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Characterization of diversity and community structure of small planktonic copepods in the Kuroshio region off Japan using a metabarcoding approach

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ABSTRACT: Small copepods are important prey for fish larvae in the Kuroshio region off southern Japan. However, revealing entire community structures of small copepods is difficult using conventional methods. We applied a metabarcoding method to size-fractionated community samples of epipelagic copepods (small: 0.1–0.5 mm, medium: 0.5–1.0 mm, and large: 1.0–2.0 mm). Samples were collected from 2013 to 2016 from the shoreward (Kuroshio Slope, KS) and oceanic (Kuroshio Gyre, KG) sides of the Kuroshio Current at 138° E; the results were compared with those in the center of the subtropical gyre (SG). The KS and KG sites showed both spatial differences and seasonal changes, with distinct differences between winter–spring and summer–autumn in each size-fractionated community. Water temperature markedly influenced copepod diversity and community structure, especially in the small size fraction. Warm-water species in the SG intruded into the Kuroshio regions during high-temperature periods, leading to high diversity in summer–autumn. Inter-annual environmental variations influenced by temperature and productivity were evident in KS, leading to clear changes in the sequence proportions of dominant small copepods including *Paracalanus* sp. and immature stages of the large copepod *Calanus sinicus*. Immature stages of medium/large copepods formed a substantial proportion of small-copepod communities in the Kuroshio regions (KS: 28.8%; KG: 24.7%; SG: 11.9%; based on average proportions of sequence reads). Because of their ecological importance and sensitivity to environmental changes, monitoring communities of small copepods with high taxonomic resolution may provide further insights into marine ecosystems, including fish recruitment, in the Kuroshio region.

KEY WORDS: Small copepods · Metabarcoding · Kuroshio · Diversity · Community structure

1. INTRODUCTION

In oceanic pelagic zones, small zooplankton communities are mostly composed of planktonic copepods; small copepods (<1 mm) are considered to be among the most numerous metazoans on Earth (Turner 2004). Out of a total of >2700 marine planktonic copepod species (Razouls et al. 2005–2020), the naupliar stages of almost all species are included in the small-copepod category, in addition to copepodite and adult-stage small copepods including the genera *Acartia*, *Clausocalanus*, *Corycaeus*, *Micro-*

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setella, *Paracalanus*, *Pseudocalanus*, *Oithona*, and *Oncaea* (Gallienne & Robbins 2001, Turner 2004). Although the feeding ecology of small copepods has not been investigated thoroughly, they use a variety of food sources, including phytoplankton, protists, microzooplankton, and bacterioplankton, thereby having a substantial impact on marine ecosystems (Berggreen et al. 1988, Roff et al. 1995, Paffenhöfer 1998, Jungbluth et al. 2017). Small copepods themselves are important prey for several fish larvae, and they connect the lower to higher trophic levels via the classical food chain and microbial loop (Turner 1984). Small copepods are sensitive to environmental changes, and their abundance and community structure affect the recruitment of species that are important for fisheries (Capuzzo et al. 2018). Therefore, it is necessary to investigate small-copepod communities to increase our understanding of marine food webs.

The western subtropical North Pacific has a high diversity of pelagic taxa, including copepods (Rombouts et al. 2009, Tittensor et al. 2010). In this area, the Kuroshio, which is a strong and warm western boundary current, flows along the edge of the North Pacific Subtropical Gyre and transports diverse planktonic species from lower latitudes (Shih & Chiu 1998). The Kuroshio flows eastward off the southern coast of Japan, and numerous commercially important fish, including Japanese sardine and Pacific saury, use the Kuroshio region off Japan as their spawning and nursery grounds (Sugisaki et al. 2010). Owing to the large hydrographic changes induced by the Kuroshio Current, different copepod communities exist in regions shoreward of the Kuroshio Current compared with those in the core and southern parts of the current (Kidachi & Ito 1979, Shimode et al. 2006, Miyamoto et al. 2017, Sogawa et al. 2017). Moreover, there is an inter-annual variation of copepod biomass associated with the Southern Oscillation Index in the Kuroshio region, which might affect the stocks of commercially important fish spawning in this area (Nakata & Hidaka 2003). The abundance of small copepods has been underestimated in previous studies, because these mainly used coarse mesh sizes (≥330 µm) (Gallienne & Robbins 2001, Turner 2004). In addition, identifying small copepods at the species level is difficult. Therefore, the diversity and community structure of small copepods need to be comprehensively evaluated in the Kuroshio region.

The molecular classification method based on species-specific DNA sequences is effective for accurately identifying small copepods. Sanger sequencing (Llinás 2008) and DNA hybridization methods (Kiesling et al. 2002), along with quantitative PCR, have been applied to copepod nauplii to reveal the life history of specific copepod species (Jungbluth et al. 2013, Fujioka et al. 2015). However, these methods are labor-intensive for revealing the structure of entire communities of small copepods, owing to their abundance and diversity. In contrast, metabarcoding analysis using a high-throughput sequencer can reveal the community structure rapidly and comprehensively without depending on morphological classifications (Lindeque et al. 2013, Hirai et al. 2015). Metabarcoding analyses have already been applied to show spatial and temporal changes in zooplankton communities and diversity in the open ocean (Hirai & Tsuda 2015, Casas et al. 2017, Sommer et al. 2017, Deagle et al. 2018).

The ecosystem across the Kuroshio Current has been monitored since 2001 at 138° E off Cape Omaezaki in Japan (the 'O-line') (Fig. 1A) to investigate seasonal and annual changes in environmental and biological data, including zooplankton (Sugisaki et al. 2010, Kodama et al. 2014). A metabarcoding method for copepods based on the 28S D2 region was developed using zooplankton samples collected along this monitoring line, and ample 28S D2 DNA barcoding data have been recorded for planktonic copepods in this region (Hirai et al. 2015, 2017b). From 2013 to 2016, we collected seasonal zooplankton samples along the O-line from both the coastal side (Kuroshio Slope, KS) and the oceanic side (Kuroshio Gyre, KG) of the Kuroshio Current (Fig. 1A). Samples were also collected from the core of the Kuroshio Current (Kuroshio Core, KC) along the monitoring line in 2015. Zooplankton samples were size-fractionated and used for metabarcoding analysis to reveal the diversity and community structure of small copepods. The results were compared with those collected at a lower latitude in the North Pacific Subtropical Gyre (SG) to characterize the small-copepod communities in the Kuroshio region.

2. MATERIALS AND METHODS

2.1. Sampling

Seasonal samples of zooplankton in the Kuroshio region were collected between 2013 and 2016 as part of the ocean monitoring program of the O-line at 138° E off Japan (Fig. 1) aboard the FRV 'Soyo-maru' (Japan Fisheries Research and Education Agency). Sampling occurred in 5 different seasons (winter, early spring, late spring, summer, and autumn), pri-

