* sp. 1. CO1 sequences from two *Scina* sp. individuals that were smaller than 0.5 mm matched with specimens identified as *Scina tullbergi* (Bovallius, 1885) (100%) and were re-named accordingly. Upon re-examination of morphological characters of *Lycaeidae* sp. 1 specimens, we identified these as members of the genus *Simorhynchotus* based on the unique formation of the last few segments of pereopod #2. Specimens identified as *Vibilia stebbingi* Behning & Woltereck, 1912 in Burridge et al., (2017) matched with South Atlantic *Vibilia armata* (99%) and were re-named. CO1 sequences of *Brachyscelus* sp. 1, *Lycaea* sp. 1 and *Simorhynchotus* sp. 1 were highly distinct from those available for described species and are therefore provisionally indicated as distinct and potentially undescribed species. Finally, specimens of the genus *Primno* were primarily juveniles or subadults and therefore very difficult to identify based on underdeveloped morphological characteristics. However, adult specimens could be identified with confidence as *Primno abyssalis* (Bowman in Fulton, 1968), *Primno brevidens* Bowman, 1978, *Primno johnsoni* Bowman, 1978 and *Primno latreillei*

TABLE 1    Hyperiid amphipods from Atlantic Meridional Transect Cruise 22 (AMT22) used in this study. Species identifications are shown in the first two columns and earlier morphological identifications as reported in Burridge et al. (2017) are also listed (see text for more details). The number of mitochondrial cytochrome oxidase 1 (CO1) sequences obtained (# seq), sampling station(s), Longhurst (1998) oceanic regions, number of Operational Taxonomic Units identified by Automatic Barcode Gap Delimitation analysis (#OTUS), and the Barcode of Life Database (BOLD) BINs and IDs are listed (see also Fig 1). **Bold font** in the BOLD ID column indicates species for which this is the first CO1 sequence in the public domain (accessed 12 July 2024).

| Genus | Species | Family | Burridge et al. 2017 | # CO1 seq | Sampling station(s) | Oceanic regions | # OTUs | BOLD BIN | BOLD ID |
|---|---|---|---|---|---|---|---|---|---|
| *Amphithyrus* | *bispinosus* | Amphithyridae | *Amphithyrus bispinosus* | 1 | AMT22-29A | NATR | 1 | AFJ1974 | NBCHyAmp-001 |
| *Amphithyrus* | *sculpturatus* | Amphithyridae | *Amphithyrus sculpturatus* | 1 | AMT22-13 | NAST W | 1 | AFJ1975 | **NBCHyAmp-002** |
| *Anchylomera* | *blossevillei* | Phrosinidae | *Anchylomera blossevillei* | 2 | AMT22-47A | SATL | 1 | AFJ3991 | **NBCHyAmp-003-004** |
| *Brachyscelus* | *crusculum* | Brachyscelidae | *Brachyscelus crusculum* | 3 | AMT22-35 | WTRA | 1 | ADC7383, n.a. | **NBCHyAmp-005-007** |
| *Brachyscelus* | *globiceps* | Brachyscelidae | *Brachyscelus globiceps* | 1 | AMT22-62 | SATL-SSTC | 1 | AFJ7676 | **NBCHyAmp-008** |
| *Brachyscelus* | *macrocephalus* | Brachyscelidae | *Brachyscelus macrocephalus* | 2 | AMT22-37A, AMT22-42 | WTRA | 1 | AFK9173 | **NBCHyAmp-009-010** |
| *Brachyscelus* | sp. 1 | Brachyscelidae | *Brachyscelus* sp. 1 | 1 | AMT22-35 | WTRA | 1 | n.a. | **NBCHyAmp-011** |
| *Calamorhynchus* | *pellucidus* | Oxycephalidae | *Calamorhynchus pellucidus* | 1 | AMT22-47A | SATL | 1 | AFL1397 | NBCHyAmp-012 |

TABLE 1 Hyperiid amphipods from Atlantic Meridional Transect Cruise 22 (AMT22) (*cont.*)

| Genus | Species | Family | Burridge et al. 2017 | # CO1 seq | Sampling station(s) | Oceanic regions | # OTUs | BOLD BIN | BOLD ID |
|---|---|---|---|---|---|---|---|---|---|
| *Cranocephalus* | *scleroticus* | Oxycephalidae | *Cranocephalus scleroticus* | 2 | AMT22-29A, AMT22-43A | NATR, WTRA | 1 | AFK5945 | NBCHyAmp-013-014 |
| *Dairella* | *californica* | Dairellidae | *Dairella californica* | 2 | AMT22-37A, AMT22-62 | WTRA, SATL-SSTC | 1 | AFL1475, AFL1474 | NBCHyAmp-015-016 |
| *Eupronoe* | *maculata* | Eupronoidae | *Eupronoe maculata* | 1 | AMT22-35 | WTRA | 1 | AEE6445 | NBCHyAmp-017 |
| *Eupronoe* | *minuta* | Eupronoidae | *Eupronoe minuta* | 4 | AMT22-13 | NAST W | 2 | AEV9815, AEL5160 | NBCHyAmp-018-021 |
| *Hyperietta* | *luzoni* | Lestrigonidae | *Hyperietta luzoni* | 2 | AMT22-47A | SATL | 1 | AFJ9377, AFJ9378 | NBCHyAmp-022-023 |
| *Hyperietta* | *stephenseni* | Lestrigonidae | *Hyperietta stephenseni* | 1 | AMT22-37A | WTRA | 1 | AAF4001 | NBCHyAmp-024 |
| *Hyperietta* | *vosseleri* | Lestrigonidae | *Hyperietta vosseleri* | 2 | AMT22-42 | WTRA | 1 | AAF4001, AFJ9379 | NBCHyAmp-025-026 |
| *Hyperioides* | *longipes* | Lestrigonidae | *Hyperioides longipes* | 5 | AMT22-29A, AMT22-47A, AMT22-55 | NATR, SATL | 2 | AAX1878, AEK77568 | NBCHyAmp-028-031 |
| *Hyperoche* | *martinezii* | Hyperiidae | *Hyperoche martinezii* | 1 | AMT22-62 | SATL-SSTC | 1 | AFJ1812 | NBCHyAmp-032 |
| *Hyperoche* | *medusarum* | Hyperiidae | *Hyperoche medusarum* | 1 | AMT22-42 | WTRA | 1 | AFK6823 | NBCHyAmp-033 |

