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Morpho-molecular characterization of sand-dwelling dinoflagellate communities from the German Wadden Sea and insights into their spatiotemporal distribution

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ABSTRACT

Benthic protist communities, including sand-dwelling dinoflagellates, are still poorly characterized worldwide and there is a lack of knowledge on their diversity and spatiotemporal dynamics. In this study, sediments were obtained from several coastal locations of the German Wadden Sea during the summer months of 2017 and 2018 to determine the diversity and spatiotemporal distribution of sand-dwelling dinoflagellates. The analyses consisted of light microscopy and the metabarcoding of small subunit (SSU) rRNA gene V4 region amplicons. Additionally, SSU rRNA sequences were newly obtained for 15 observed taxa included in a reference database and were used to taxonomically assign the obtained amplicon sequence variants (ASVs). The combination of morphological and molecular approaches allowed a deep characterization of dinoflagellate communities. Seventy-three morphospecies were detected by microscopy, while metabarcoding ASVs (amplicon sequence variants) were assigned to 116 species, resulting in a more comprehensive biodiversity inventory than achieved with either method alone. Pfiesteriaceae, *Togula compacta* and *Thecadinium yashimaense* predominated in the different sampling locations. Communities from Wilhelmshaven diverged from the others, consistent with its muddier sediment. A high level of resemblance was determined for the communities from each location, regardless of the sampling month and year, indicating a high summertime stability of benthic communities. Further efforts will be needed to characterize benthic protist diversity, as a first step in obtaining a complete picture of the habitat characteristics and physico-chemical conditions that influence benthic community composition.

HIGHLIGHTS

- Metabarcoding and microscopy observations provide complementary results.
- Much of the diversity of sand-dwelling dinoflagellates is uncharacterized.
- Benthic dinoflagellate communities were stable at each location in summer.

Introduction

Dinoflagellates represent one of the main components of marine protist communities in terms of diversity and abundance and they have been studied in detail for centuries (Le Bescot *et al.*, 2016). Most dinoflagellates are planktonic, but many species, mainly those that are heterotrophic, are benthic, inhabiting sandy sediments, or grow epiphytically on macroalgae (Taylor *et al.*, 2008). In contrast to their planktonic counterparts, sand-dwelling dinoflagellate communities have received little attention. Studies have been conducted in only a few areas, such as the North German Wadden Sea, where their diversity has been thoroughly characterized, e.g. (Hoppenrath, 2000a, 2000b, 2000c, 2000d), with less information available from Australia (Murray, 2003) and Kuwait (Saburova *et al.*, 2009; Al-Yamani & Saburova, 2010). Elsewhere, the diversity of sand-dwelling dinoflagellates remains poorly explored (Hoppenrath *et al.*, 2014), evidenced by the relatively large number of new sand-dwelling species still being identified, including *Amphidiniopsis bulla*, *A. crumena*, *A. erinacea*, *A. nileribanjensis*, *A. selene*, *Dinotrix phymatodea*, *D. pseudoparadoxa* (Yamada *et al.*, 2020; Reñé *et al.*, 2020a; Yamaguchi *et al.*, 2023), and the new genera still being erected, such as *Bindiferia* gen. nov., *Carinadinium* gen. nov., *Coutea* gen. nov., *Laciniporus* gen. nov. and *Pachena* gen. nov. (Saburova & Chomérat, 2019; Hoppenrath *et al.*, 2020, 2021; Borchhardt *et al.*, 2021; Chomérat *et al.*, 2023). Nonetheless, molecular information is lacking for many known morphospecies, due to the difficulties in separating cells from sediments or the inability to grow heterotrophic species in culture. Consequently, inferences regarding the phylogenetic position and relationships of sand-dwelling dinoflagellates cannot be made and the structure and dynamics of their communities remain largely unknown.

The amplification and massive sequencing of marker genes using high-throughput sequencing (metabarcoding) have opened up new opportunities to characterize marine protist communities while overcoming the difficulties related to morphological species identification (Santoferrara *et al.*, 2020). Metabarcoding analyses of benthic protist communities from sediments by SSU rRNA gene amplification has proven useful in determining dinoflagellate diversity (Bik *et al.*, 2012; Gong *et al.*, 2015; Forster *et al.*, 2016). However, for benthic dinoflagellates, few metabarcoding studies have been conducted so far (Kohli *et al.*, 2014a, 2014b; Smith *et al.*, 2017). The morphological characterization of sand-dwelling dinoflagellates has traditionally been conducted using cells eluted from sediments according to the melting seawater-ice method (Uhlir, 1964; Hoppenrath *et al.*, 2014). Metabarcoding data obtained from DNA purified from the eluted cells have been compared with the data obtained following direct lysis within sediments (Reñé *et al.*, 2020b). The elution method resulted in the enrichment of ciliates and dinoflagellates, and their diversity was higher than that determined by direct lysis, mainly due to the larger sample volumes processed. Metabarcoding of cells eluted from sediments was also employed to study the composition and temporal dynamics of the sand-dwelling dinoflagellate community at three

Mediterranean locations (Reñé *et al.*, 2021). The results demonstrated the presence of characteristic species for each location and the temporal dynamics of some of them. Although a high diversity of dinoflagellate species was detected, a large fraction of the obtained sequences were those of unknown taxa, a consequence of the lack of studies on sand-dwelling dinoflagellates diversity in the Mediterranean Sea and the unavailability of reference molecular sequences.

By contrast, the diversity of dinoflagellates, including benthic species, in the North German Wadden Sea has been thoroughly characterized (Drebes & Elbrächter, 1976; Hoppenrath, 2000d, 2004; Hoppenrath *et al.*, 2009) such that there is robust background knowledge of their morphological diversity. In this study, we analysed the community of sand-dwelling dinoflagellates from different

locations in the German Wadden Sea by combining traditional light microscopy (LM) identification with metabarcoding of V4 SSU rRNA gene amplicons. The results obtained from these two methods were then compared. Our exploration of species diversity in different intertidal habitats of the German Wadden Sea provides insights into the spatiotemporal variability of dinoflagellate communities in sediments from the studied locations.