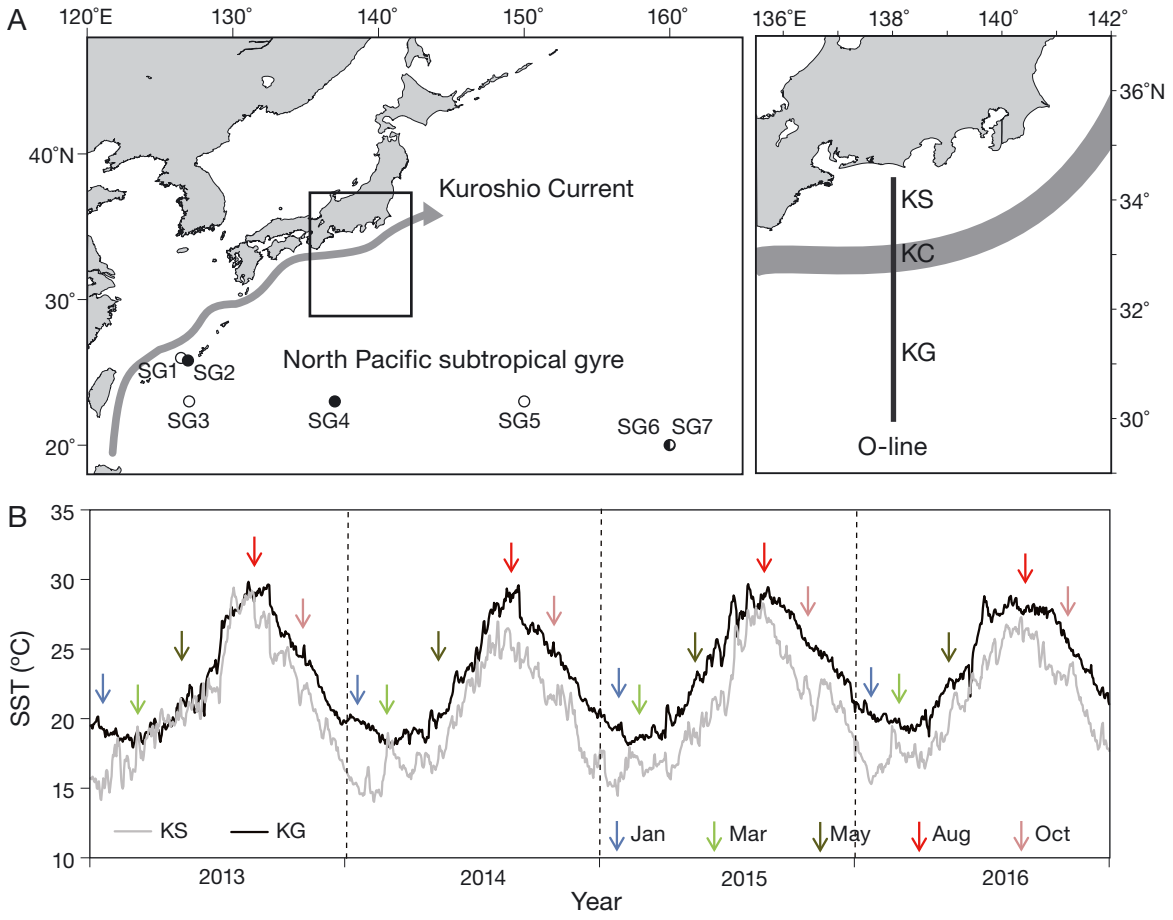

Fig. 1. (A) Sampling locations. Seasonal samples in the Kuroshio region off Japan were collected shoreward of the Kuroshio Current (Kuroshio Slope, KS) and on the oceanic side (Kuroshio Gyre, KG) from 2013 to 2016 along the monitoring line at 138° E (O-line). A typical Kuroshio Current pattern during this period is indicated, and seasonal sampling for the core of the Kuroshio Current (Kuroshio Core, KC) was performed in 2015. Seven samples (SG1–7) were collected at 6 stations at lower latitudes of the North Pacific Subtropical Gyre (SG). Black (white) circles show samples obtained at night (day). (B) Temporal variation of satellite data of sea surface temperature (SST) in KS and KG during 2013 and 2016. Detailed sampling information is provided in Table S1

marily in January, March, May, August, and October. Zooplankton samples were collected from the epipelagic layer (0–200 m) using a North Pacific Standard Plankton (NORPAC) net with a 100 µm mesh and a mouth opening of 0.16 m^2, mainly at night (37/44 samples). We analyzed 17 samples from KS (shoreward of the Kuroshio Current) at 33° 45′ to 34° 30′ N and 15 samples from KG (oceanic side of the Kuroshio Current) at 30° 00′ to 32° 00′ N (Table S1 in the Supplement at www.int-res.com/articles/suppl/m657p025_supp.pdf). The location of the Kuroshio axis at 138° E was determined based on a water temperature of 15°C at 200 m depth (Kawai 1972). Only 5 samples from 2015 were analyzed for KC. Seven zooplankton samples from SG (low latitude in the North Pacific SG) at 20° 00′ to 25° 59′ N were collected during 4 research cruises aboard the RV 'Hakuhomaru' (Japan Agency for Marine-Earth Science and Technology). Sampling in SG was also conducted using a NORPAC net. Zooplankton samples were size-fractionated using nylon meshes of 0.1, 0.5, 1.0, and 2.0 mm. We classified the zooplankton as small (0.1–0.5 mm), medium (0.5–1.0 mm), or large (1.0–2.0 mm). The zooplankton samples were preserved in 99% ethanol and the ethanol was replaced within 24 h. Ethanol-preserved samples were stored in the dark at 4°C until molecular analysis was carried out in the laboratory.

During each research cruise, the vertical water temperature profile was obtained using a CTD system (SBE-911 Plus; Sea-Bird Electronics). Water samples were collected using Niskin bottles attached to the CTD system and were filtered using Whatman GF/F filters (GE Healthcare) for chlorophyll *a* (chl *a*) analysis. Chl *a* was extracted with *N*,*N*-dimethylformamide, and its concentration was analyzed using

a Turner fluorometer (Turner Designs). We obtained environmental data for all seasons during sampling periods, although zooplankton samples were not collected during some cruises. When zooplankton samples were not available, we mainly used environmental data at 34° N in KS and 30° N in KG. Daily satellite data of sea surface temperature (SST) were also obtained for KS and KG during 2013 and 2016. The high-resolution National Oceanic and Atmospheric Administration (NOAA) optimum interpolation SST (OISST) version 2 dataset, which was described by Reynolds et al. (2007), was used. This dataset has a horizontal grid size of 0.25° × 0.25° and a temporal resolution of 1 h. We obtained the SST data at KS and KG by averaging the SST data within a 0.25° × 0.25° rectangle, centered on 34° and 30° N at 138° E, respectively.

2.2. High-throughput sequencing and bioinformatics analysis

Total genomic DNA was extracted from each size-fractionated sample. Only small and medium samples were analyzed for KG and SG, because there were not enough samples of large copepods. We prepared the libraries for metabarcoding analyses according to the methods of Hirai et al. (2017b). Briefly, DNA was extracted from each zooplankton size fraction using the Gentra Puregene Cell and Tissue Kit (Qiagen). The large ribosomal subunit (LSU) D2 region (approximately 400 bp in copepods) was amplified by PCR using the KOD Plus Version 2 (Toyobo) and the primer pair LSU Cop-D2F (5′-AGA CCG ATA GCA AAC AAG TAC-3′) and LSU Cop-D2R (5′-GTC CGT GTT TCA AGA CGG-3′; Hirai et al. 2015). We added adaptor and index sequences to the second and third PCRs, for multiplex sequencing. Sequencing runs were carried out using a MiSeq Reagent Kit v3 on the Illumina MiSeq in FASMAC, and 2 × 300 bp paired-end sequence reads were obtained from a total of 110 zooplankton samples. Raw reads are available in the NCBI/EBI/DDBJ Sequence Read Archive (BioProject accession PRJDB8486).

The detailed commands for bioinformatics analysis followed the method of Hirai et al. (2020). They are available in the Dryad repository (doi:10.5061/dryad.x95x69pdt). Trimmomatic was used to carry out the initial quality-filtering for the raw sequence reads (Bolger et al. 2014). Adaptor and low-quality sequences (<100 bp and average <30 quality in every 30 bp) were removed. Paired-end sequences were merged, and primer regions and reads containing ambiguous bases (Ns) were removed using 'mothur' (Schloss et al. 2009). Copepod sequences were selected in this study based on a naïve Bayesian classifier (Wang et al. 2007) using the custom database containing 28S D2 sequences from 257 copepods and 36 other metazoan taxa. After standardization of sequence reads based on a minimum number of reads per sample, all sequence reads were aligned using MAFFT with the 'addfragments' option (Katoh & Standley 2013). We performed further quality-filtering to remove improperly aligned, chimeric, singleton, and non-copepod reads. Possible chimeric sequences were determined using UCHIME (Edgar et al. 2011). We used a 98.5% similarity threshold for sequence clustering into operational taxonomic units (OTUs) using OptiClust (Westcott & Schloss 2017) after a single-linkage pre-clustering and standardization of reads. Minimum sequences of ≥4 were applied to OTUs, and standardized sequences with the same read numbers were used as the final data for the community analyses. The criteria for similarity and abundance thresholds followed Hirai et al. (2020), who validated the bioinformatics analyses using artificial communities containing morphologically identified copepods. For detailed taxonomic assignment, a BLAST search was conducted for representative sequences of copepod OTUs, and OTUs were assigned to species in cases where they showed ≥99% sequence similarity to a single copepod species in the NCBI database.

2.3. Data analyses for diversity and community structure of copepods

The total number of OTUs and the Shannon (H) and Simpson ($1 - D$) diversity indices were calculated to assess spatial and temporal changes in copepod diversity in each size fraction using PRIMER version 7 (Clarke & Gorley 2015). Data on diversity were analyzed with Kruskal-Wallis and Mann-Whitney tests using SPSS (IBM), with Bonferroni corrections for Mann-Whitney tests in multiple comparisons. Regression analysis was performed in Excel (Microsoft) using the numbers of OTUs in each size fraction as the dependent variable and temperature (at a depth of 10 m) as the independent variable for each station. The numbers of OTUs shared with SG were investigated in each season in the Kuroshio region.