TABLE 1   Hyperiid amphipods from Atlantic Meridional Transect Cruise 22 (AMT22) (*cont.*)

| Genus | Species | Family | Burridge et al. 2017 | # CO1 seq | Sampling station(s) | Oceanic regions | # OTUs | BOLD BIN | BOLD ID |
|---|---|---|---|---|---|---|---|---|---|
| *Lanceola* | *sayana* | Lanceolidae | *Lanceola* sp. 1 | 1 | AMT22-66 | SSTC | 1 | AEI4162 | NBCHyAmp-034 |
| *Laxohyperia* | *vespuliformis* | Hyperiidae | *Laxohyperia vespuliformis* | 1 | AMT22-17 | NAST W | 1 | AFK9796 | NBCHyAmp-035 |
| *Leptocotis* | *tenuirostris* | Oxycephalidae | *Leptocotis tenuirostris* | 2 | AMT22-58A | SATL | 1 | AAX5358 | NBCHyAmp-036-037 |
| *Lestrigonus* | *bengalensis* | Lestrigonidae | *Lestrigonus bengalensis* | 1 | AMT22-29A | NATR | 1 | AAF7277 | NBCHyAmp-038 |
| *Lestrigonus* | *schizogeneios* | Lestrigonidae | *Lestrigonus* sp. 1 | 1 | AMT22-42 | WTRA | 1 | n.a. | NBCHyAmp-039 |
| *Lycaea* | *pulex* | Lycaeidae | *Lycaea pulex* | 13 | AMT22-31, AMT22-37A, AMT22-62 | NATR, WTRA, SATL-SSTC | 4 | ADG7359, FK8738, AFJ5035, AFJ5036, | NBCHyAmp-040-052 |
| *Lycaea* | *serrata* | Lycaeidae | *Lycaea serrata* | 1 | AMT22-42 | WTRA | 1 | AFJ5034 | NBCHyAmp-053 |
| *Lycaea* | sp. 1 | Lycaeidae | *Lycaea* sp. 1; *Lycaea* sp. 2 | 4 | AMT22-39 | WTRA | 1 | AFJ5033 | NBCHyAmp-054-057 |
| *Lycaeopsis* | *themistoides* | Lycaeopsidae | *Lycaeopsis themistoides* | 3 | AMT22-35, AMT22-53, AMT22-55 | WTRA, SATL | 2 | AFL4386, AFJ8486, n.a. | NBCHyAmp-058-060 |
| *Oxycephalus* | *piscator* | Oxycephalidae | *Oxycephalus piscator* | 2 | AMT22-56A | SATL | 1 | AEL2037 | NBCHyAmp-061-062 |
| *Parapronoe* | *campbelli* | Eupronoidae | *Parapronoe campbelli* | 2 | AMT22-35 | WTRA | 1 | n.a. | NBCHyAmp-063-064 |

TABLE 1 Hyperiid amphipods from Atlantic Meridional Transect Cruise 22 (AMT22) (*cont.*)

| Genus | Species | Family | Burridge et al. 2017 | # CO1 seq | Sampling station(s) | Oceanic regions | # OTUs | BOLD BIN | BOLD ID |
|---|---|---|---|---|---|---|---|---|---|
| *Parapronoe* | *parva* | Eupronoidae | *Parapronoe parva* | 2 | AMT22-29A, AMT22-45 | NATR, WTRA | 1 | AFK1938, AFL0074 | NBCHyAmp-065-066 |
| *Phronima* | *atlantica* | Phronimidae | *Phronima atlantica* | 2 | AMT22-42 | WTRA | 2 | ADC7384, AAI2995 | NBCHyAmp-067-068 |
| *Phronima* | *sedentaria* | Phronimidae | *Phronima sedentaria* | 2 | AMT22-37A | WTRA | 1 | AFK4779, AFK4969 | NBCHyAmp-069-070 |
| *Phronima* | *solitaria* | Phronimidae | *Phronima solitaria* | 2 | AMT22-37A, AMT22-39 | WTRA | 1 | AAI2995, n.a. | NBCHyAmp-071-072 |
| *Phronima* | *stebbingi* | Phronimidae | *Phronima stebbingi* | 1 | AMT22-29A | NATR | 1 | AAF5021 | NBCHyAmp-073 |
| *Phronimella* | *elongata* | Phronimidae | *Phronimella elongata* | 3 | AMT22-35, AMT22-42 | WTRA | 1 | AEL4527, AFK3387, n.a. | NBCHyAmp-074-076 |
| *Phronimopsis* | *spinifera* | Lestrigonidae | *Phronimopsis spinifera* | 17 | AMT22-29A, AMT22-35, AMT22-45, AMT22-47A, AMT22-58A, AMT22-60A | NATR, WTRA, SATL | 2 | AEK4936, AFJ1834, AFJ1835, AFJ1837, AFJ1833, AFJ1834, AFJ1838, AFJ1836 | NBCHyAmp-077-093 |

TABLE 1 Hyperiid amphipods from Atlantic Meridional Transect Cruise 22 (AMT22) (cont.)

| Genus | Species | Family | Burridge et al. 2017 | # CO1 seq | Sampling station(s) | Oceanic regions | # OTUs | BOLD BIN | BOLD ID |
|---|---|---|---|---|---|---|---|---|---|
| *Phrosina* | semilunata | Phrosinidae | *Phrosina semilunata* | 13 | AMT22-09, AMT22-29A, AMT22-45, AMT22-47A, AMT22-64A | NAST E, NATR, WTRA, SATL, SATL-SSTC | 4 | AFG2629, AFJ2916, AFJ2915, AFJ2916, AFJ2917 | NBCHyAmp-094-106 |
| *Platyscelus* | armatus | Platyscelidae | *Platyscelus armatus* | 2 | AMT22-45 | WTRA | 1 | AFL2448 | **NBCHyAmp-107-108** |
| *Platyscelus* | crustulatus | Platyscelidae | *Platyscelus crustulatus* | 1 | AMT22-42 | WTRA | 1 | AFL0351 | **NBCHyAmp-109** |
| *Platyscelus* | ovoides | Platyscelidae | *Platyscelus ovoides* | 6 | AMT22-51, AMT22-62, AMT22-64A | SATL, SATL-SSTC | 3 | AFJ8730, AFJ8731, ADZ3111 | NBCHyAmp-110-115 |
| *Primno* | abyssalis | Phrosinidae | *Primno evansi* | 39 | AMT22-66, AMT22-68A, AMT22-70, AMT22-72, AMT22-74 | SSTC, SANT-FKLD | 1 | AFK9804, n.a. | **NBCHyAmp-116-154** |
| *Primno* | brevidens | Phrosinidae | *Primno johnsoni* | 2 | AMT22-31, AMT22-62 | NATR, SATL-SSTC | 2 | AFK9803, n.a. | NBCHyAmp-155-156 |

TABLE 1 Hyperiid amphipods from Atlantic Meridional Transect Cruise 22 (AMT22) (cont.)