Materials and methods

Sampling, sample preparation and microscopic identifications

Intertidal sandy sediment samples were obtained at different locations from the German Wadden Sea (German Bight, North Sea) during the summer (June to September) of 2017 and 2018 (Fig. 1, Supplementary table S1). Samples from Wilhelmshaven were taken at the 'Fliegerdeich' site of the south beach (53°30'36"N, 8°07'43"E) on 15 and 26 June 2017 (left side of the slipway), and on 30 August and 18 September 2018 (left and right sides of the slipway). The site is located within Jade Bay (Lower Saxony Wadden Sea), a shallow tidal bay that is connected to the North Sea through the Jade Channel, which enters the bay east of Wilhelmshaven (Schückel *et al.*, 2013, 2015). This bay is a macrotidal flat system distinguished by its land-locked character and its large mudflat belt (Schückel *et al.*, 2013). A connection via groins and a slipway results in small sandy corners; these sand patches were sampled in this study. Samples from Schillig (53°42'23"N, 8° 01'56"E) were taken on 12 and 20 June 2017, 22 August 2018 and 13 September 2018. Schillig is located at the mouth of Jade Channel. Tidal flats at the western side of the channel are gently sloping and show a fining-up zonation from the low to the high- water line (Hertweck *et al.*, 2005). Sand samples were taken in a mixed flat area and in rippled sand flat parts. Sand samples from the East Frisian Island Wangerooge (53°46'52"N, 7°51'21"E) were obtained on 5 September 2018. Wangerooge is a barrier island that faces northwards, to the German Bight of the North Sea, and the sampling location was in its south-western part. Samples from the North Frisian Island Sylt were taken at the Oddewatt (55°01'55"N, 8°25'52"E) and List Fähranleger (List ferry pier) (55° 00'54"N, 8°26'20"E), both of which are located in the north-east, between Sylt and the mainland (Hoppenrath, 2000d), on 13 September 2018. Additional exploratory samplings were conducted in Schillig on 9 June 2017 and in Maade Siel (53° 33'45"N, 8°9'10"E) on 14 June 2017. Sampling and cell extractions were done as described in Reñé & Hoppenrath (2019). Briefly, sandy sediment samples were obtained with a spoon during low tide and immediately transferred to the laboratory, where 100–150 g of wet sediment was used to elute dinoflagellate cells according to the melting seawater-ice method (Uhlig, 1964). Cell extractions were conducted in triplicate. Cells recovered from two of the replicates were gravity-filtered in 3 µm polycarbonate filters and then immediately frozen until further processing. For the third replicate, seawater containing living eluted cells was recovered, collected in a Petri dish, and used for LM observations. The complete dish was scanned using a Leica DMIL inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) and all observed cells were identified to the species level, when possible. The naming of so far undescribed species followed (Hoppenrath, 2000d) whenever possible, with attempts made to link names to morphological descriptions, e.g., '*Gyrodinium*' spec1 (a *Gyrodinium* sensu lato taxon, possibly a new genus). Specimens of interest were photographed using a Leica DFC290 digital camera.

DNA extraction, PCR amplification and sequencing of cell isolates

An initial check was made in the NCBI database for the presence of rRNA gene sequences corresponding to benthic dinoflagellate species. Then, individual cells observed belonging to taxa lacking molecular sequences were manually isolated under LM using glass capillary pipettes, successively washed in filtered seawater, photographed and placed into 0.2 ml PCR- tubes. When the

target taxon was present at high cell numbers, 20–50 cells were isolated and placed in the same tube. The samples were immediately frozen at -20°C and stored until processed. Single-cell samples were directly used for subsequent PCRs after three rounds of freezing and thawing to facilitate cell breakage. For samples containing many cells, total DNA was extracted using the NucleoSpin Plant II extraction kit (Macherey Nagel, France) following the manufacturer's instructions and then used as a template for subsequent PCRs.

The primers used in this study are listed in Supplementary table S2. A first 50 μl multiplexing PCR was conducted for samples obtained in 2017, using the primers 18ScomF1-18ScomR1 to amplify the SSU rRNA gene fragment and the primers D1R- D3B to amplify the large-subunit (LSU) rRNA gene fragment. The PCRs contained 0.5 M of GC melt reagent, 1 \times GC buffer, 0.2 mM of each dNTP, 0.25 μM of each primer and 1 \times Advantage GC2 DNA polymerase mix (Takara Bio). PCR conditions were as follows: initial denaturation for 5 min at 94°C , followed by 30 cycles of 30s at 94°C , 30s at 55°C and 2 min at 68°C , followed by a final extension for 3 min at 68°C . In the second round, short fragments of the target genes were amplified in 25 μl PCRs using 0.5 μl of PCR product from the first round as the template and the primer pairs 18ScomF1-Dino18SR1, G10F-G18R, Dino18SF2- 18ScomR1 and D1R-D3B (Supplementary table S2). The composition and the PCR conditions were the same as in the first round. For the 2018 samples, the protocol described in Reñé & Hoppenrath (2019) was followed. Briefly, a first touch-down PCR was conducted using primers EK-82F and 28S-1611R. Subsequent nested PCRs were conducted to amplify the region of interest. The SSU rRNA gene was obtained using the EK-82F–EK-1520R primer pair and, when necessary, a subsequent PCR was conducted using the primers DIN464F–EK-1520R. LSU rRNA gene sequences were obtained using the D1R- D3B primers. Some of the nested PCRs were conducted using puReTaq Ready-to-go PCR beads (GE Healthcare Bio-Sciences, Inc.) and 0.4 μM of each primer to amplify the complete or partial SSU and LSU rRNA gene regions.

All PCR products were electrophoresed in an agarose gel and then visualized under UV illumination. Sanger sequencing was done at external services (Genewiz, UK; Macrogen, the Netherlands; and Genoscreen, France) using forward and reverse primers and an ABI 3730XL DNA analyser. The resulting fragments were merged using Geneious v. R6. All sequences obtained were deposited in GenBank under the accession numbers ON015051–ON015066 for the SSU rRNA gene and ON015067–ON015075 for the LSU rRNA gene sequences (Supplementary table S3).

Metabarcoding and sequence analysis

A total of 26 samples were analysed, corresponding to samples collected at different sites and dates in replicate (Supplementary table S4). DNA from filters retaining seawater-ice sediment-eluted cells was purified as previously described (Reñé *et al.*, 2020b, 2021). The V4 SSU rRNA gene (amplicon length ca. 490 bp) was PCR amplified using the universal eukaryotic EK565F forward primer (5'-GCA GTT AAA AAG CTC GTA GT-3') and the eukaryotic biased against metazoans 18S-EUK-1134R-Unon-Met reverse primer (5'-TTT AAG TTT CAG CCT TGC G-3') tagged with part of the Illumina adapters. For the 2018 samples, 5 μl of the first-round PCR products were purified with 2 μl of ExoSAPIT (ThermoFisher) and a second PCR using Nextera XT Index Kit v2 was conducted to tag each sample with unique indexes. Equal amounts of the indexed libraries were pooled and purified from PCR components and dimers using AMPure XP beads (Beckman Coulter). Amplicon paired-end Illumina MiSeq (2 \times 300 bp) sequencing was carried out on multiplexed samples as described elsewhere (Reñé *et al.*, 2020b, 2021). Samples from 2017 were sequenced by Eurofins Genomics (Ebersberg, Germany), and those from 2018 at the

Senckenberg am Meer Molecular and Metabarcoding Laboratory (Wilhelmshaven, Germany). The sequences were demultiplexed according to their respective indexes; primers were removed from paired-end reads using cutadapt v.1.14 (Martin, 2011) and processed following the DADA2 v.1.10 pipeline (Callahan *et al.*, 2016). Forward and reverse reads were truncated to 260 and 250 nucleotides, respectively, to retain the best-read quality region; the maximum number of expected errors was set to 4 and 6, respectively, and the minimum overlap to 10 nucleotides. Sequence analyses were run at the Marine Bioinformatics Service (MARBITS) of the Institut de Ciències del Mar (ICM-CSIC) in Barcelona. The resulting amplicon sequence variants (ASVs) were assigned taxonomically using the VSearch global alignment algorithm (Rognes *et al.*, 2016) against PR2 v.4.12.0 (Guillou *et al.*, 2013), including the new reference sequences obtained in this study, with an identity cut-off of 60%. ASVs corresponding to dinoflagellates (class Dinophyceae) were extracted from the complete data set and analysed in the present study. The dinoflagellate ASVs were re-classified at the species level using BLAST v2.13, with default parameters (Altschul *et al.*, 1990), but the classification was retained only for ASVs with taxonomic assignment showing > 90% identity; those with 60–90% identity were manually labelled as 'Unknown'. The raw sequence data were publicly deposited in the NCBI's SRA database under accession number PRJNA785761.

The metabarcoding data were analysed using the package 'phyloseq' (McMurdie & Holmes, 2013) from RCRan (R Core Team, 2019), and all graphs were constructed using 'ggplot2' (Wickham, 2016). The data were normalized by computing the relative abundance for each sample. Non-metric multidimensional scaling (NMDS) using Bray–Curtis distances was performed to analyse the dissimilarity between locations. For the NMDS comparing LM and metabarcoding identifications, only those morphospecies with molecular sequences available and ASVs showing > 98% identity to any reference sequence were used.