We identified the copepod community structures in all samples based on the Bray–Curtis similarity index using cluster and multidimensional scaling (MDS) analyses in PRIMER. The proportions of sequence

reads in each OTU were $\log_{x+1}$-transformed. The differences among cluster groups were tested using permutational multivariate analysis of variance (PERMANOVA) using the PERMANOVA+ add-on in PRIMER (Anderson et al. 2008). In each clustered group, the key species contributing to the group were evaluated using similarity percentage (SIMPER) analysis. SIMPER analysis was also performed to identify the species contributing to the differences in cluster groups in small-copepod communities. The compositions of copepods were investigated further with the Jaccard similarity index using the presence/absence of OTUs.

Seasonal changes in the dominant copepods in KS and KG were evaluated based on mean proportions of sequence reads in major OTUs in each sampling season, and they were compared to mean values in SG. The top 5 dominant OTUs in the mean sequence reads in each size category (small, medium, and large), season (January, March, May, August, and October), and sampling location (KS, KG, and SG) were defined as the major OTUs. Other copepod OTUs were treated as the non-major OTUs. Cluster analyses were conducted using the same method in PRIMER, and major OTUs were clustered based on their distribution patterns.

In addition to seasonal changes, inter-annual changes in small-copepod communities were evaluated between 2013 and 2016 in KS. We focused on OTUs contributing to small-copepod communities in KS detected by SIMPER analyses. Anomalies in the proportions of sequence reads in each OTU were calculated using the following formula:

$$P'_x = \log_{10}(P_x/P) \quad (1)$$

where P_x is the proportion of sequence reads in OTU x in each small-copepod community in KS, and P is the mean proportion throughout the sampling periods. Anomalies were also calculated for water temperature, chl *a* concentration, and total number of OTUs. Pearson's product moment correlation coefficients (r) were also calculated using SPSS for proportions of sequence reads and water temperature or chl *a* concentration at 10 m depth in the OTUs in small-copepod communities in KS.

Finally, we investigated the contributions of immature stages of medium-sized/large copepods. The proportions of sequence reads for major OTUs of medium-sized/large copepods were calculated in each small-copepod community. The differences of the mean proportions of sequence reads were tested among sampling areas using Mann-Whitney *U*-tests in SPSS.

3. RESULTS

3.1. Water temperature and chl *a* concentration

In the Kuroshio region, SST obtained by satellite data showed the same seasonal patterns in each year from 2013 to 2016, with the highest SST around August and the lowest SST around January in KS or March in KG (Fig. 1B). Throughout the sampling period, SST was generally higher in KG than in KS.

The vertical profiles of CTD data showed that the highest water temperatures at 10 m were 25.7–28.5°C in August, followed by 21.1–24.4°C in October, both in KS (Fig. 2). The water temperatures were relatively low in other seasons; the surface water temperature (at 10 m) tended to increase from January (13.6–16.0°C) to March (14.9–17.6°C) through May (16.7–21.0°C). A clear thermocline was observed in August and October, whereas in other seasons the water temperature steadily decreased with depth. Seasonal changes of chl *a* concentrations were evident in KS, and chl *a* concentrations were higher than those in KG in each season. The surface (at 10 m) chl *a* concentration was lowest in August (0.15–0.28 µg l^{-1}), with a distinct subsurface chl *a* maximum (0.74–1.50 µg l^{-1}). The highest chl *a* concentrations were generally observed in May in KS (up to 1.81 µg l^{-1}).

Smaller seasonal and inter-annual environmental changes were observed in KG. The water temperature (at 10 m) was highest in August (28.2–29.3°C) and October (23.9–26.0°C). The water temperatures were >17.9°C at a depth of 10 m, and mixed layers developed during winter and spring, particularly in January and March. The surface (at 10 m) chl *a* concentrations were relatively high in January (0.33–0.41 µg l^{-1}) and March (0.35–0.55 µg l^{-1}), and distinct subsurface chl *a* maximums were observed in May (0.39–0.49 µg l^{-1}), August (0.36–0.49 µg l^{-1}), and October (0.35–0.42 µg l^{-1}). The maximum subsurface chl *a* was deeper in KG than in KS.

The KC temperature profile showed the same seasonal patterns as those in KS, although the surface water temperature (at 10 m) was higher in winter (>18.4°C) than in KS. As in KG, there was a subsurface chl *a* maximum, particularly from May–October (0.59–0.76 µg l^{-1}), with peak levels higher than those in KG. The SG temperature profile was consistently high (25.1–30.3°C at 10 m), which was similar to the summer KG temperature profile. The chl *a* concentration was low in SG, with a distinct subsurface chl *a* maximum of 0.21–0.55 µg l^{-1}.

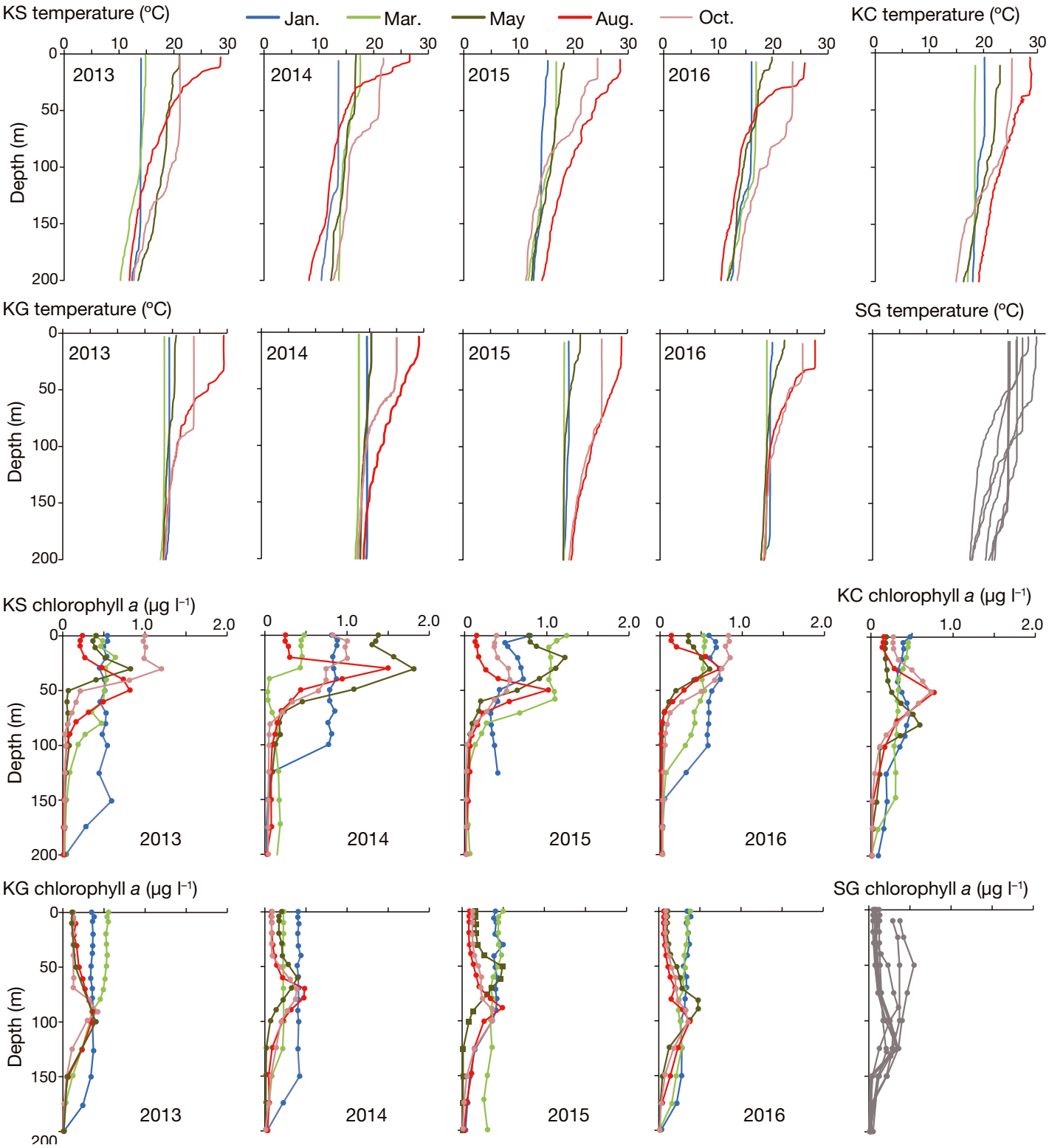

Fig. 2. Vertical water temperature profiles and chlorophyll *a* concentration at 0–200 m. Vertical profiles for different seasons (indicated by different colors) in each year (2013–2016) and region (KS: Kuroshio Slope; KG: Kuroshio Gyre; KC: Kuroshio Core; SG: Subtropical Gyre) in the Kuroshio region. Note that KC sampling was carried out only in 2015. Detailed sampling information for environmental data is listed in Table S1