| Genus | Species | Family | Burridge et al. 2017 | # CO1 seq | Sampling station(s) | Oceanic regions | # OTUs | BOLD BIN | BOLD ID |
|---|---|---|---|---|---|---|---|---|---|
| *Primno* | *johnsoni* | Phrosinidae | *Primno evansi; Primno latreillei* | 39 | AMT22-27, AMT22-29A, AMT22-45, AMT22-47A, AMT22-53, AMT22-55 | NATR, WTRA, SATL | 1 | AAX8046, AFK9805, n.a. | NBCHyAmp-157-195 |
| *Primno* | *latreillei* | Phrosinidae | *Primno evansi; Primno latreillei* | 13 | AMT22-55, AMT22-62, AMT22-64A | SATL-SSTC | 1 | AAX8046, AFK9805, n.a. | NBCHyAmp-196-208 |
| *Pronoe* | *capito* | Pronoidae | *Pronoe capito* | 2 | AMT22-35, AMT22-42 | WTRA | 1 | AFJ5773 | NBCHyAmp-209-210 |
| *Rhabdosoma* | *minor* | Oxycephalidae | *Rhabdosoma minor* | 2 | AMT22-29A, AMT22-37A | NATR, WTRA | 1 | AFK7752, n.a. | NBCHyAmp-211-212 |
| *Scina* | *curilensis* | Scinidae | *Scina curilensis* | 3 | AMT22-29A, AMT22-37A | NATR, WTRA | 1 | AFJ2759, n.a. | NBCHyAmp-213-215 |
| *Scina* | *spinosa* | Scinidae | *Scina spinosa* | 2 | AMT22-35 | WTRA | 1 | ADX0941 | NBCHyAmp-216-217 |
| *Scina* | *tullbergi* | Scinidae | *Scina tullbergi; Scina* sp. | 4 | AMT22-66, AMT22-68A | SSTC | 1 | AFK4294 | NBCHyAmp-218-221 |
| *Simorhynchotus* | sp. 1 | Lycaeidae | *Lycaeidae* sp. 1 | 2 | AMT22-35, AMT22-45 | WTRA | 2 | ADO9548, n.a. | NBCHyAmp-222-223 |

TABLE 1 Hyperiid amphipods from Atlantic Meridional Transect Cruise 22 (AMT22) (cont.)

| Genus | Species | Family | Burridge et al. 2017 | # CO1 seq | Sampling station(s) | Oceanic regions | # OTUs | BOLD BIN | BOLD ID |
|---|---|---|---|---|---|---|---|---|---|
| *Streetsia* | challengeri | Oxycephalidae | *Streetsia challengeri* | 2 | AMT22-58A | SATL | 1 | AFJ6076 | NBCHyAmp-224-225 |
| *Streetsia* | mindanaonis | Oxycephalidae | *Streetsia mindanaonis* | 1 | AMT22-42 | WTRA | 1 | AFJ1524 | NBCHyAmp-226 |
| *Streetsia* | porcella | Oxycephalidae | *Streetsia porcella* | 2 | AMT22-27, AMT22-29A | NATR | 1 | ADC7582 | NBCHyAmp-227-228 |
| *Tetrathyrus* | forcipatus | Platyscelidae | *Tetrathyrus forcipatus* | 2 | AMT22-29A, AMT22-42 | NATR, WTRA | 1 | AFL3082 | NBCHyAmp-229-230 |
| *Themisto* | gaudichaudi | Hyperiidae | *Themisto gaudichaudi* | 4 | AMT22-09, AMT22-62, AMT22-68A | NAST E, SATL-SSTC, SSTC | 1 | AAG2099, AFL3673, ADZ2163, AFK2165 | NBCHyAmp-231-234 |
| *Tryphana* | malmii | Tryphanidae | *Tryphana malmii* | 10 | AMT22-31, AMT22-35 (2), AMT22-42 (2), AMT22-45, AMT22-62 (2), AMT22-64A (2) | NATR, WTRA, SATL-SSTC | 2 | AFJ1632, AFJ1633 | NBCHyAmp-235-244 |
| *Tullbergella* | cuspidata | Oxycephalidae | *Tullbergella cuspidata* | 1 | AMT22-55 | SATL | 1 | AEL0165 | NBCHyAmp-245 |

TABLE 1    Hyperiid amphipods from Atlantic Meridional Transect Cruise 22 (AMT22) (cont.)

| Genus | Species | Family | Burridge et al. 2017 | # CO1 seq | Sampling station(s) | Oceanic regions | # OTUs | BOLD BIN | BOLD ID |
|---|---|---|---|---|---|---|---|---|---|
| Vibilia | armata | Vibilidae | Vibilia armata; Vibilia stebbingi | 19 | AMT22-29A (5), AMT22-31 (2), AMT22-62 (2), AMT22-64A (5), AMT22-68A (5) | NATR, SATL-SSTC, SSTC | 1 | ACQ0666, AFJ8448, n.a. | NBCHyAmp-246-264 |
| Vibilia | australis | Vibilidae | Vibilia australis | 2 | AMT22-62, AMT22-70 | SATL-SSTC, SSTC | 2 | AFJ8449, AES4783 | NBCHyAmp-265-266 |
| Vibilia | borealis | Vibilidae | Vibilia borealis | 2 | AMT22-39 (2) | WTRA | 1 | AFJ8450 | NBCHyAmp-267-268 |
| Vibilia | propinqua | Vibilidae | Vibilia propinqua | 2 | AMT22-64A (2) | SATL-SSTC | 1 | AFJ8452 | NBCHyAmp-269-270 |
| Vibilia | pyripes | Vibilidae | Vibilia pyripes | 1 | AMT22-17 | NAST W | 1 | AFJ8451 | NBCHyAmp-271 |
| Vibilia | sp. 1 | Vibilidae | Vibilia sp. 1 | 2 | AMT22-37A (2) | WTRA | 1 | AEK6501, AEK6502 | NBCHyAmp-272-273 |

Stebbing, 1888 and by combining morphological and genetic information, we revised some of the species names with respect to Burridge et al., (2017) (see table 1).