Phylogenetic analyses

A concatenated alignment of 239 representatives and 7270 positions, including SSU, ITS and LSU rRNA gene sequences and comprising all major dinoflagellates lineages and 10 outgroups, was used to construct the phylogenetic tree (Chacón & Gottschling, 2020). Additional sequences of interest and the new sequences obtained in this study, previously concatenated when needed, were added to the alignment using MAFFT v.7 (Kato & Standley, 2013), resulting in 288 sequences and 7499 positions. The alignment was then trimmed to remove poorly aligned regions using trimAL v1.2 under the gappyout option (Capella-Gutierrez *et al.*, 2009), resulting in a final alignment of 4701 positions (1779 for SSU + 612 for ITS + 2319 for LSU rRNA gene). Standard best-fit model selection was conducted for each partition using jModelTest (Darriba *et al.*, 2012), implemented in IQ-TREE v.2.1.2 (Nguyen *et al.*, 2015), and a maximum-likelihood phylogenetic tree was constructed with RAxML-ng (Kozlov *et al.*, 2019). Bootstrap statistical support (% BS) was evaluated using 1000 replicates. A Bayesian analysis was carried out with MrBayes v. 3.2 (Ronquist *et al.*, 2012) using four MCMC chains and 1 000 000 generations. The consensus tree was obtained from post burn-in trees and Bayesian posterior probabilities (BPP) were evaluated. The original alignment was then used to include ASVs sequences, using MAFFT v.7 under the –add option, and the previously obtained tree then served as a constrained tree for the phylogenetic placement of ASVs using the evolutionary placement algorithm implemented in EPA-ng (Barbera *et al.*, 2019). The final trees were visualized and annotated using FigTree v1.4.4 and iTOL v4 (Letunic & Bork, 2019).

The phylogenetic distances between the sand-dwelling dinoflagellate communities detected in Wadden Sea samples were compared using the UniFrac distance metric. For that purpose, singletons (ASVs represented only by a single read) were removed, resulting in 2002 ASVs. All sequences were

aligned and trimmed as previously described, and a maximum-likelihood phylogenetic tree was computed using IQ-TREE v2.1.2. A distance matrix was then created using the unweighted (presence/absence information) Unifrac function implemented in the 'phyloseq' package from RCrAn. The statistical significance of phylogenetic distances between the dinoflagellate communities was evaluated by global and pairwise adonis tests by location, and adjusted by Bonferroni correction with 1000 permutations using the 'vegan' package (Oksanen *et al.*, 2019). The significance cut-off for the p value was 0.05.

Results

Characterization of the community composition in the studied locations

Dinoflagellate cells eluted from the different samples using the seawater-ice method were examined by LM to determine the identity of the occurring morpho- species. Up to 73 different morphospecies were identified, many of which corresponded to non-described species (Table 1). Images of the most representative morphospecies are provided in Figs 2–50. The number of species detected in a single sample ranged from 12 to 38. The richness values were lowest in samples from Wilhelmshaven and highest in those from Wangerooge, List ferry pier and Oddewatt (Sylt) (Table 1). Some species were recurrently detected in all samples and locations, e.g. the '*Katodinium*' *asymmetricum* species complex or *Amphidinium incoloratum*, while others were characteristic of a given location, e.g. *Amphidiniopsis rotundata* and *Planodinium* spp. in Wilhelmshaven, *Apicoporus* spp. in Wangerooge, *Polykrikos lebouriae* in Schillig and Oddewatt, and *Togula compacta* in Schillig and Wangerooge (Table 1).

The dinoflagellate community was also characterized by metabarcoding of the SSU rRNA gene V4 region. Independently of the total number of reads retrieved, the reads corresponding to dinoflagellates greatly varied between samples, from <1% to >95% of the protist community obtained from cells eluted from the sediments (Supplementary table S4). Among the 2259 ASVs belonging to dinoflagellates retrieved in this study, only 10% could not be assigned taxonomically (thus classified as Unknown because they showed a sequence identity <90% with reference sequences), representing a relatively low fraction of the community. The 2030 ASVs showing >90% identity with reference sequences were taxonomically assigned to 116 different species. The reduction in species assignments was caused by the large number of ASVs with little variation between them and thus assigned to the same species; for example, 632 ASVs were assigned to *Togula compacta*, 419 ASVs to *Thecadinium yashimaense* and 120 ASVs to *Ankistrodinium semilunatum*. An additional 299 ASVs were assigned to environmental dinoflagellate sequences, labelled as Dinophyceae_XXX.

The taxonomic composition varied significantly between locations (Fig. 51). Schillig samples were characterized by the presence of the autotrophic naked dinoflagellate *Togula compacta*, which completely dominated the community in September 2018, and *Polykrikos herdmaniae*, which was mainly detected in June 2017. Wilhelmshaven samples were dominated by species belonging to the genera '*Katodinium*' and *Aduncodinium*. Samples from Wangerooge and List ferry pier (Sylt) were of similar composition and characterized by a remarkable presence of *Ankistrodinium semilunatum*. Finally, the sample from Oddewatt (Sylt) was dominated by ASVs belonging to the autotrophic thecate dinoflagellate *Thecadinium yashimaense*. That species was also detected at List ferry pier (Sylt), albeit at lower relative abundances. Differences in community composition were evident in the NMDS results (Fig. 53), which showed the close clustering of samples obtained at each location (and their replicates) regardless of the sampling year or month. All samples obtained from Wilhelmshaven were of low dissimilarity, as were those obtained from Schillig, except the two replicates of sample Schillig 4, which had a higher dissimilarity. The samples obtained from Oddewatt (Sylt) diverged from those

obtained at the other locations whereas the samples from Wangerooge and List ferry pier (Sylt) clustered closer to each other.

Our analysis revealed significant statistical differences in the phylogenetic composition of communities from the Wadden Sea (Table 2), accounting for 39% of the variance ($p < 0.001$). Pairwise tests showed that the Schillig communities differed from the Wilhelmshaven communities whereas the communities from Wangerooge, Oddewatt, and List ferry pier were not statistically different at the phylogenetic level.

Comparison of the two identification methods

Taxa observed by LM and/or detected by metabarcoding were compared (Supplementary table S5, Fig. 52). Among the 73 morphospecies observed, the correspondence between LM and metabarcoding detections could be evaluated only for 45, which represented morphospecies with reference SSU rRNA gene sequences either already available or newly obtained in this study. Of these, 27 morphospecies were detected by both methods and 18 only by LM. The morphospecies not detected by metabarcoding included, most significantly, *Amphidiniopsis* spp. and *Herdmania litoralis* representatives, *Amphidinium bipes*, *Sabulodinium undulatum* and *Testudodinium testudo*. Those species were present in many analysed samples but never appeared in the metabarcoding results. After evaluating the ability of metabarcoding to detect the different morphospecies, we compared the correspondence of the two methods for dinoflagellate species detections along the different samples. While 47% of dinoflagellate species were detected by both methods, 42% were detected only by LM and the remaining 11% only by metabarcoding. Additional dinoflagellate species were detected only by metabarcoding and did not correspond to LM-observed species. Sequences belonging to 43 additional species were detected in the samples when taking into account ASVs with $> 98\%$ identity to the reference sequences and thus assigned to a given species with certainty. The addition of these newly detected species to those observed by LM resulted in 31% of the detected species confirmed by both methods, 29% observed only by LM and 40% only obtained by metabarcoding. However, many of the observed morphospecies lacked a reference sequence. Consequently, some of the ASVs that lacked high identity with reference sequences likely represented the molecularly uncharacterized species. The dissimilarity of the results obtained using the two approaches and only including the presence/absence of ASVs with $> 98\%$ identity to the reference sequences is depicted in Fig. 54. At each location, the dissimilarity of the communities obtained by microscopy was lower than that of the communities obtained by metabarcoding. However, the patterns were the same as those that emerged when the whole dinoflagellate metabarcoding dataset was taken into account. Samples from each location were of low dissimilarity while those from Wilhelmshaven were more distant to samples from the other locations. There was also a distance between communities characterized using the two approaches, but the general distribution of the samples was congruent.