3.2. Diversity pattern of copepods in the Kuroshio region

The final data for community analyses contained 6589 sequence reads (total 724 790 reads) and 735 OTUs. The numbers of sequence reads filtered during bioinformatics analysis are shown in Table S2. The number of OTUs in each sample varied from 25 (large copepods in May 2014 in KS) to 202 (small copepods in August 2013 in KS), and this number increased with increasing water temperature at a depth of 10 m, with significant correlations for small and large copepods ($p < 0.005$; Fig. 3A). The increase in OTUs with higher water temperature was especially evident in the fraction of small copepods. In each sample, the diversity of copepods decreased with increasing copepod size. The mean number of OTUs was highest for small copepods in all sampling seasons and regions, and most of the copepod communities showed the same pattern in diversity indices (Fig. 3B; Table 1). Significant differences ($p < 0.05$) were only detected between small and medium copepods in August in KG

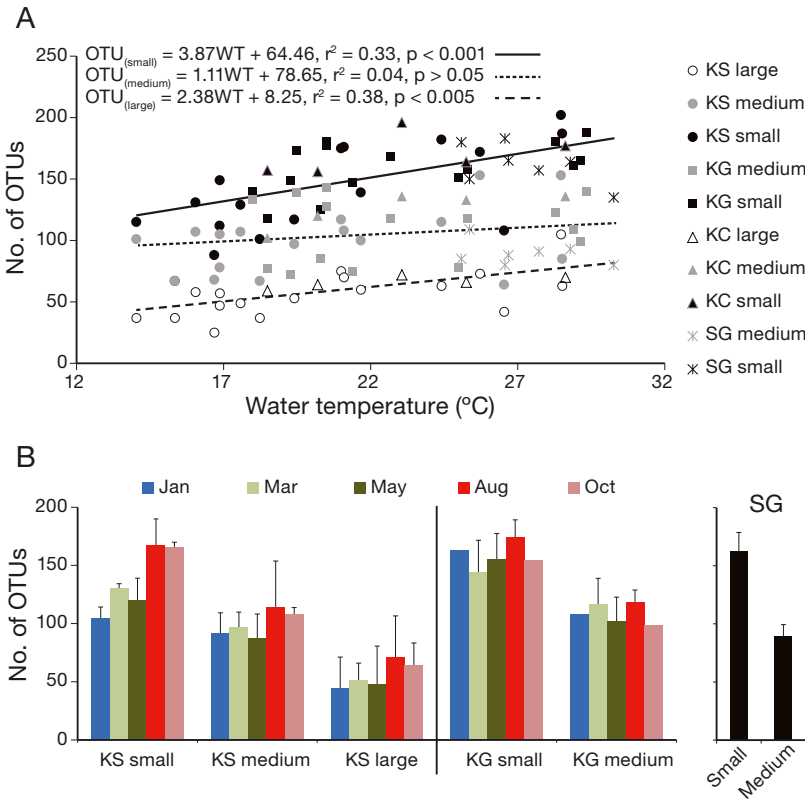

Fig. 3. (A) Relationships between the number of operational taxonomic units (OTUs) and water temperature at a depth of 10 m. A regression analysis was performed for each copepod size category. (B) Seasonal changes in the number of OTUs in the Kuroshio Slope (KS) and the Kuroshio Gyre (KG). Mean number of OTUs are presented for each sampling season. Mean values were used for the Subtropical Gyre (SG). Error bars indicate SD

Table 1. Average diversity indices (Shannon: H; Simpson: $1 - D$) for copepods of different size classes in the Kuroshio region. Also shown are the average numbers of operational taxonomic units (OTUs) shared with the Subtropical Gyre (SG). KS: Kuroshio Slope; KG: Kuroshio Gyre; KC: Kuroshio Core; ND: no data

Area	Month	N	Small			Medium			Large		
			H	$1 - D$	Shared/total OTUs	H	$1 - D$	Shared/total OTUs	H	$1 - D$	Shared OTUs
KS	Jan	3	2.16	0.76	54.1/104.3	2.22	0.70	32.5/91.7	2.14	0.82	ND
	Mar	3	2.38	0.77	67.6/130.0	2.41	0.74	31.2/96.7	2.20	0.80	ND
	May	4	2.74	0.85	62.6/120.3	2.35	0.74	29.4/87.3	2.07	0.78	ND
	Aug	4	3.62	0.94	84.4/167.3	2.76	0.81	39.2/113.8	2.54	0.86	ND
	Oct	3	3.39	0.92	82.0/165.7	3.00	0.88	36.8/107.7	2.79	0.91	ND
KC	Jan	1	3.52	0.94	92.3/156	3.40	0.95	45.1/120.0	2.43	0.87	ND
	Mar	1	3.79	0.97	92/157	3.16	0.93	42.7/102.0	2.55	0.89	ND
	May	1	4.11	0.97	111.6/196	3.37	0.93	56.4/136.0	2.55	0.88	ND
	Aug	1	3.79	0.96	100.9/177	3.52	0.95	50.1/136.0	2.41	0.82	ND
	Oct	1	3.43	0.92	80.3/164	3.08	0.91	44.6/133.0	2.66	0.87	ND
KG	Jan	2	3.59	0.95	91.9/163	3.28	0.93	42.9/107.5	ND	ND	ND
	Mar	3	3.20	0.88	81.8/143.7	2.91	0.87	43.8/116.3	ND	ND	ND
	May	4	3.67	0.94	87.6/155.3	3.05	0.91	42.4/101.5	ND	ND	ND
	Aug	4	3.91	0.97	105.8/173.8	3.21	0.92	53.2/117.8	ND	ND	ND
	Oct	2	3.69	0.95	103/154.5	3.27	0.94	45.9/98.0	ND	ND	ND
SG		7	3.53	0.93	ND	3.04	0.91	ND	ND	ND	ND

(OTU numbers and Shannon and Simpson indices) and in SG (OTU numbers and Shannon index).

The same seasonal changes were observed in all size categories in KS, with relatively low copepod diversity in January, March, and May. High diversity was observed in August and October in KS, although no significant seasonal variations were observed. The same patterns of copepod diversity were observed in KG, although the seasonal changes were less distinct than in KS. Higher copepod diversity in KG than KS were evident from January to May, but differences were not significant. Similar or higher diversity was observed in small-copepod communities in August and October in KS and KG, compared with those in SG. The proportions of OTUs shared with SG were larger in small (49.5–66.7%) than medium (32.3–46.9%) copepods in each area (Table 1). The number of shared OTUs with SG was larger in KG (81.8–105.8) than KS (54.1–84.4) in each season, and the shared OTUs were especially evident in August and October in small copepods in KS and KG. High copepod diversity was also observed in KC throughout the year in 2015, with a large number of OTUs shared with SG in small copepods (80.3–111.6).

3.3. Community structures of copepods in the Kuroshio region

Copepod community structures were categorized into 3 major groups (Groups I, II, and III) including 11 significantly different clusters ($p < 0.005$; PERMANOVA) (Fig. 4A). The clusters were greatly affected by sampling area, sampling season, and copepod size. The community changes were visualized using multidimensional scaling (MDS) analyses for different copepod sizes and sampling regions (Fig. 4B) and for the effect of water temperature (Fig. 4C). In each size category for both KS and KG, we observed winter–spring and summer–autumn cluster groups. Although there were some inter-annual changes, copepod communities in January, March, and May were clustered into the winter–spring groups, whereas those in August and October were clustered into the summer–autumn groups. Group I included all size categories during winter–spring (clusters 2–4) as well as the large copepod community during summer–autumn (cluster 1) in KS. The copepod communities in KG and SG were categorized into Groups II (medium size) and III (small size). Groups II and III also included copepod communities during summer–autumn in KS (clusters 5 and 11). The small-copepod community during summer–autumn in KG (cluster 10) formed the same cluster group as that in SG. In KC, copepod communities showed seasonal changes, and these communities were placed between the KS and KG communities in each copepod size in the MDS analysis (Fig. 4B). No distinct community structures were observed among SG sampling stations. The sampling time (day or night) was not clearly associated with the major patterns of 11 clusters. The medium- and large-copepod communities in January in 2013 collected during the day were not categorized into any clusters (indicated by grey in Fig. 4). The same regional and seasonal changes described above were also observed in the copepod compositions based on the presence/absence of OTUs (Fig. S1).

3.4. Key species in small-copepod communities

A total of 36 OTUs were selected as major copepod OTUs, which included both small and medium-sized/large copepods, based on dominance in each cluster in the community analysis (Fig. 4). Although distribution patterns were OTU-specific, the cluster analysis based on the distribution patterns of major OTUs also showed 4 distinct groups affected by sampling area, sampling season, and copepod size (Fig. 5). These included dominant medium-sized/large copepods in winter–spring in KS (Group A), dominant small copepods in winter–spring in KS (Group B), dominant copepods in summer–autumn in KS (Group C), and dominant copepods in KG and SG (Group D).