### Genetic diversity

The CO1 barcode region contained enough variation for hyperiid species identification, but was too rapidly evolving to resolve most deeper nodes in a phylogenetic analysis with high support (fig. 2). Nevertheless, the families Brachyscelidae, Cyllopodidae, Cystisomatidae, Dairellidae, Lestrigonidae, Lycaeopsidae, Phronimidae, Pronoidae, Tryphanidae and Vibiliidae were supported as monophyletic clades
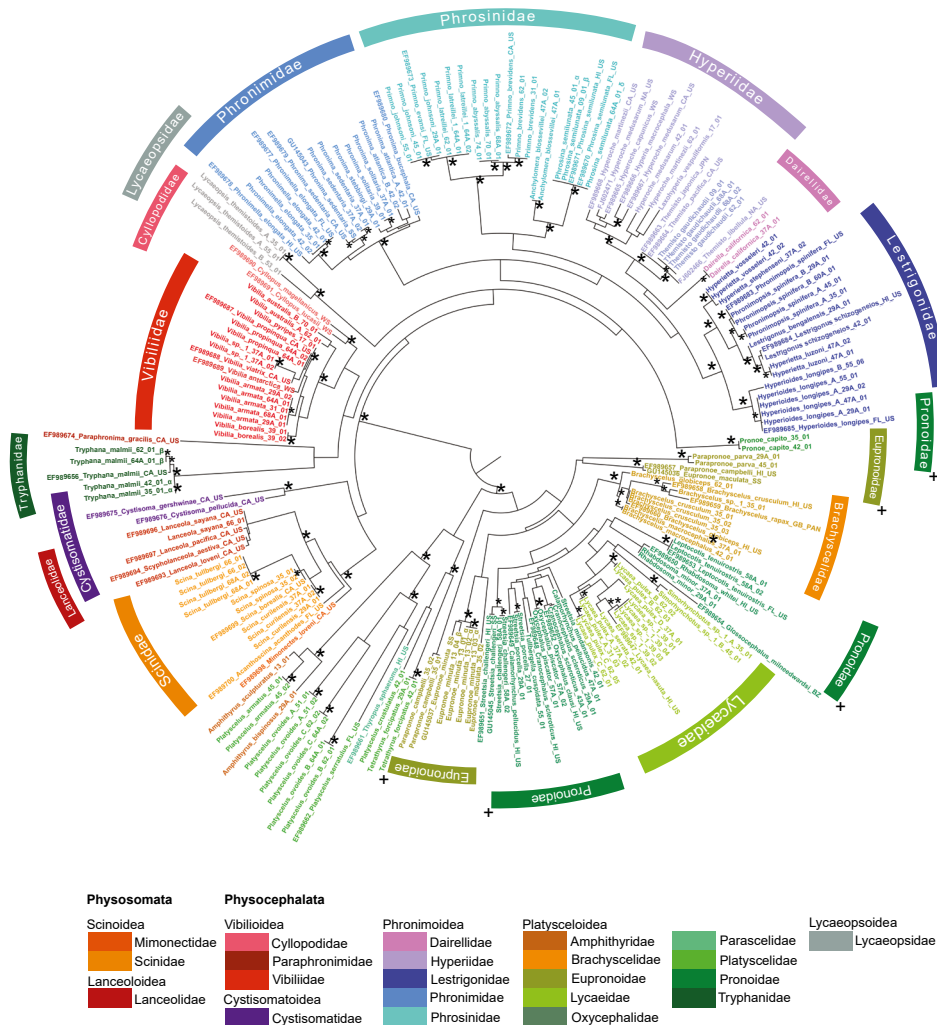


FIGURE 2     Mitochondrial maximum likelihood phylogeny for hyperiid amphipods. Analysis based on partial cytochrome c oxidase 1 sequences representing 63 identified species from this study (table 1) as well as 56 sequences from Genbank (table S2). Bootstrap values >80% are indicated with an asterisk (*). Families are indicated by color and arranged by infraorder and superfamily of the suborder Hyperiidea (taxonomy as in Horton et al., 2016; Zeidler 2016; see also table S3).

(>80% bootstrap support; fig. 2). The average genetic distance between specimens within families was 18.9% and ranged from rather low in Dairellidae (3%, range 2–4%), Pronoidae (0.4%, range 0–1%) and Tryphanidae (3.6%, range 3–4%) to highest in Platyscelidae (40.4%, 38–43%), but note the uneven sampling of individuals and species across taxa (table 1; table S3).

Average genetic distances within and between morphologically identified species were 4.4% (0.6% s.d.) and 41.9% (3.5% s.d.), respectively. However, many morphologically identified species contained variation indicative of (cryptic) species complexes and were considered as distinct OTUs based on ABGD analysis (table 1; table S4). All OTUs identified by ABGD were also separated in different 'bins' in the Barcode of Life Database (BOLD) and these analyses were largely congruent, except in some cases where no bin could be assigned (e.g. *Brachyscelus* sp. 1, *Lestrigonus* sp. 1) or when more bins were assigned than OTUs (e.g. *Phronimopsis spinifera*, *Phrosina semilunata*). Within these species complexes, between two and four mitochondrial clades were observed in phylogenetic analyses that sometimes were congruent with morphological features and/or geography. We have provisionally indicated these clades with letters (A, B, C, D), but further research is required to verify their population or (cryptic) species status. Within nine morphospecies, we found substantial average sequence divergence between clades, namely in *Hyperioides longipes* Chevreux, 1900 (A, B: 17.2%, range 15–19%), *Lycaea pulex* (A, B, C, D: 24.3%, range 19–28%), *Lycaeopsis themistoides* Claus, 1879 (A, B: 26.7%, range 24–29%), *Phronima atlantica* Guérin-Méneville, 1836 (A, B: 21.4%,

range 19–24%), *Phronimopsis spinifera* (A, B: 13.6%, range 12–15%), *Platyscelus ovoides* (Risso, 1816) (A, B, C: 36.1%, range 26–45%), *Primno brevidens* (A, B: 11.4%, range 10–13%), *Simorhynchotus* sp. 1 (A, B: 13.6%, range 12–15%), *Vibilia australis* Stebbing, 1888 (A, B: 15.4%, range 14–17%) (table S4). In three other morphospecies, we observed moderate sequence divergences between clades that were still resolved as separate OTUs in the ABGD analysis, namely *Eupronoe minuta* Claus, 1879 (A, B: 6.1%, range 5–7%), *Phrosina semilunata* (A, B, C, D: 6.4%, range 3–10%) and *Tryphana malmii* (A, B: 5.2%, range 4–6%) (table S4). Overall, we observed high levels of sequence divergence within and between described species and there was no clear barcode gap. If we exclude variation between divergent clades within morphospecies, the overall average sequence divergence within species was 1.4% (range 0–7%) (table S4).