Molecular characterization and phylogenetic relationships of taxa

To allow further phylogenetic assignments of metabarcoding SSU rRNA gene sequences, we focused our effort on determining sequences for undescribed species and for known species lacking reference sequences. LSU rRNA gene data were additionally generated to place the corresponding organisms more robustly in phylogenetic trees (Supplementary table S3, Fig. 55).

New sequences were obtained for several naked dinoflagellates. Although not detected in metabarcoding studies, *Amphidinium bipes* was present in the samples (Fig. 12) and its SSU rRNA gene sequence was successfully generated from single-cell isolations. Likewise, SSU and LSU rRNA gene sequences of *A. herdmanii* (Fig. 14) were determined, confirming their proximity with those already available in GenBank for this species. The two species clustered together (- % BS/0.99 BPP) and were included in a clade containing Kareniaceae members, i.e. *Karlodinium*, *Karenia* and *Takayama*, but without statistical support. Thus, they showed no relationship with other members of the *Amphidinium* genus. SSU and LSU rRNA gene sequences were also obtained for *A. incoloratum* (Fig. 15), which clustered with other *Amphidinium* representatives, such as *A. carterae* and *A. massarti* (100%/1). The SSU rRNA gene sequence of specimens identified as *Amphidinium corpulentum* were also determined (Fig. 13). Although GenBank contains an available sequence labelled *A. corpulentum*, it actually corresponds to *Togula jolla* (Flø Jørgensen *et al.*, 2004). Therefore, our sequence represents the first bona fide sequence available for this species. The sequence of *A. corpulentum* AR61 clustered within the *Gymnodinium sensu stricto* clade in the phylogenetic tree, confirming that this species does not belong to the genus *Amphidinium*. It formed a sister branch with sequences corresponding to undetermined gymnodinioid representatives. The LSU rRNA gene sequence of *Togula compacta* (Fig. 46) was previously available and the SSU rRNA gene sequence was determined in this study. All sequences corresponding to *Togula* species clustered together with maximum statistical support. Finally, the SSU rRNA gene of a morphospecies resembling *Apicoporus* representatives was also sequenced (Fig. 21). The phylogenetic tree confirmed its close relationship with *A. glaber*, albeit without statistical support, implying a new species yet to be described. The sequence of *A. parvidiaboli* clustered in a sister branch together with *Togula* representatives.

The newly sequenced SSU rRNA gene of a morphospecies initially identified as *Gymnodinium danicans* (Fig. 22) clustered within a clade that included sequences of *Durinskia* representatives (89%/1), forming a sister group with *D. agilis*. This suggests that *G. danicans* is not a naked species but a thecate, and probably a novel species of the genus *Durinskia*. This awaits confirmation in further studies, including observations of the thecal tabulation. The newly determined SSU and LSU rRNA gene sequences of the Gonyaulacales species *Psammadinium inclinatum* (= *Thecadinium inclinatum*), placed close to *Fukuyoa* and *Gambierdiscus* in the phylogenetic tree, allowed the reclassification of that species into a new genus (Reñé & Hoppenrath, 2019). The SSU and LSU rRNA gene sequences of a morphospecies identified as '*Katodinium*' *asymmetricum* (Fig. 25) showed a close relationship with the sequences of *Aduncodinium glandulum* (93%/1), within the Pfiesteriaceae family (Peridinales). The newly determined SSU rRNA gene sequence of *Planodinium striatum* (Figs 31, 32) clustered with those already available (100%/1) and occupied a basal position in the family Podolampadaceae, albeit without statistical support. Additional LSU and SSU rRNA gene sequences that were missing when we started this work were generated for other Podolampadaceae representatives, including *Roscoffia capitata* (Fig. 35) and *R. minor* (Figs 36, 37). The sequences of these two species formed sister clades, but again statistical support was poor.

rRNA gene sequences were also determined for several novel morphospecies that could not be identified based on the phenotypic characters observed by LM. However, their phylogenetic relationships did not provide any insights into their identity. The morphospecies identified as '*Gyrodinium*' spec 1 *sensu* Hoppenrath (2000d) (Fig. 48) did not show a close phylogenetic relationship with other dinoflagellate sequences. Thus, its identity remains elusive and it probably represents a new genus. The rRNA gene sequences of another two undetermined morphospecies corresponded to isolates AR06 (Fig. 49) and AR59 (Fig. 50). The sequence of isolate AR06 clustered within Podolampadaceae representatives but their relationships remain unclear. The sequence of isolate

AR59 formed a sister branch with *Moestrupia* representatives in a clade also including *Akashiwo sanguinea* sequences, but these relationships did not necessarily clarify its identity.

The phylogenetic positions of ASVs obtained by metabarcoding (Fig. 56) were used to determine the community composition of sand-dwelling dinoflagellates. Many ASVs corresponded to gymnodinioid representatives, including members of the Gymnodiniales sensu stricto clade and Amphidinales. Among Dinophysales, only *Sinophysis* members were detected. Suessiales members were scarcely detected, and those from Gonyaulacales were significantly missing from the samples analysed, with their occurrences mainly corresponding to *Thecadinium yashimaense*. Benthic representatives of Prorocentrales were also present in the samples as were those of different clades within the Peridinales, principally represented by Pfiesteriaceae members but also members of Podolampadaceae or Heterocapsaceae. Finally, ASVs attributable to naked dinoflagellates of unknown affiliation were relatively abundant in the sand-dwelling dinoflagellate communities, including those assigned to the genera *Ankistrodinium*, *Apicoporus* and *Togula*.

Discussion

Combining morphological and molecular approaches to characterize protist communities

Studies focused on determining protist diversity were traditionally based on microscopy observations but they have been gradually replaced by studies using molecular approaches. They allow better estimates of protist richness, even though they strongly rely on the previous availability of molecular information, while morphological identifications provide more accurate estimates of abundance and biomass (Santi *et al.*, 2021). Thus, many studies have combined microscopy observations with DNA metabarcoding to characterize protist communities. A strong correspondence between the two methods with respect to the diversity and relative abundance of different groups, such as dinoflagellates (Gran-Stadniczeňko *et al.*, 2019), has been reported, but significant differences have been described as well, attributed to incomplete reference databases for metabarcode classifications, differences in the copy number of ribosomal genes between taxonomic groups, and biases caused by primers and PCR amplification (Abad *et al.*, 2016; Santi *et al.*, 2021). The efforts to obtain SSU rRNA sequences for the taxa observed in the samples resulted in a low fraction of taxonomically unassigned metabarcodes, in most cases caused by incomplete database coverage. Additionally, problems related to low resolution of V4 SSU rRNA were not detected in the taxonomic assignments. This was also the case in a previous study, where the same amplicon used in this study satisfactorily identified 374 of the 422 dinoflagellate species with available molecular sequences (Mordret *et al.*, 2018). Nevertheless, there remains a need to combine and complement the results obtained by LM and metabarcoding in order to obtain a reliable characterization of the sample composition, as noted in several studies (Groendahl *et al.*, 2017; Gran-Stadniczeňko *et al.*, 2019; Santi *et al.*, 2021; Caracciolo *et al.*, 2022).