In small-copepod communities, the group in KS in winter–spring (cluster 4 in Fig. 4) was distinct from the other groups of small copepods. The key OTU identified as '*Paracalanus* sp. (NWP)', which is one of the cryptic species of the *Paracalanus parvus* species complex (Cornils & Held 2014), was the largest contributor to this cluster, with a mean of 26.7–41.3% of sequence reads from January to March, followed by the OTUs of the large copepod *Calanus sinicus*, with 14.9–24.8% of sequence reads (Fig. 5, Table 2). Two OTUs identified as *Oithona similis* also helped to discriminate cluster 4 from other small-copepod communities (Table 3).

In the cluster group of small copepods in summer–autumn in KS (cluster 11), the contributions of *Paracalanus* sp. (NWP) and *C. sinicus* decreased (mean: 6.8–9.7% and 7.1–10.7% of sequence reads, respectively). However, these 2 species were still selected in order to discriminate this group from small-copepod communities in KG and SG, in addition to *Temora turbinata*, *Paracalanus* sp. (OTU 31), and the large copepods *Subeucalanus* spp. (Table 3). As the similarity between SG and the Kuroshio regions gradually in-

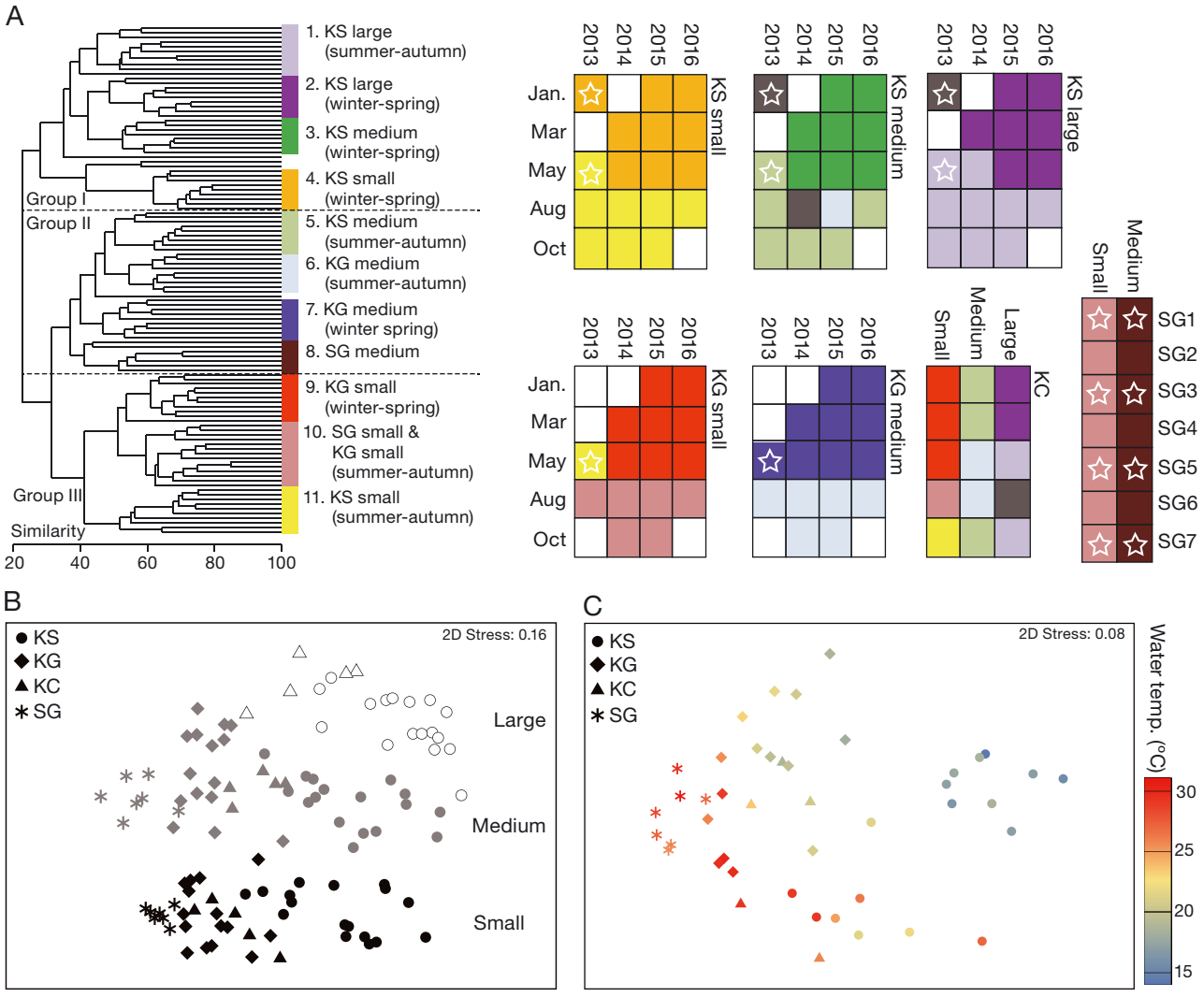

Fig. 4. (A) Cluster analysis of all samples. The 3 groups including 11 clusters are indicated by different colors. Copepod community samples that are not categorized into any clustered groups are indicated in grey. Samples collected during the day are represented by stars. (B) Multidimensional scaling (MDS) analysis for all copepod community samples. Large copepods are presented in white, medium-sized copepods in grey, and small copepods in black. (C) MDS analysis for small-copepod communities. Colors for each sample indicate the water temperature at a depth of 10 m. KS: Kuroshio Slope; KG: Kuroshio Gyre; KC: Kuroshio Core; SG: Subtropical Gyre

creased with increasing water temperature (Fig. 4C), the Paracalanidae species *P. aculeatus* and *Acrocalanus longicornis*, which were common in KG, became dominant in cluster 11 (Table 2).

The contributions of specific dominant species were not as evident in KG and SG. *Delibus nudus*, *A. longicornis*, *Cosmocalanus darwinii*, *Clausocalanus furcatus*, *P. aculeatus*, and *Calocalanus minutus* were the main small copepod species that discriminated the small-copepod communities of KG and SG from that of KS (Table 3). They characterized cluster 10 (small-copepod community in KG in summer–autumn and SG), differentiating it from cluster 9 (small-copepod community in KG in winter–spring). Cluster 9 showed a high dominance of *O. atlantica*, *Mesocalanus tenuicornis*, *P. tropicus*, *O. similis*, and *Paracalanus* sp. (NWP), which were primarily observed in KS.

3.5. Inter-annual change of small copepods in KS

In addition to typical seasonal patterns of copepod community and diversity in the Kuroshio region, the relatively large inter-annual changes observed in this study were investigated for 11 OTUs of small copepods between 2013 and 2016 in KS (Fig. 6).