### *Genus* Lycaea

A total of 18 specimens were sequenced from the genus *Lycaea*, of which 13 were morphologically identified as *L. pulex* Marion, 1874, one was identified as *L. serrata* Claus, 1879, and four specimens were named *Lycaea* sp. 1 (Burridge et al., 2017; fig. 3). *Lycaea pulex* was sampled from 12 °N to 34 °S and appears to have a broad latitudinal distribution in the Atlantic Ocean (table 1; fig. 3). This species was composed of four mitochondrial clades, designated here as *Lycaea pulex* A, B, C and D. *Lycaea pulex* A (N = 7) was restricted to tropical waters and the southern edge of the North Atlantic Gyre (NATR and WTRA). *Lycaea pulex* B (N = 3) and *L. pulex* C (N = 2) were sympatric and sampled at a single station in the transition zone between the South
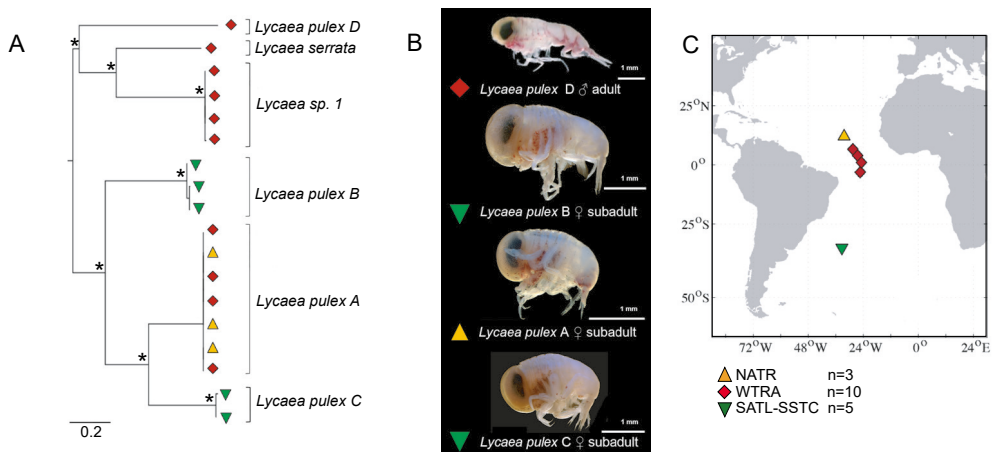
FIGURE 3    Phylogenetic analysis of the genus *Lycaea*. A) Maximum likelihood tree based on CO1
sequences showing relationships between clades supported by ABGD analyses: *Lycaea pulex* A,
*Lycaea pulex* B, *Lycaea pulex* C, *Lycaea pulex* D, *Lycaea serrata* and *Lycaea sp. 1*. ML bootstrap
values >80% are indicated with an asterisk (*). B) Lateral views of *Lycaea pulex* A, *Lycaea
pulex* B, *Lycaea pulex* C, and *Lycaea pulex* D (scale bar is 1 mm). C) Map showing collection
locations, with Longhurst oceanic provinces marked by symbols and abbreviations (see also
fig. 1 and table 1).

Atlantic Gyre and the South subtropical
convergence (SSTC), and *L. pulex* D (N =
1) was sampled only once in the equato-
rial upwelling region (WTRA; fig. 3). The
genetic distance between these four clades
ranged from 19% to 32%, and within clades
from 0 to 2% (table S4).

## *Phronimopsis spinifera*

The only member of the genus
*Phronimopsis*, *P. spinifera*, differs mark-
edly from other genera in the family
Lestrigonidae in the unique structure of
pereopod #2 (P2) and the phronimid-look
of females (Claus 1879). The 17 CO1
sequences obtained from *P. spinifera* speci-
mens were separated into two OTUs (table
1, fig. 4). The genetic distance between the
clades was 13.6% and ranged from 0 to 4%
within clades (table S4). *Phronimopsis spi-
nifera* A (N = 4) was restricted to the meso-
trophic tropical regions of the Atlantic,
while *P. spinifera* B (N = 13) occurred

mostly in both the North and South oligo-
trophic subtropical gyres.

## *Phrosina semilunata*

We observed morphological variation
among specimens of *Phrosina semilunata*,
mainly in denticle formation on the carpus
of pereopods #4 and #5 and the shape of
the head. This species was sampled from
39 °N to 35 °S across subtropical and tropi-
cal waters in the Atlantic Ocean (table 1;
fig. 5). ABGD and phylogenetic analyses
placed the 13 *P. semilunata* CO1 sequences
into four OTUs or clades (fig. 5). Pairwise
genetic distances between these clades
ranged from 3–10% and within clades
from 0 to 1% (table S4). Clades A (N = 5)
and C (N = 2) were restricted to subtropi-
cal and tropical regions in the Atlantic
Ocean. Clade B (N = 3) was only found
in the North Atlantic Ocean and grouped
with a sequence of *P. semilunata* from
Florida. Clade D (N = 3) was only found in
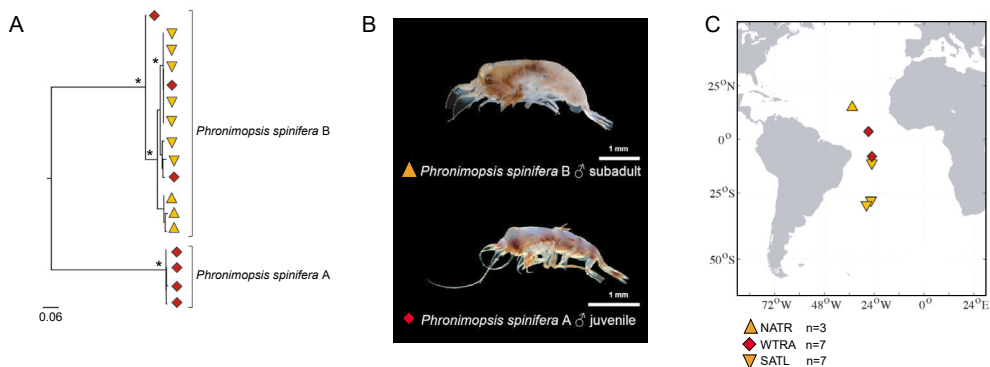
FIGURE 4    Phylogenetic analysis of *Phronimopsis spinifera*. A) Maximum likelihood tree based on CO1 sequences showing relationships for clades A and B identified by ABGD analysis. Bootstrap values >80% are indicated with an asterisk (*). B) Specimen lateral views (scale bar is 1 mm). C) Map showing collection locations, with Longhurst oceanic provinces marked by symbols and abbreviations (see also fig. 1 and table 1).
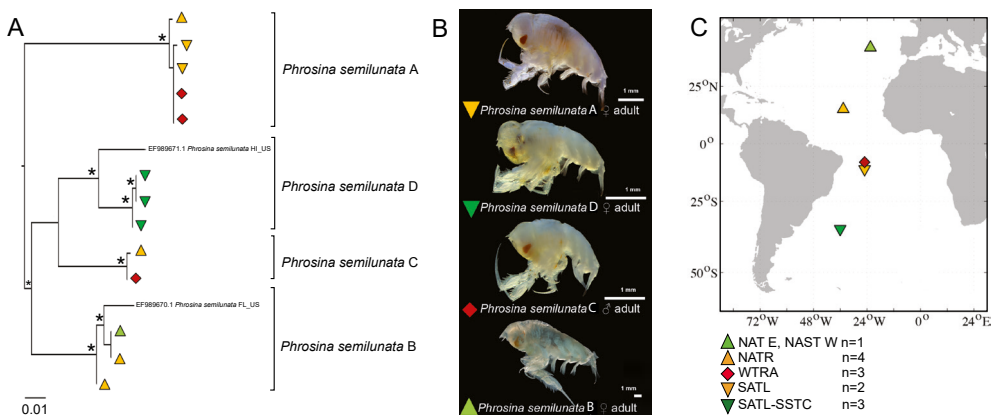


FIGURE 5    Phylogenetic analysis of *Phrosina semilunata*. A) Maximum likelihood tree based on CO1 sequences showing four divergent clades supported by ABGD analyses. Bootstrap values >80% are indicated with an asterisk (*). B) Lateral views of *Phrosina semilunata* A, *Phrosina semilunata* B, *Phrosina semilunata* C, *Phrosina semilunata* D (scale bar is 1 mm). C) Map showing collection locations, with Longhurst oceanic provinces marked by symbols and abbreviations (see also fig. 1 and table 1).

the convergence region (SATL-SSTC) and interestingly, grouped with a sequence of the same species from Hawaii (fig. 5, table S2).