The difference in the communities characterized by LM and metabarcoding in this study reflected the intrinsic properties of each method. Microscopy identifications are highly dependent on the morphological characters observed, the recognition of (semi-) cryptic diversity, and the detection limits for low-abundance taxa. For metabarcoding, many biases can occur, due to inefficient cell lysis, biases in primers coverage, biased PCR amplifications, incomplete reference databases, or a lack of taxonomic resolution of the molecular barcode (Burki *et al.*, 2021). Nonetheless, the results of the two methods were complementary and in general agreement regarding sample groupings and differences between locations. The finding that 30% of the detected sand-dwelling dinoflagellates in the different

samples corresponded to species observed by LM but not by metabarcoding reflected the lack of a molecular signal, which can be due to many factors. Although an elution method was used to separate cells from sediments, the percentage of dinoflagellate reads was highly variable in the samples processed. In some of them, dinoflagellates represented < 5% of the total protist community. Consequently, morphospecies with low abundances were unlikely to have been recovered, due to stochastic effects. Additionally, metabarcoding and LM analyses were carried out from replicated samples. Given the low specimen numbers observed for some morphospecies by LM, the respective abundances may not have been the same in the three sample replicates; alternatively, the sequencing depth may not have sufficed to detect low-abundance taxa. However, species consistently absent in the metabarcoding dataset but observed by LM merit particular consideration. *Sabulodinium undulatum* was never detected by sequence, despite its presence in many of the analysed samples. Likewise, *Amphidiniopsis* representatives, including the closely related *Herdmania litoralis*, were also not detected even though they were regularly observed by LM, sometimes in significant cell numbers. Other remarkable cases included the naked species *Testudodinium testudo* and *Amphidinium bipes*. Since it is unlikely that all of these species were not detected because of their low abundance, the differential efficiency of the cell lysis may have played a role, especially for benthic dinoflagellates with thick protective thecae. However, several heavily armoured dinoflagellates were successfully lysed, and some of the missing species were naked. Another potential reason may be due to nucleotide mismatches with the eukaryotic universal primers used to amplify the V4 SSU rRNA gene sequences. However, ASVs corresponding to *Sabulodinium undulatum*, *Amphidiniopsis bulla* and *A. erinacea* were detected in a previous study using the same primer pair (Reñé *et al.*, 2021). Therefore, the reason for the lack of detection of some dinoflagellates by metabarcoding remains elusive and may well be the product of a confluence of factors.

Conversely, almost 40% of dinoflagellates were detected by metabarcoding but not by LM. Some of them corresponded to planktonic dinoflagellates and others to species never observed in the study area. Sediments harbour and accumulate extracellular environmental DNA that can be transported from distant locations (Pawlowski *et al.*, 2022). Thus, many detections could correspond to DNA from dead specimens or extracellular (e) DNA (Nagler *et al.*, 2018) rather than species inhabiting the sampling site. Our results show that, although a large fraction of the diversity of sand-dwelling dinoflagellates was detected only by metabarcoding, some important components of that community are still missing from the molecular inventory. The inclusion of LM observations avoided the significant biases caused by the presence of large amounts of eDNA in sediments. These results demonstrate the importance of combining LM-based detections and identifications with high-throughput sequencing to obtain a complete and reliable characterization of dinoflagellate diversity.

Diversity of the sand-dwelling dinoflagellate community

Early studies focusing on sand-dwelling dinoflagellate diversity observed and described a limited number of species, and many observed taxa were later reclassified. Nonetheless, those descriptions established the basis for subsequent studies. The locations of those early studies included the Isle of Man, UK (Herdman, 1922), the Brittany Coast, France (Balech, 1956; Dragesco, 1965) and British Columbia, Canada (Baillie, 1971). Later studies conducted in additional locations, including Japan (Montani & Huang, 1998), the Danish Wadden Sea (Larsen, 1985) and diverse tropical areas (Larsen & Patterson, 1990), provided further knowledge on benthic and sand-dwelling dinoflagellates. The numerous taxonomic studies describing new species present in sediments have been accompanied by thorough characterizations of the respective communities. Compared with other studies, the sampling effort in our study yielded a relatively large number of morphospecies (73), suggesting that the

sampling and observation efforts enabled us to detect most representative taxa present in the area. In a study conducted in the north-east English Channel (Gómez & Artigas, 2014) and the Russian Sea of Japan (Selina, 2016), 70 and 67 morphospecies were detected, respectively, but studies at other locations resulted in fewer morphospecies: 36 in Botany Bay, Australia (Murray, 2003), 32 in Japan (Tamura, 2005), 24 along Malaysian coastal areas (Mohammad-Noor *et al.*, 2004), 43 in Kuwait sediments (Saburova *et al.*, 2009; Al-Yamani & Saburova, 2010) and 27 along the west coast of Korea (Kim *et al.*, 2015).

Epiphytic dinoflagellate communities from Australia and New Zealand were previously characterized using metabarcoding (Kohli *et al.*, 2014a, 2014b; Smith *et al.*, 2017). In both cases, the number of operational taxonomic units attributed to dinoflagellates was quite limited, suggesting that those communities were less diverse than the communities inhabiting sediments. Another study used metabarcoding to characterize epiphytic communities from Japanese coastal waters, but the analyses of dinoflagellates were limited to the diversity of *Gambierdiscus* representatives (Funaki *et al.*, 2022). The community composition of sand-dwelling dinoflagellates present along the Catalan coast, NW Mediterranean Sea, was also determined by metabarcoding (Reñé *et al.*, 2021). ASVs were assigned to 98 different known species, a diversity similar to that obtained in this study. The proportion of ASVs classified as 'Unknown', because they could not be assigned to any described species or genus, ranged from 17–30% at Mediterranean locations, but 'Unknown' ASVs accounted for only 10% of the samples from the German sites, despite the use in the latter study of conservative thresholds to assign the ASVs. This difference reflects the fact that the components of sand-dwelling communities from the Wadden Sea are well known, given the many taxonomic studies conducted in the area, while the diversity of Mediterranean communities remains mostly unexplored, and molecular reference sequences are accordingly missing for many of the species present in those communities. Yet, an especially diverse benthic Mediterranean dinoflagellate community can be expected, as a preliminary investigation of samples from Elba (Italy) revealed 90 morphospecies (Hoppenrath *et al.*, 2014), including undescribed species (Hoppenrath, pers. comm.)

The well-characterized diversity of sand-dwelling dinoflagellates in the German Wadden Sea includes a study that determined the species composition of benthic dinoflagellates at the Sylt locations Oddewatt and List ferry pier over a 2 year period, which yielded observation of 52 and 51 different morphospecies, respectively (Hoppenrath, 2000d). The presence of 27–29 different morphospecies at Oddewatt and 24–31 at List ferry pier was determined in samples taken during September 1997 and 1998. Those values agree with the number of morphospecies observed by LM in this study for the samples obtained at the same locations in September (30 and 31, respectively). The similarity suggests that the richness of sand-dwelling dinoflagellates has not changed significantly during the 20 years separating the studies.