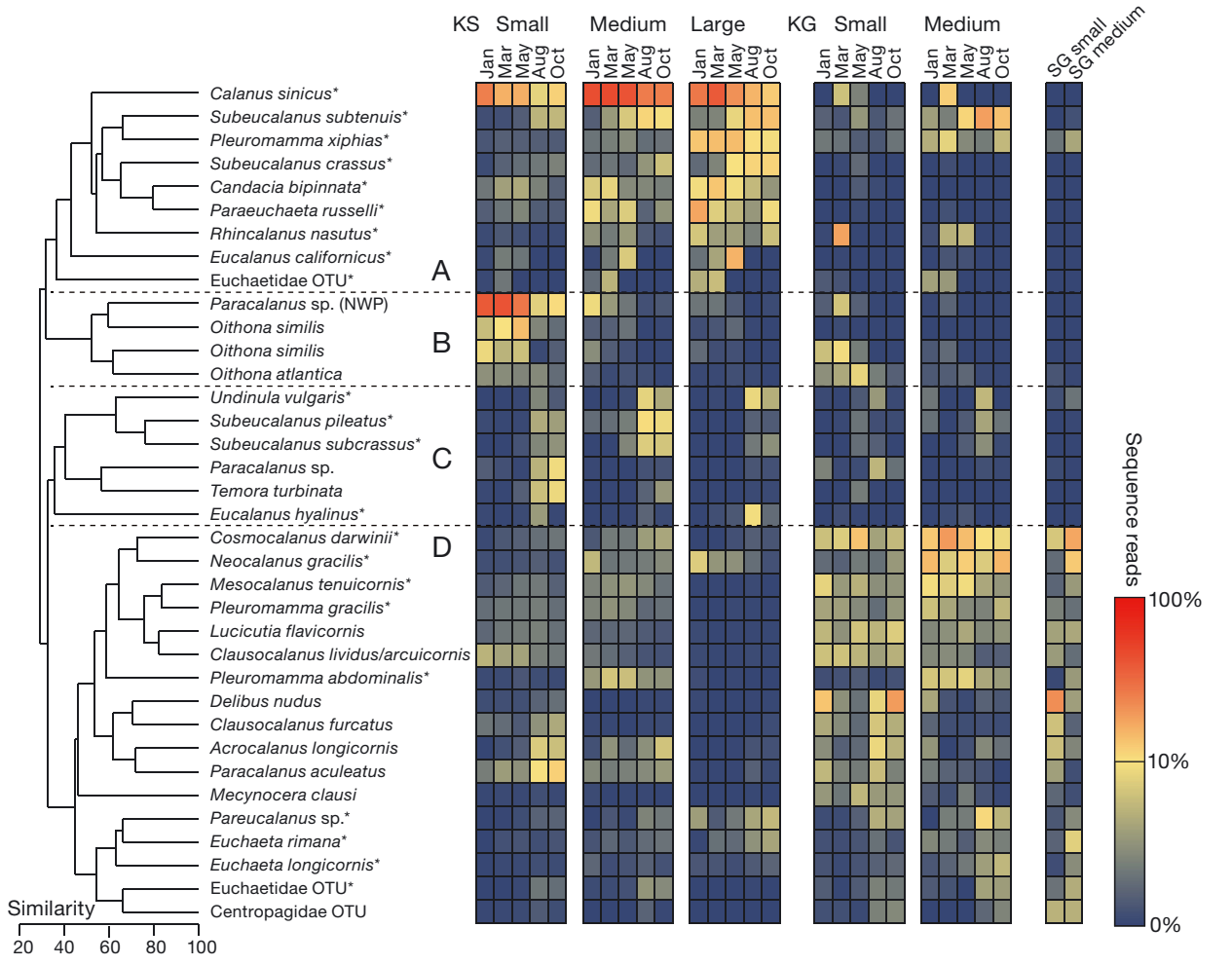

Fig. 5. Seasonal changes of major operational taxonomic units (OTUs) in the Kuroshio region. The proportions of sequence reads in each major OTU are averaged by sampling seasons in the Kuroshio Slope (KS) and the Kuroshio Gyre (KG) from 2013–2016. All samples are averaged for the Subtropical Gyre (SG). Mean proportions of sequence reads were log-transformed. The major OTUs are clustered into Groups A–D based on their distribution patterns. The major OTUs are selected from the top 5 dominant OTUs in each size category, season, and sampling location. Asterisks indicate major OTUs identified for major medium-sized/large copepods. Other OTUs are treated as major small copepods

These included 5 OTUs (OTUs 1, 4, 19, 25, and 26) which were mainly dominant in winter–spring in KS, showing negative and positive correlations to temperature and chl *a*, respectively. The other 6 OTUs (OTUs 3, 8, 10, 20, 31, and 36) were mainly dominant in summer–autumn, with positive and negative correlations to temperature and chl *a*, respectively.

Relatively lower water temperatures and higher chl *a* concentrations were observed from late spring to autumn in 2014 (indicated by arrows in Fig. 6). During this period, low numbers of OTUs were observed compared to other sampling periods, and relatively low OTU numbers continued until the early spring in 2015. Although the annual changes in each species differed, these environmental variables in 2014 influenced the dominant copepods in KS in winter–spring, OTU 1 *C. sinicus* and OTU 4 *Paracalanus* sp. (NWP). In particular, the large copepod *C. sinicus* showed the highest positive correlations with chl *a* and continued its relative dominance in the small-copepod community until the early spring in 2015. However, lower proportions of these species were observed during periods of high temperature and low chl *a* concentration in each season, such as summer 2013 and 2015. Other dominant OTUs in winter–spring in KS showed no clear responses in 2014. The influence of low temperature and high chl *a* in 2014 were also not clear in dominant OTUs during summer–autumn. However, decreases of populations were sporadically observed in August in OTU 10 *P. aculeatus*, OTU 20 *Subeucalanus pileatus*, and OTU 31 *Paracalanus* sp., and in October in OTU 36 *T. turbinata* in 2014.

Table 2. Top 5 operational taxonomic units (OTUs) contributing to similarity in each cluster group of small copepods using similarity percentage (SIMPER) analysis. The cluster groups are presented in Fig. 4A. AS: average similarity (%) within a cluster group. Medium-sized/large copepods are indicated by an asterisk. Results of SIMPER analyses in other cluster groups are listed in Table S3 in the Supplement. KS: Kuroshio Slope; KG: Kuroshio Gyre; SG: Subtropical Gyre

Cluster	OTUs	Blast-hit species	Contribution (%)
Cluster 4	OTU 4	*Paracalanus* sp. (NWP)	18.61
AS: 66.46	OTU 1	*Calanus sinicus**	13.09
KS small	OTU 26	*Oithona similis*	9.08
Winter–spring	OTU 25	*Oithona similis*	7.24
	OTU 19	*Clausocalanus lividus/arcuicornis*	6.26
Cluster 9	OTU 2	*Cosmocalanus darwinii**	5.68
AS: 57.77	OTU 19	*Clausocalanus lividus/arcuicornis*	5.12
KG small	OTU 28	*Oithona atlantica*	4.35
Winter–spring	OTU 16	*Lucicutia flavicornis*	4.26
	OTU 13	*Mesocalanus tenuicornis**	3.89
Cluster 10	OTU 7	*Delibus nudus*	7.36
AS: 63.57	OTU 8	*Acrocalanus longicornis*	5.3
KG & SG small	OTU 24	*Clausocalanus furcatus*	5.25
Summer–autumn	OTU 2	*Cosmocalanus darwinii*	4.4
	OTU 16	*Lucicutia flavicornis*	4.09
Cluster 11	OTU 10	*Paracalanus aculeatus*	6.06
AS: 59.02	OTU 8	*Acrocalanus longicornis*	5.2
KS small	OTU 36	*Temora turbinata*	4.57
Summer–autumn	OTU 4	*Paracalanus* sp. (NWP)	4.34
	OTU 3	*Subeucalanus subtenuis**	4.09

3.6. Contributions of medium and large copepods to small-copepod communities

Metabarcoding revealed the contributions of immature medium and large copepods to the OTUs in small-copepod communities. The proportions of sequence reads of major medium-sized/large copepods in communities of small copepods were 15.3–49.7, 18.8–65.9, 10.9–23.9, and 7.7–19.7% in KS, KG, KC, and SG, respectively (Fig. 7). The proportions of sequence reads in KS (mean 28.8%) and KG (mean 24.7%) were significantly higher than those in SG (mean 11.9%; $p < 0.01$), but there were no significant differences between KS and KG (Table S4). In KS, the proportions of sequence reads of major medium-sized/large copepods in small-copepod communities were strongly influenced by *C. sinicus*, which showed 0.2–40.8% of sequence reads in small-copepod communities (Fig. 7). The proportions of sequence reads of *C. sinicus* tended to be high during both early and late spring in KS. The high contribution of medium and large copepods in small-copepod communities in the Kuroshio region was demonstrated in SIMPER analysis as well, with *C. sinicus*, *Subeucalanus subtenuis,* and *S. pileatus* in KS and *C. darwini* and *M. tenuicornis* in KG (Tables 2 & 3). Small-copepod communities in SG were predominantly characterized by major small (mean 44.6%) and non-major (mean 43.5%) copepod OTUs.

4. DISCUSSION

4.1. Metabarcoding as a tool to investigate the small-copepod community

Seasonal and inter-annual changes in zooplankton communities are commonly used to track changes in marine ecosystems at various global ocean sampling stations (Mackas & Beaugrand 2010). Although the importance of small copepods has been recognized for improving time-series analysis of zooplankton, difficulty in identifying species using conventional microscopic analysis hampers investigation of small copepods, including nauplii (Pitois et al. 2009). In this study, we applied a molecular-based metabarcoding approach using the 28S D2 region to investigate communities of small copepods in the Kuroshio region of Japan, where there is a large standing stock of copepod nauplii (Nakata 1988, Kobari et al. 2018). The single primer pair can amplify the 28S D2 region of various planktonic copepods with high diversity, and most copepods in the Kuroshio region can be analyzed at the species level by metabarcoding analysis (Hirai et al. 2015). For example, the metabarcoding approach successfully classified cryptic species of copepods with taxonomic challenges, including important small copepod species in the genus *Paracalanus* (Hidaka et al. 2016). Sequence reads in OTUs are known to be a proxy of biomass in zooplankton including copepods (Lindeque et al. 2013, Hirai et al. 2015). This study evaluated the importance of immature stages of medium-sized/large copepods in small-copepod communities, including *Calanus sinicus* in KS. Although the metabarcoding method is still developing, the approach in this study effectively characterized spatial, seasonal, and annual changes in small-copepod communities in the Kuroshio region, eliminating the need to use morphological characteristics.