### *Tryphana malmii*

The 10 CO1 sequences obtained from *T. malmii* specimens consisted of two clades with average genetic distance of 5.2%

with separate geographic distributions. *Tryphana malmii* A (N = 4) was only found in the tropical equatorial upwelling region (WTRA) and *Tryphana malmii* B (N = 6) was restricted to the colder transition zone between the South Atlantic gyral (SATL) and the Southern subtropical convergence (SSTC) province (fig. 6). Pairwise genetic distances between these clades ranged
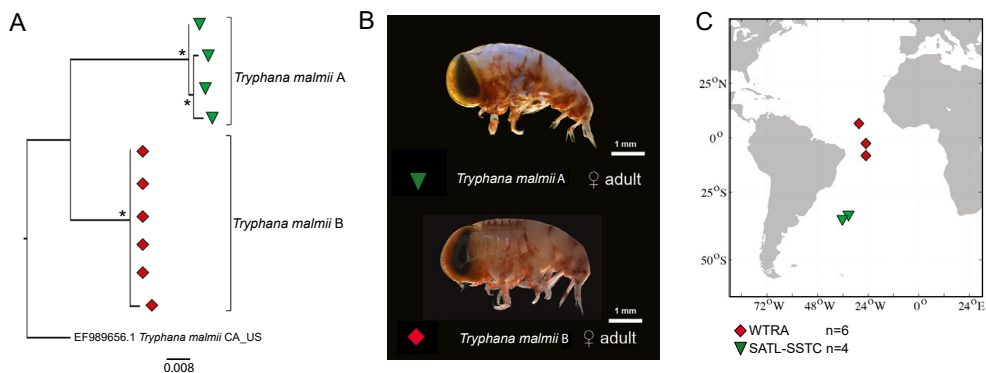
FIGURE 6    Phylogenetic analysis of *Tryphana malmii*. A) Maximum likelihood tree based on CO1
            sequences showing two divergent clades supported by ABGD analyses. Bootstrap values >80%
            are indicated with an asterisk (*). GenBank sequence EF989656.1 was used to root the tree
            (collection location: California, U S A from Browne et al., 2007). B) Lateral views of *Tryphana
            malmii* A and *Tryphana malmii* B (scale bar is 1 mm). C) Map showing collection locations,
            with Longhurst oceanic provinces marked by symbols and abbreviations (see also fig. 1 and
            table 1)
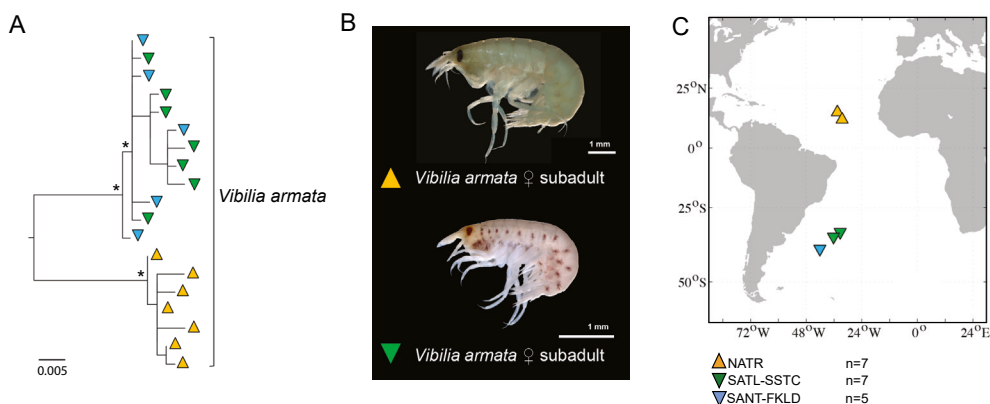


FIGURE 7    Phylogenetic analysis of *Vibilia armata*. A) Maximum likelihood tree based on CO1 sequences
            showing two geographic clades. Bootstrap values >80% are indicated with an asterisk (*).
            B) Lateral views of *Vibilia armata* female subadult specimens belonging to distinct clades
            are shown (scale bar is 1 mm). C) Map showing collection locations, with Longhurst oceanic
            provinces marked by symbols and abbreviations (see also fig. 1 and table 1).

from 4–6% and within clades from 0 to 1%
(table S4).

## *Vibilia armata*

CO1 sequences were obtained from 19
specimens of *Vibilia armata* collected in
the Northwest Atlantic subtropical gyral

(NATR) and Southern subtropical conver-
gence (SSTC) provinces (fig. 7). Although
all specimens were placed within the
same OTU by ABGD analyses, a ML phy-
logeny supports two clades with average
genetic distance of 2.2% between them
(range 2–3%). There is clear geographic

separation between these two clades with one clade restricted to the North Atlantic subtropical gyre (NATR) and one clade restricted to the colder transition and southern convergence waters (SATL-SSTC, SANT-FLKD).