Information available from the study area prior to our study did not include records of '*Katodinium*'/ *Aduncodinium glandulum* and '*Katodinium*' sp., *Amphidinium bipes* and *A. carterae*, or *Apicoporus glaber*, all of which were newly recorded in our study. This does not necessarily imply a change in species composition but rather the fact that in previous studies only individual samples were investigated despite the known patchiness of species distributions in sediments. *Speroidium fungiforme* was not documented in an earlier report (Hoppenrath, 2000d), most likely due to the misidentification of these tiny, fast-moving cells. Other taxa were recorded only after a thorough study (Hoppenrath, 2000d) while for *Thecadinium yashimaense* its invasion and establishment have been documented and discussed (Hoppenrath *et al.*, 2007). A new *Adenoides* species was discovered in 2009 (Hoppenrath, pers. obs.), and *Spiniferodinium galeiforme*, previously documented in the

Netherlands (Haupt & Hoppenrath, 2006), has now been newly detected in the East Frisian Wadden Sea, at Wangerooge (this study). The metabarcoding record of an ASV with 99% identity to *S. palustre*, a freshwater species (Kretschmann *et al.*, 2015), is curious. As the samples were obtained from fully marine sites, no explanation for the detection can be offered yet. As mentioned above, *Nusuttodinium* morphospecies were recorded, but metabarcoding ASVs did not show a match with available sequences of two freshwater *Nusuttodinium* species. In that case, we proposed unknown species diversity. In addition, *Blixaea quinquecorne* is a new finding in the German Wadden Sea. The most recently described genus *Bindiferia* (Borchhardt *et al.*, 2021) was presumed to be present in the German Wadden Sea but it was not recorded before this study. Likewise, the detection by metabarcoding of *Stylodinium litorale* represents a new record for the German Wadden Sea.

Detection of unknown diversity

Many of the dinoflagellate species detected in our study remain to be described. Some of the morphospecies could be assigned to a given genus but represented an unknown species, such as members of the genera *Apicoporus*, '*Katodinium*', *Roscoffia* and *Planodinium*, while others probably belong to new genera, including the morphospecies identified as Podolampadaceae gen. nov. and cf. *Amphidiniopsis* gen. nov. The morphological characters of other specimens did not suffice for a tentative classification, such as unidentified AR06, AR59, '*Gyrodinium*' spec1 *sensu* (Hoppenrath, 2000d), and their phylogenetic positions did not provide robust information about their taxonomic assignment. Further taxonomic studies are needed to complete the characterization of the diversity present at the study sites. Furthermore, some morphospecies were assigned to known species but the molecular barcodes did not show a 100% correspondence with the available sequences, only a close identity (95–98%), suggesting unknown (pseudo)cryptic diversity for some of them. This may have been the case for the morphospecies identified by LM as *Amphidinium carterae*. The identity of its closest ASVs with reference sequences was only 95.5–98.8%, indicating the occurrence of an as-yet unidentified species diversity of small phototrophic *Amphidinium sensu stricto* species as well as the need for further descriptive work. For the morphospecies identified as *Nusuttodinium latum*, *N. poecilochroum*, *Sinophysia stenosoma* and *S. ebriola*, none of the ASVs could be assigned to them, but some showed a close identity (92–95%) with sequences of members of the *Nusuttodinium/Pellucidodinium* group and *Sinophysia* representatives. Those ASVs may thus have corresponded to the morphospecies observed by LM but representing unknown phylogenetic diversity. Surprisingly, *Polykrikos lebouriae* and *P. herdmaniae* were observed by LM but only the latter species was detected by metabarcoding, including in samples in which only *P. lebouriae* was observed microscopically. As both species occurred in a sample from Schillig, it is likely that they were also present in other samples.

Since the V4 barcode can separate the two species, the results cannot convincingly be explained. Molecular information obtained from single-cell sequencing and from metabarcoding also suggested that two new *Durinskia* species were present in the samples. One corresponded to the morphospecies identified as *Gymnodinium danicans* but the phylogenetic analysis placed that species within the *Durinskia* genus. The other, observed in the Oddewatt sample, was previously referred to as gen. et sp. nov. in Hoppenrath (2000d) but later identified as *Durinskia baltica* (Hoppenrath *et al.*, 2014). An ASV showing 98.5% identity with *Durinskia dybowsky* probably corresponded to the observed morphospecies. A better taxonomic assignment of metabarcodes awaits the generation of more SSU rRNA gene sequences.

Numerous ASVs were also assigned to the same few species, such as *Togula compacta*, *Thecadinium yashimaense* and *Ankistrodinium semilunatum*, and represented intraspecific variability. This was also

the case in previous studies and it mostly involved species detected at higher relative abundances (Reñé *et al.*, 2021), as in the present study. Intraspecific variability in dinoflagellate ribosomal genes has been documented for representatives of the genera *Protooperidinium*, *Preperidinium*, *Diplopsalis* and Dinophysales (Gribble & Anderson, 2007; Handy *et al.*, 2009).

Spatiotemporal variability of benthic dinoflagellate communities

The geographic distribution and temporal variability of benthic protist communities have been explored in studies that used metabarcoding to determine the factors and processes shaping those communities. In some of those studies, temperature was proposed to be a main driver of community structure and dynamics (Gong *et al.*, 2015; Kong *et al.*, 2019; Salonen *et al.*, 2019) whereas other studies implicated processes such as distance-decay or a combination of deterministic and stochastic processes (Chen *et al.*, 2017; Zhang *et al.*, 2018; Pan *et al.*, 2019). Our study assessed the community of sand-dwelling dinoflagellates in different locations of the German Wadden Sea. Comparative studies of community composition at three Mediterranean locations previously showed low dissimilarity (Reñé *et al.*, 2021). By contrast, the communities from some locations explored in this study differed remarkably, with comparative analyses of their phylogenetic composition showing statistical differences between Schillig and Wilhelmshaven samples but not between samples from the other locations. While many features of the Mediterranean locations are similar (sediment grain-size, beach morphology), those of locations from the Wadden Sea are more divergent, reflected in the associated communities. Wilhelmshaven is located in Jade Bay, whose mudflats have a mud content as high as 76% (Schückel *et al.*, 2013) and a total organic carbon content (mean 1.05%) up to twice as high as that of North Sea locations such as the Otzumer Balje (mean 0.5%) (Reiss & Kröncke, 2001; Schückel *et al.*, 2015). The bay is considered to be a tidal bay rather than an estuary because freshwater discharge has little influence on the salinity of its waters (Schückel & Kröncke, 2013). The connection of Jade Bay with the North Sea is through a tidal channel. The margins of the bay's northern entrance, where Schillig is located, are sandflats, and the same habitat features characterize the island of Wangerooge, located northwards (Schückel *et al.*, 2013). Sediments from the Sylt locations are predominantly sandy, but coarse-grained sediments are deposited by wind-blown dunes (Reise *et al.*, 1994). The significant differences in sediment composition between Wilhelmshaven and the rest of the sampling locations suggest this feature as the main driver of community assembly. In fact, sediment composition has also been suggested as the main factor shaping different macrofaunal communities in Jade Bay compared with other Wadden Sea areas (Schückel *et al.*, 2013). Similarly, in a study of benthic protist communities from the sandy beaches of British Columbia, Canada, grain size was proposed as a main factor determining diversity and community composition (Okamoto *et al.*, 2022).

Sampling at the study sites was conducted during the summer months (June–September) and during two different years (2017 and 2018) for Schillig and Wilhelmshaven. Our results suggest that, during summer, the communities at each location are stable. As noted above, temperature may be a major factor shaping dinoflagellate benthic communities over time (Huang *et al.*, 2020). This conclusion is supported by the greater similarity and reduced diversity of communities from three Mediterranean sites during warm months (Reñé *et al.*, 2021) and the high similarity of the samples obtained during the summer months from Schillig and Wilhelmshaven, regardless of the sampling year. The similarity of the interannual composition of protist communities during the summer months was likewise reported in a previous study (Okamoto *et al.*, 2022).