Despite the usefulness of metabarcoding in zooplankton evaluation, there are still technical limita-

Table 3. Operational taxonomic units (OTUs) contributing to dissimilarity between cluster groups of small copepods using similarity percentage (SIMPER) analyses. Top 10 OTUs are selected in each comparison between Groups 1 and 2. OTUs in **bold** indicate larger proportions of sequence reads in Group 2 than in Group 1. AD: average dissimilarity (%) between Groups 1 and 2. Medium-sized/large copepods are indicated by an asterisk. KS: Kuroshio Slope; KG: Kuroshio Gyre; SG: Subtropical Gyre

Cluster	OTUs	Blast-hit species	Contribution (%)
Group 1: cluster 4	OTU 4	*Paracalanus* sp. (NWP)	5.43
(KS winter–spring)	OTU 1	*Calanus sinicus**	4.62
Group 2: clusters 9–11	OTU 26	*Oithona similis*	3.62
(other small clusters)	**OTU 7**	***Delibus nudus***	**2.99**
AD: 67.93	**OTU 8**	***Acrocalanus longicornis***	**2.79**
	OTU 25	*Oithona similis*	2.76
	OTU 2	***Cosmocalanus darwinii****	**2.65**
	OTU 24	***Clausocalanus furcatus***	**1.73**
	OTU 10	***Paracalanus aculeatus***	**1.71**
	OTU 40	***Calocalanus minutus***	**1.54**
Group 1: clusters 9 & 10	**OTU 36**	***Temora turbinata***	**2.93**
(KG & SG small clusters)	OTU 7	*Delibus nudus*	2.93
Group 2: cluster 11	**OTU 4**	***Paracalanus* sp. (NWP)**	**2.89**
(KS summer–autumn)	**OTU 1**	***Calanus sinicus****	**2.84**
AD: 57.84	**OTU 3**	***Subeucalanus subtenuis****	**2.3**
	OTU 31	***Paracalanus* sp.**	**2.1**
	OTU 20	***Subeucalanus pileatus****	**2.03**
	OTU 2	*Cosmocalanus darwinii**	1.97
	OTU 10	*Paracalanus aculeatus*	1.75
	OTU 40	*Calocalanus minutus*	1.67
Group 1: cluster 9	**OTU 7**	***Delibus nudus***	**2.98**
(KG winter–spring)	OTU 25	*Oithona similis*	2.62
Group 2: cluster 10	**OTU 8**	***Acrocalanus longicornis***	**2.53**
(KG & SG summer–autumn)	OTU 28	*Oithona atlantica*	2.46
AD: 49.61	**OTU 55**	***Calocalanus curtus***	**2.2**
	OTU 34	**Centropagidae OTU**	**2.13**
	OTU 4	*Paracalanus* sp. (NWP)	2.08
	OTU 13	*Mesocalanus tenuicornis**	1.82
	OTU 24	***Clausocalanus furcatus***	**1.75**
	OTU 10	***Paracalanus aculeatus***	**1.67**
	OTU 37	*Paracalanus tropicus*	1.67

tions, such as differences in genome size, copy numbers of target genes, primer mismatches, sequencing errors, and sequence resolutions, depending on marker choices (Bucklin et al. 2016). These biases should be considered when interpreting metabarcoding data. For example, the 28S D2 region is conserved more than common mitochondrial markers, such as cytochrome *c* oxidase subunit I (*COI*), for species identification of copepods (Blanco-Bercial et al. 2011). Because the highly variable *COI* is currently a standard gene for DNA barcoding of zooplankton (Bucklin et al. 2010), the use of multiple markers including both nuclear mitochondrial genes would lead to a better understanding of zooplankton diversity (Berry et al. 2019). There are also improvements needed in the methodology, such as the implementation of technical replicates. However, we followed the strict procedure of quality-filtering of bioinformatics described by Hirai et al. (2020), which used mock communities including known copepod species for validating data analysis. The strict bioinformatics analysis may be one reason for the relatively lower numbers of OTUs compared with those in other metabarcoding studies of zooplankton in subtropical areas (Sommer et al. 2017, Blanco-Bercial 2020). However, the total number of copepod OTUs in the present study was considerably larger than those of detected through microscopic observations in the Kuroshio region (Lee et al. 2009, Miyamoto et al. 2017). These findings indicate that metabarcoding effectively revealed undescribed and cryptic species especially in communities of small copepods, suggesting high regional species diversity among copepods in the Kuroshio region.

4.2. Unique copepod communities and diversity in the Kuroshio region off Japan

The Kuroshio Current is a strong western boundary current, providing distinct environmental structures across the current. For instance, different water temperature and chl *a* concentrations were observed between KS and KG. Although a large meander of the Kuroshio Current sometimes occurs off the southern coast of Japan (Usui et al. 2013), this pattern was not observed during our study period (2013–2016) (Table S1). In addition to spatial patterns of environmental changes, both satellite data of SST and vertical profiles of CTD showed typical patterns of seasonal changes similar to other studies (Sugisaki et al. 2010, Kodama et al. 2014). Thus, the changes in copepod community structure and diversity in this study are responses to common seasonal environmental variations in the Kuroshio region.

In a previous metabarcoding study of copepods, different community structures were observed in the Kuroshio region, the center of SG, and the Pacific equatorial region (Hirai & Tsuda 2015). However,

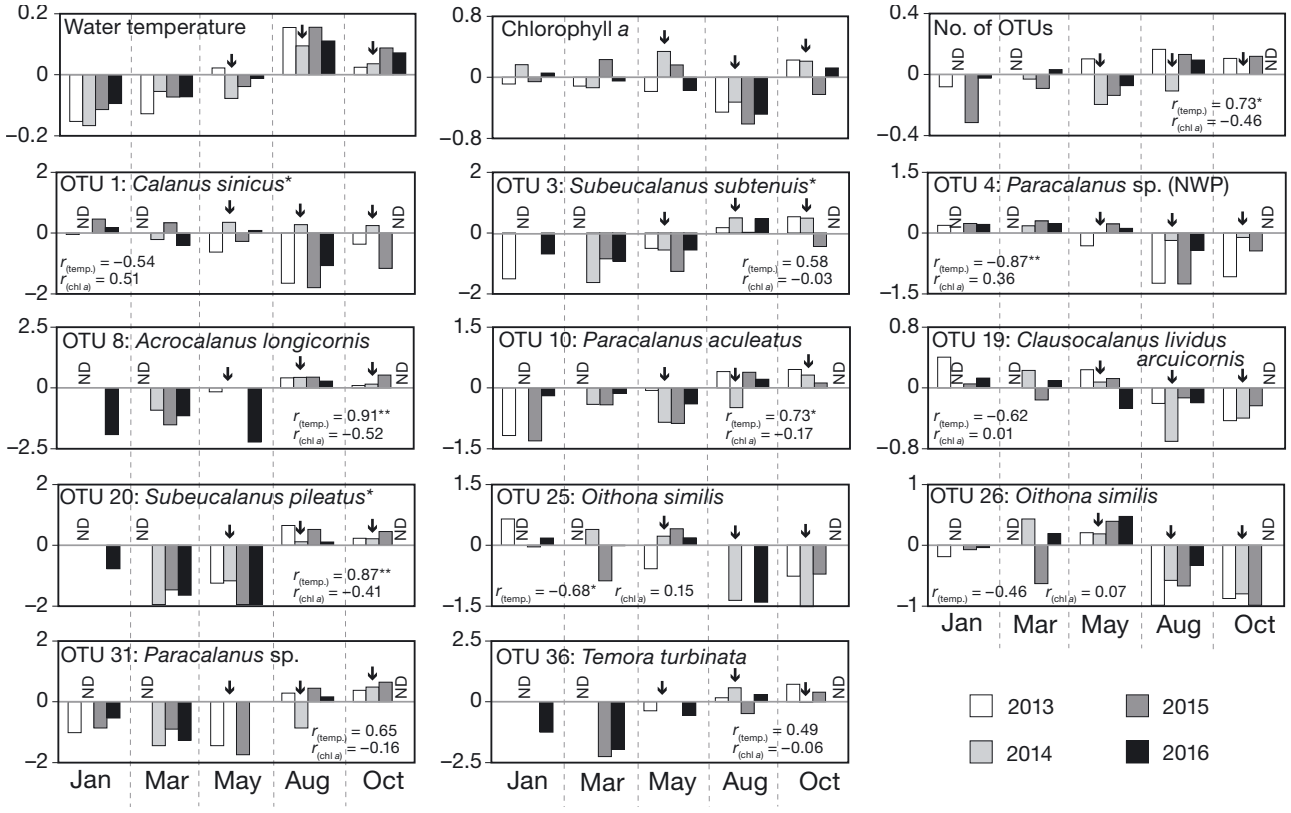

Fig. 6. Inter-annual anomalies of environmental variables, the number of operational taxonomic units (OTUs), and proportion of sequence reads in key OTUs of small copepods in the Kuroshio Slope (KS). OTUs contributing to small communities in KS were selected by SIMPER analyses. Water temperature and chlorophyll *a* (chl *a*), at a depth of 10 m, were used to study the inter-annual anomalies of environmental variables. Arrows represent the periods with low temperature and high chl *a* in 2014. ND: no data. Medium-sized/large copepods are indicated by an asterisk after their name. Pearson′ correlation coefficients (r) represent the proportions of reads and temperature or chl *a* in each major OTU; significant correlations are indicated by asterisks (*$p < 0.05$; **$p < 0.01$)