### Genus Primno

Juvenile specimens of the genus *Primno* are difficult to identify due to the highly variable claw-like structure and denticle patterns on the carpus of pereopod #5 (P5). We identified two specimens as *Primno brevidens*, which had 11.4% sequence divergence between them, but also grouped with a Genbank sequence of the same nominal species from the Pacific. We further recovered three additional distinct clades in phylogenetic analyses of CO1 sequences from 91 *Primno* specimens
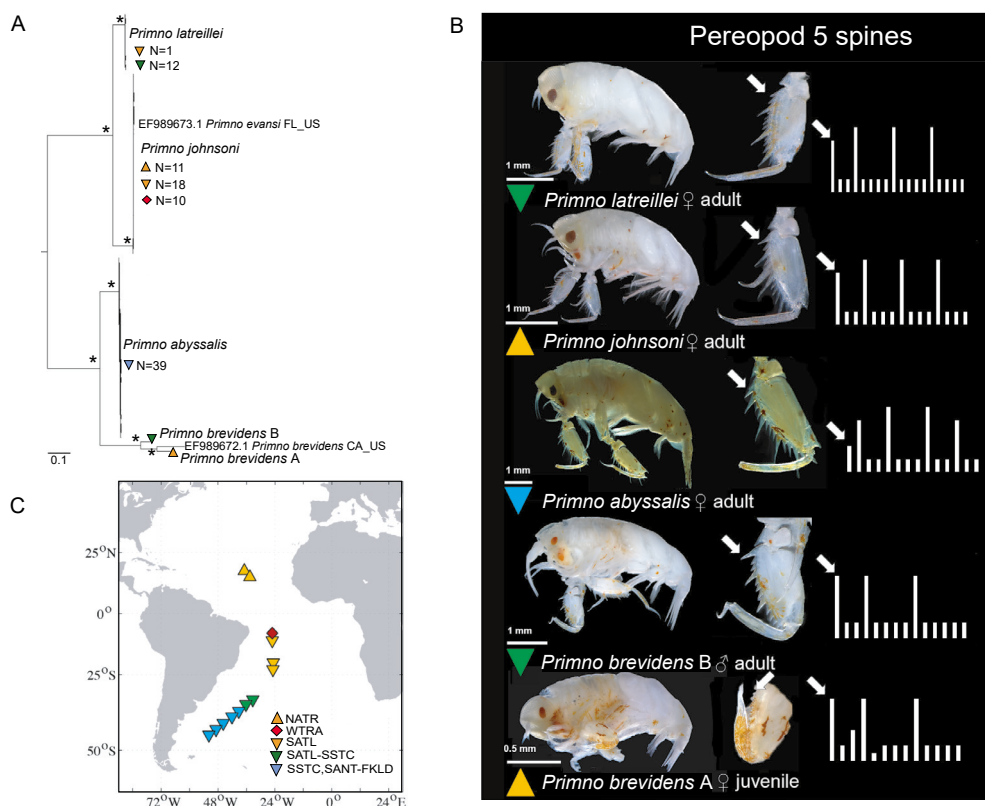


FIGURE 8 Phylogenetic analysis of the genus *Primno*. A) Maximum likelihood tree based on CO1 sequences showing relationships between *Primno abyssalis*, *Primno brevidens* A, *Primno brevidens* B, *Primno johnsoni* and *Primno latreillei*, including GenBank sequences EF989672.1_Primno_brevidens and EF989673.1_Primno_evansi from Browne et al., (2007). Bootstrap values >80% are indicated with an asterisk (*). B) Lateral views of each species with images and bar-graphs of the denticle patterns on the carpus of pereopod #5 next to specimen photographs to highlight distinctive characters for species in the genus *Primno* (scale bar is 1 mm). C) Map showing collection locations, with Longhurst oceanic provinces marked by symbols and abbreviations (see also fig. 1 and table 1).

(fig. 8). Average genetic distance between these clades was 16.9% (range 9.1–20.9%), with limited variation observed within the clades (0.4%, range 0.2–0.9%). Re-examining the morphology of adult specimens of each clade resulted in identifying the three clades as *Primno johnsoni*, *Primno latreillei* and *Primno abyssalis*, each with distinct denticle patterns on the carpus of pereopod #5 corresponding to the original descriptions. Specimens of *P. johnsoni* were found across subtropical and tropical Atlantic waters (NATR, WTRA, SATL). *Primno latreillei* was abundant in the southern convergence waters (SATL, SSTC) and *Primno abyssalis* was found in the colder and eutrophic waters of the subantarctic province (SANT-KFLD, fig. 8). According to Bowman (1985), *P. abyssalis* is restricted to the subarctic province of the North Pacific and is closely related to *Primno macropa* Guérin-Méneville, 1836. However, the denticle pattern on the carpus of P5 observed in adult specimens in our material corresponded with the original description.

## Discussion

We report here on a spatially extensive collection of new CO1 DNA barcodes developed for the ecologically important suborder Hyperiidea in the Atlantic Ocean. These taxonomically vouchered CO1 barcodes expand existing knowledge by 30 species for which no CO1 data is currently available (table 1), increasing total CO1 database coverage by 10.5% of hyperiid species globally. New CO1 sequences for 17 hyperiid species are the first barcodes available from the Atlantic Ocean, expanding this ocean's taxonomically vouchered database by 15.1%. Species newly reported for CO1 include representatives from 13 hyperiid familes, including for example three of the six *Vibilia* species, two of three *Streetsia* species, all three *Scina* species, two of four *Primno* species, two of three *Platyscelus* species, all *Lycaea* species, two of three *Hyperietta* species, and three of four *Brachyscelus* species that were sampled study-wide. Our new CO1 records are particularly valuable in that morphological identification was to species by an expert amphipod systematist (R. Vonk), and specimens and DNA were vouchered into the collection at Naturalis Biodiversity Center, creating a permanent repository of material that can be re-examined by future workers on hyperiid biodiversity. While we recognize that there are instances where incomplete specimen information may accelerate species discovery and description (Rogers et al., 2023), full expert identification of vouchered material remains the gold standard for linking morphological to genetic observations at the species level. Finally, development of a locally relevant and vouchered CO1 barcode database enhances taxonomic classification of sequence information in metabarcoding studies (Pappalardo et al., 2021; Questel et al., 2021), largely by reducing the incomplete taxon sampling that plagues most reference databases. Reference databases used to classify genetic data often contain a minority (< 50%) of the species known to be present in any given ecosystem (Hestetun et al., 2020; Keck et al., 2022), a problem exacerbated in communities with small-bodied organisms and/or at deeper depths in the ocean. New CO1

sequences reported here greatly enhance existing knowledge on the genetic diversity of the hyperiids in the Atlantic, and support ongoing and future research to better incorporate this taxonomic group into large-scale biodiversity surveys.

Though the CO1 barcode region was highly variable and suitable for hyperiid species recognition and discovery, phylogenetic relationships at higher taxonomic levels were largely unresolved (fig. 2) and should be treated with caution. Earlier multi-locus phylogenetic analyses based on three nuclear (18S, 28S, H3) and one or two mitochondrial loci (CO1, 16S) (Hurt et al., 2013; Copilaş-Ciocianu et al., 2020; Biancani 2019) focussed on resolving these higher level relationships and found that some taxonomic revisions are in order because of convergent evolution of morphological structures (especially appendages). Particularly, the genera *Cystisoma* and *Paraphronima* group more closely to Physosomata than Physocephalata, which is what we found based on including published sequences (fig. 2). However, as we did not sample new specimens from these species we cannot shed new light on this issue. There has also been a discussion as to whether the hyperiids represent a single colonization of the open water column. Two studies that included both hyperiid and non-hyperiid outgroups recovered the suborder Hyperiidea (Physocephalata + Physosomata) as a monophyletic clade (Browne et al., 2007; Biancani 2019). However, interestingly, Copilaş-Ciocianu et al., (2020) conclude that the pelagic environment was independently colonized twice based on an analysis of ecological transitions and the inclusion of three benthic genera (*Leucothoe*, *Amphilochus*,

and *Colomastix*) within Physosomata. We agree with previous authors that a phylogenomic approach (e.g. as in Wolfe et al., 2019) would be most valuable to provide a robust phylogeny on which to base systematic revisions as well as conclusions about hyperiid and amphipod evolution.