In summary, our study demonstrates the benefits of combining morphological and molecular approaches to fully characterize benthic protist communities. The complementary results obtained with these two methods revealed the presence of uncharacterized diversity among sand-dwelling dinoflagellates and thus the need for greater efforts aimed at obtaining molecular sequences of known representatives. However, despite insights into the spatio-temporal distribution of species, the information is incomplete, and further studies are needed, including year-round community surveys at selected sites to determine community dynamics and a characterization of the abiotic factors that may underlie differences in community compositions. As all of the samples in our study were obtained from coastal intertidal locations, additional sampling locations, representing different sediment types and different depths (also sublittoral), would provide a more complete picture of benthic dinoflagellate communities from the German Bight.

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Supplementary information

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Author contributions

A. Reñé: original concept, sampling, microscopy, phylogenetics, metabarcoding, drafting and editing manuscript; N. Timoneda: metabarcoding, editing manuscript; S. Khodami: metabarcoding, editing manuscript; P. López-García: funding, editing manuscript; P. Martínez Arbizu: funding, editing manuscript; M. Hoppenrath: original concept, sampling, microscopy, funding, drafting and editing manuscript.

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Table 1. List of morphospecies detected (shaded areas) by light microscopy, and the total number of morphospecies observed in each sample. WHV: Wilhelmshaven, SCH: Schillig, WGR: Wangerooge, LIST: List ferry pier, ODD: Oddewatt.

Morphospecies	WHV1	WHV2	WHV3	WHV4	WHV5	WHV6	SCH1	SCH2	SCH3	SCH4	WGR	LIST	ODD
<i>Adenoides</i> sp. nov.													
<i>Aduncodinium</i> / <i>Katodinium</i> ' <i>asymmetricum</i> complex													
<i>Aduncodinium glandulum</i> (sensu Hoppenrath 2000d)													
<i>Aduncodinium</i> / <i>Katodinium</i> ' sp. nov.													
<i>Amphidiniella</i> sp. (sensu Murray 2003)													
<i>Amphidiniopsis</i> cf. <i>arenaria</i>													
<i>Amphidiniopsis dragescoi</i>													
<i>Amphidiniopsis hirsuta</i>													
<i>Amphidiniopsis</i> cf. <i>konovalovae</i>													
<i>Amphidiniopsis rotundata</i>													
<i>Amphidiniopsis</i> sp.													
<i>Amphidiniopsis swedmarkii</i>													
cf. <i>Amphidiniopsis</i> gen. nov. spec1 (sensu Hoppenrath 2000d)													
cf. <i>Amphidiniopsis</i> gen. nov. spec2 (sensu Hoppenrath 2000d)													
<i>Amphidinium bipes</i>													
<i>Amphidinium carterae</i> / <i>massartii</i>													
<i>Amphidinium</i> cf. <i>steinii</i> (<i>A. operculatum</i> sensu Hoppenrath 2000d)													
<i>Amphidinium corpulentum</i>													
<i>Amphidinium herdmanii</i>													
<i>Amphidinium incoloratum</i>													
<i>Amphidinium mootonorum</i>													
<i>Amphidinium psittacus</i>													
<i>Amphidinium scissum</i>													
<i>Amphidinium</i> sp. 1 (sensu Hoppenrath 2000d)													
<i>Amphidinium</i> sp. 3 (sensu Hoppenrath 2000d)													
<i>Amphidinium</i> sp. (heterotroph A)													
<i>Amphidinium</i> sp. (heterotroph B)													
<i>Amphidinium</i> sp. (heterotroph C)													
<i>Amphidinium</i> sp. (heterotroph D)													
<i>Amphidinium</i> sp. (phototroph E)													
<i>Ankistrodinium semilunatum</i>													
<i>Apicoporus glaber</i>													
<i>Apicoporus</i> sp.													
<i>Bindiferia</i> sp.													
<i>Blixaea quinquecorne</i>													
<i>Carinadinium acanthium</i>													
<i>Durinskia</i> sp. (= <i>Durinskia baltica</i> sensu Hoppenrath et al. 2014)													
<i>Durinskia</i> sp. (= <i>Gymnodinium danicans</i>)													
<i>Gymnodinium</i> sp. 1 (sensu Hoppenrath 2000d)													
<i>Gymnodinium venator</i> (syn. <i>Amphidinium pellucidum</i>)													
' <i>Gyrodinium</i> ' spec. 1 (sensu Hoppenrath 2000d)													
<i>Hemidinium</i> sp.													
<i>Herdmania litoralis</i>													
<i>Herdmania</i> sp.													
<i>Nusuttodinium latum</i>													
<i>Nusuttodinium poecilochroum</i>													
cf. <i>Nusuttodinium</i> sp.													
<i>Planodinium striatum</i>													
<i>Planodinium</i> sp. nov.													
<i>Polykrikos herdmaniae</i>													
<i>Polykrikos lebouriae</i>													
<i>Prorocentrum</i> cf. <i>foraminosum</i>													
<i>Prorocentrum fukuyoi</i>													
<i>Prorocentrum</i> cf. <i>fukuyoi</i> (small)													
<i>Psammodinium inclinatum</i>													

Table 1. (Continued).

Morphospecies	WHV1	WHV2	WHV3	WHV4	WHV5	WHV6	SCH1	SCH2	SCH3	SCH4	WGR	LIST	ODD
<i>Pseudadenoides kofoidii</i> (syn. <i>Adenoides eludens</i> Balech)													
<i>Roscoffia capitata</i>													
<i>Roscoffia minor</i>													
<i>Roscoffia</i> sp. nov.													
<i>Sabulodinium undulatum</i>													
<i>Sabulodinium</i> sp. nov.													
<i>Sinophysis ebriola</i>													
<i>Sinophysis grandis</i>													
<i>Sinophysis</i> cf. <i>stenosoma</i>													
<i>Sinophysis</i> cf. <i>vesperilio</i>													
<i>Sinophysis</i> sp.													
<i>Speroidium fungiforme</i>													
<i>Spiniferodinium galeiforme</i>													
<i>Testudodinium testudo</i>													
<i>Thecadinium neopetasatum</i>													
<i>Thecadinium yashimaense</i>													
<i>Thecadinium</i> sp. nov. (<i>sensu lato</i>)													
<i>Togula britannica</i>													
<i>Togula compacta</i>													
Undetermined AR06													
Undetermined AR59													
TOTAL MORPHOSPECIES OBSERVED	20	12	18	25	14	18	22	22	17	19	38	30	31

Table 2. Statistical values obtained in global and pairwise tests of phylogenetic distance between locations. Statistically significant relationships ($p < 0.05$) are shown in bold.