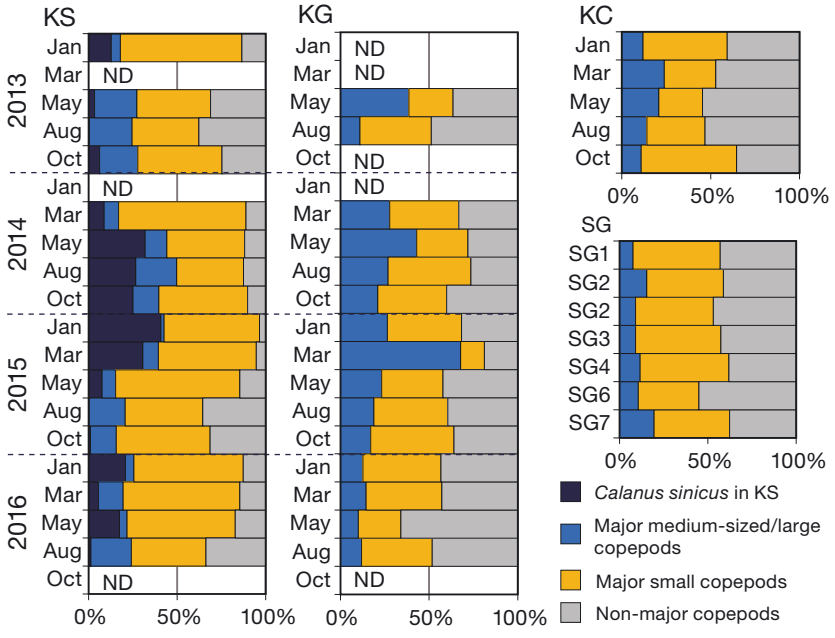

Fig. 7. Proportions of sequence reads of major medium-sized/large and small copepods in small-copepod communities. The major copepods are the 36 dominant operational taxonomic units (OTUs) indicated in Fig. 5, including both major small and medium-sized/large copepods. These OTUs are selected from the top 5 dominant OTUs in each size category, season, and sampling location. Other OTUs belong to the non-major 699 OTUs. The proportions of sequence reads of the large copepod *Calanus sinicus* are represented separately from major medium-sized/large copepods in the Kuroshio Slope (KS). ND: no data; KG: Kuroshio Gyre; KC: Kuroshio Core; SG: Subtropical Gyre

that study only analyzed community samples in the Kuroshio region during one research cruise (May 2011), and copepod communities were not categorized into size fractions. We analyzed seasonal samples for 4 years and showed that copepod communities in the Kuroshio region are unique, for each size fraction, regardless of the year and season. As shown in the morphology-based approach along the same transect line at 138° E (Sogawa et al. 2019), different environmental conditions led to distinct copepod community structures between KS and KG in all size fractions. Although unique copepod communities have been reported in the north-frontal area of the Kuroshio Current and Kuroshio axis areas (Miyamoto et al. 2017), we focused on seasonal changes inside or outside the Kuroshio region. Further sampling efforts would elucidate the small-scale spatial changes of copepod communities around the Kuroshio Current.

Characterization of seasonal changes in copepod communities have mainly focused on the shoreside of the Kuroshio Current (e.g. Shimode et al. 2006). Although environmental changes were less clear in KG than in KS, we observed seasonal changes of the copepod community and diversity in both areas, including distinct differences between winter–spring and summer–autumn. Although there were no significant differences, possibly due to inter-annual changes and small sample numbers, we detected seasonal changes in copepod diversity that were largely due to changes in water temperature, as observed by Miyamoto et al. (2017). The highest correlation between water temperature and diversity occurred in the small-copepod community. The proportion of small copepods increased with increasing water temperatures (Ikeda 1985), and the major species characterizing copepod communities in SG were mostly small copepod species. These small species, such as *Delibus nudus* and *Acrocalanus longicornis*, intruded into the Kuroshio region during high-temperature periods, when the number of OTUs shared with SG increased. Although copepod diversity is high in the center of the oligotrophic SG (Rombouts et al. 2009), we showed a comparable, and occasionally higher, copepod diversity in summer in the Kuroshio region. A well-developed thermocline provides vertical niches for zooplankton (Rutherford et al. 1999); thus, copepods generally unique to either the Kuroshio region or the center of the SG could coexist during summer in the Kuroshio region, leading to the highest levels of copepod diversity in this region. Because temporal changes were not distinct in the SG, seasonal variation in the copepod community structure is a key characteristic in the Kuroshio region.

4.3. Effects of inter-annual environmental changes on small copepods

We analyzed data collected over 4 years; however, the sequence read proportions changed annually for major copepod species in the small-copepod communities. We identified periods with unseasonably low temperature and high productivity from the late spring to autumn in 2014, particularly in KS. The fluctuation in sequence reads may well reflect the egg production of *C. sinicus*, one of the major large copepods in KS, which is dependent on water temperature as well as prey availability (Uye 2000). Temperature-dependent egg production rates have also been reported for *Paracalanus parvus* (represented as *Paracalanus* sp. [NWP] in this paper), the other important species in the community (Jang et al. 2013). We showed the strong influence of both temperature and chl *a* especially on the population dynamics of both *C. sinicus* and *Paracalanus* sp. (NWP). This indicates that annual environmental changes are important for dominant copepods in the Kuroshio region as well as for overall epipelagic communities. These annual changes may influence the recruitment success of commercially important fish such as Japanese sardine, which are affected by ambient water temperature in spawning locations in the Kuroshio region off Japan (Nishikawa 2019). Inter-annual variations have been reported in the Kuroshio region (Sugisaki et al. 2010, Kodama et al. 2014), and annual changes in local environmental factors, including water temperature, are known to affect copepod biomass during the winter (Hidaka & Nakata 2010). Additionally, increasing temperatures have already been observed in the Kuroshio region in the context of rapid climate change (Shimizu et al. unpubl. data). Considering the ecological importance of small copepods and their sensitivity to environmental variations, it is important to monitor these copepod species at high taxonomic resolutions to obtain an in-depth understanding of the changes in marine ecosystems in the Kuroshio region off the southern coast of Japan.

4.4. High contribution of medium-sized/large copepods in small-copepod communities

An area of interest was the substantial contribution of immature stages of medium-sized/large copepods to small-copepod communities in both KS and KG, indicating that the community of small copepods is largely supported by the reproduction of medium and large copepods in the Kuroshio region. One major

large copepod, *C. sinicus*, greatly affected the proportions of major medium-sized/large species in small communities in KS. A low egg production rate has been reported for *C. sinicus* during high-temperature and low-productivity periods (Zhang et al. 2005), which corresponded to the high contribution of *C. sinicus* to small-copepod communities in the winter–spring period. Although ontogenetic vertical migrations (OVMs) are known in large copepod species at high latitudes with distinct seasonal changes (Miller et al. 1984), *C. sinicus* also performs OVMs during CV copepodite stages, and some populations of *C. sinicus* are distributed in the mesopelagic layer to avoid high temperatures in the Kuroshio region (Nonomura et al. 2008). The seasonal occurrence of the large copepods *Eucalanus californicus* and *Rhincalanus* species observed in this study results from OVM for spawning during the high productivity period (Shimode et al. 2012a,b). Although life cycles are not well studied, the present study showed that large copepods in the genus *Subeucalanus* were dominant in small-copepod communities during high temperature periods in summer–autumn in the Kuroshio region. On the other hand, *Neocalanus gracilis*, which greatly contributed to the copepod community in the oligotrophic SG, has no distinct seasonal reproduction and undergoes small-scale ontogenetic migration in subtropical areas (Shimode et al. 2009). Thus, different patterns of reproduction associated with seasonal environmental changes could explain the high contribution of immature stages of medium and large copepods in the Kuroshio region. Because the nauplii of large copepods are important prey for fish larvae in the Kuroshio region (Hirai et al. 2017a), the contribution of medium-sized/large copepods to small-copepod communities might be one of the key factors that make this region a good nursery ground for fish.

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