Phylogenetic and ABGD analyses of hyperiid CO1 sequences revealed substantial within-species diversity in several cases. Overall, we detected a total of 29 distinct clades or OTUs within twelve described species identified by morphotaxonomy (table 1). For nine morphospecies we recovered high levels of genetic divergence between clades ranging from 10 to 45% (e.g. average divergence between clades for *Lycaea pulex*: 24.3% and *Platyscelus ovoides*: 36.1%) and for three morphospecies we recovered moderate genetic divergence between clades ranging from 3 to 10% (e.g. average divergence between clades for *Tryphana malmii*: 5.2%, table S4). Compared to sequence divergences reported for the CO1 gene between cryptic clades/lineages reported in other pelagic crustaceans (ranging from 1.8 to 55.6% for copepods, 3–24% for euphausiids, 2–17.7% for mysids, and 2–32.9% for ostracods, reviewed in Sprong, Peijnenburg, in preparation) our estimates fall on the high end of this scale. Within the hyperiid genus *Themisto* comparable genetic distances were reported between Atlantic and Pacific *T. libellula* 9.7%) as between Atlantic *T. libellula* and *T. compressa* (10.0%), which was interpreted as evidence for a species complex (Murray et al., 2023). Similar distances between other *Themisto* species were also reported by Tempestini et al., (2017). Another example by Kulagin et al., (2021)

examined species boundaries within a complex of krill species and found that intraspecific COI distances ranged from 2.7–4.3%, and interspecific distances ranged from 9.7–14.6%. However, they also found an intermediate case where two clades of *N. megalops* differed by 5.8%. In a thorough follow-up study, Kulagin et al., (2024a) examined additional information from two nuclear markers and morphological analysis of 131 specimens from the Atlantic, and concluded that six clades within this complex represent distinct species of varying evolutionary ages and that taxonomic surprises can be expected even in ostensibly well studied groups. Similarly, in the hyperiid genus *Paraphronima* Stenvers et al., (2021) uncovered at least five distinct genetic clades with congruent support from morphology in most cases, which led to a description of a new species. Considering the uneven and limited sampling of morpho-species composed of distinct OTUs in this study, ranging from only two individuals (e.g. *Primno brevidens*, *Phronima atlantica*) to a maximum of 17 individuals (*Phronimopsis spinifera*), our estimates of genetic divergences within and between OTUs are preliminary and OTUs should be considered as species hypotheses that need to be tested with additional data.

For several species sampled at a range of locations in the Atlantic Ocean, including *Lycaea pulex*, *Phronimopsis spinifera*, *Phrosina semilunata*, and *Tryphana malmii*, we found congruent support from ABDG analyses, reciprocal monophyly among clades, and evidence of geographic specialization for all or nearly all lineages (figs. 3, 4, 5, 6). These four species complexes have lineages with apparent geographic specialization to tropical (*Phronimopsis spinifera* A; *Tryphana malmii* B), subtropical (*Phronimopsis spinifera* B) or temperate waters (*Lycaea pulex* B and C; *Phrosina semilunata* D; *Tryphana malmii* B), suggesting more restricted biogeographic distributions than currently described for the inclusive morphospecies (Vinogradov et al., 1996; Lima & Valentin 2001; Gasca et al., 2009). Other planktonic crustacean species complexes that exhibited this level of congruence among diverse observations have nearly always been found to be true species following examination of additional genome regions or morphological characters (Andrews et al., 2014; Bode et al., 2017; Cornils et al., 2017; Hirai 2020; Kulagin et al., 2024a, Kulagin et al., 2024b). Within *Vibilia armata*, we also found two well-supported clades with 2–3% divergence even though these were not considered separate OTUs in the ABGD analysis. Here we also find a clear geographic separation with one clade restricted to the Northern and the other clade restricted to the Southern subtropical gyre (fig. 7). Similar patterns of phylogeographic structure with dispersal barriers across the equatorial upwelling region in the Atlantic have also been reported in species of copepods (Norton & Goetze 2013; Andrews et al., 2014), euphausiids (Kulagin et al., 2021), decapods (Kulagin et al., 2024b), pteropods (Burridge et al., 2015; Choo et al., 2021), and heteropods (Wall-Palmer et al., 2016). Based on hyperiid species assemblages Burridge et al., (2017) found similar divisions into gyral, equatorial, transitional, and subantarctic provinces, which were largely congruent with Longhurst's

biogeochemical provinces. These patterns may be driven, at least in part, by the distribution and diversity of gelatinous hosts for these species. However, both the particular associations and the distribution of gelatinous zooplankton remain poorly characterized. For instance, *Lycaea* and *Vibilia* species are known associates of different salps species (Madin & Harbison 1977), while *Phronimopsis spinifera* is associated with rhizarians (Nakamura et al., 2019). It would therefore be of interest to better understand the diversity and distribution patterns of these gelatinous hosts. Because hyperiids often feed on host tissues (Laval 1980), using a metabarcoding approach to examine the gut contents of hyperiids could be one way of identifying and mapping the gelatinous hosts (e.g. Pompanon et al., 2012).

Collectively, divergent clades or OTUs identified within nominal hyperiid species may represent new species, subspecies or isolated populations, however, this is impossible to determine based on our limited sampling and mitochondrial divergence alone. To verify species boundaries and formally describe new species, evidence is needed for reproductive isolation from independent characters such as nuclear genetic markers and/or morphological examinations. There were also a few cases in which Burridge et al., (2017) reported morphological differentiation among specimens and included these as putative unknown species. We report new genetic observations for these taxa and found that indeed *Lycaea* sp. 1, *Brachyscelus* sp. 1 and *Simorhynchotus* sp. 1 were highly distinct from any described species warranting further taxonomic examination. A large number of cryptic lineages were discovered despite modest sequencing effort of within-species diversity in this study (up to a maximum of N = 39 specimens for *Primno johnsoni* and *P. abyssalis*). Recent studies on integrative taxonomy and phylogeny of *Paraphronima* and *Themisto* yielded similar outcomes (Tempestini et al., 2017; Stenvers et al., 2021), with (likely) cryptic species identified despite low sampling effort. Altogether, these results highlight the extent of unknown biodiversity within the Hyperiidea and the need for more extensive work on this group of enigmatic pelagic crustaceans.

## Acknowledgements

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