Global test	Location			
$R^2 = 0.389$ $p < 0.001$	Wangerooge	Wilhelmshaven	Sylt Oddewatt	Sylt List ferry pier
Schillig	$R^2 = 0.19, p = 0.42$	$R^2 = 0.23, p = 0.01$	$R^2 = 0.269, p = 0.39$	$R^2 = 0.261, p = 0.35$
Wangerooge		$R^2 = 0.194, p = 0.12$	$R^2 = 0.633, p = 1$	$R^2 = 0.611, p = 1$
Wilhelmshaven			$R^2 = 0.211, p = 0.11$	$R^2 = 0.201, p = 0.12$
Sylt Oddewatt				$R^2 = 0.607, p = 1$

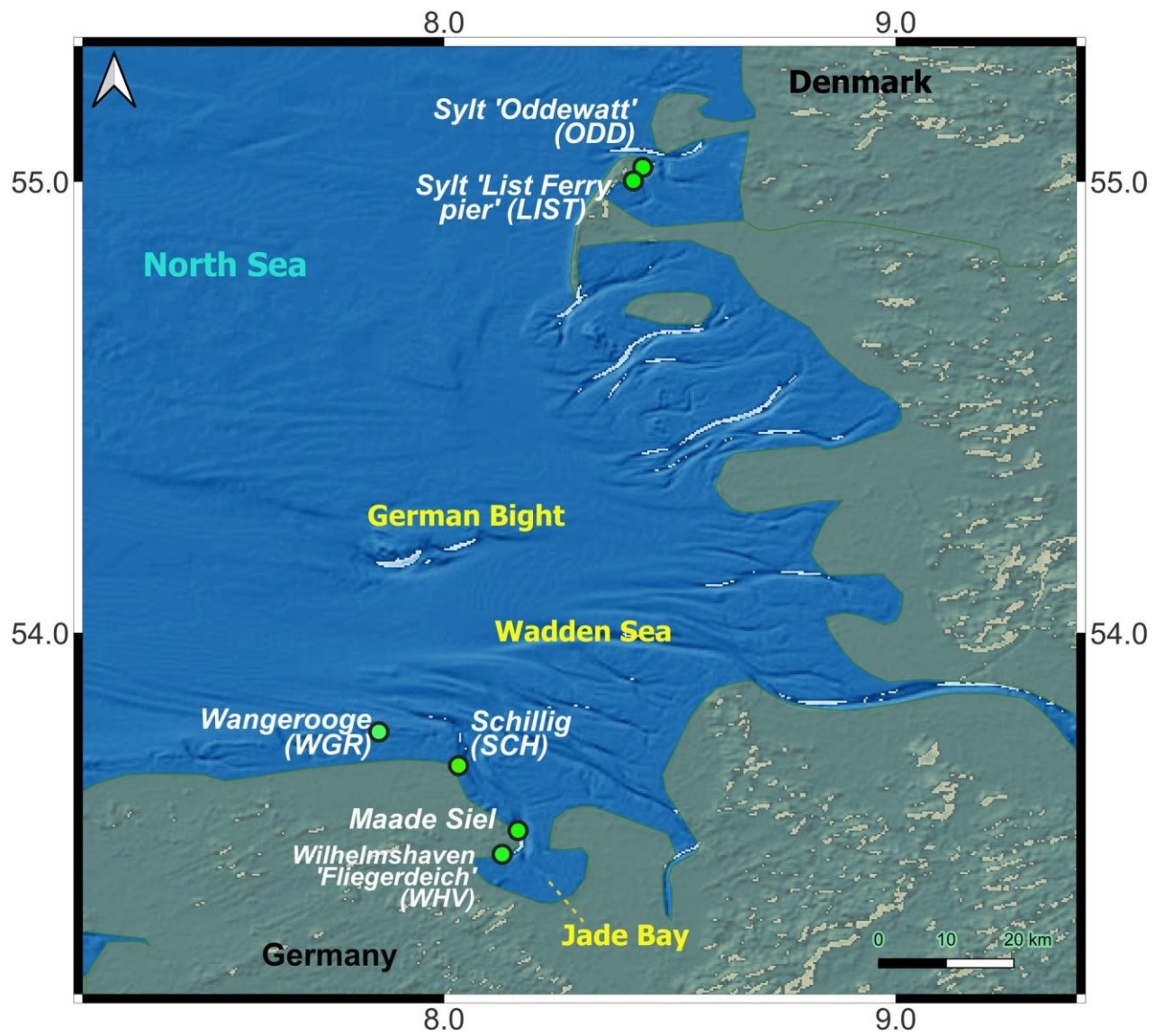
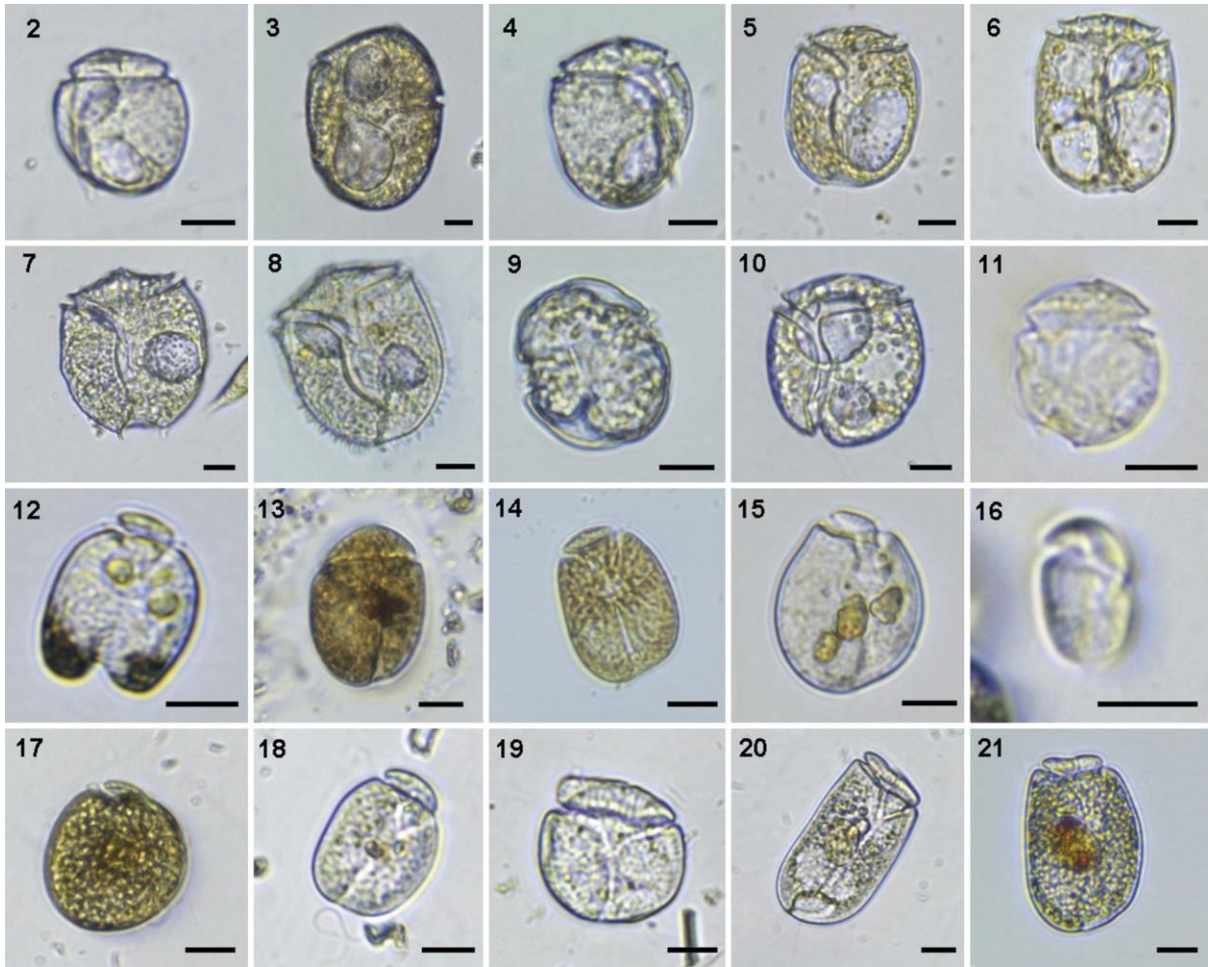
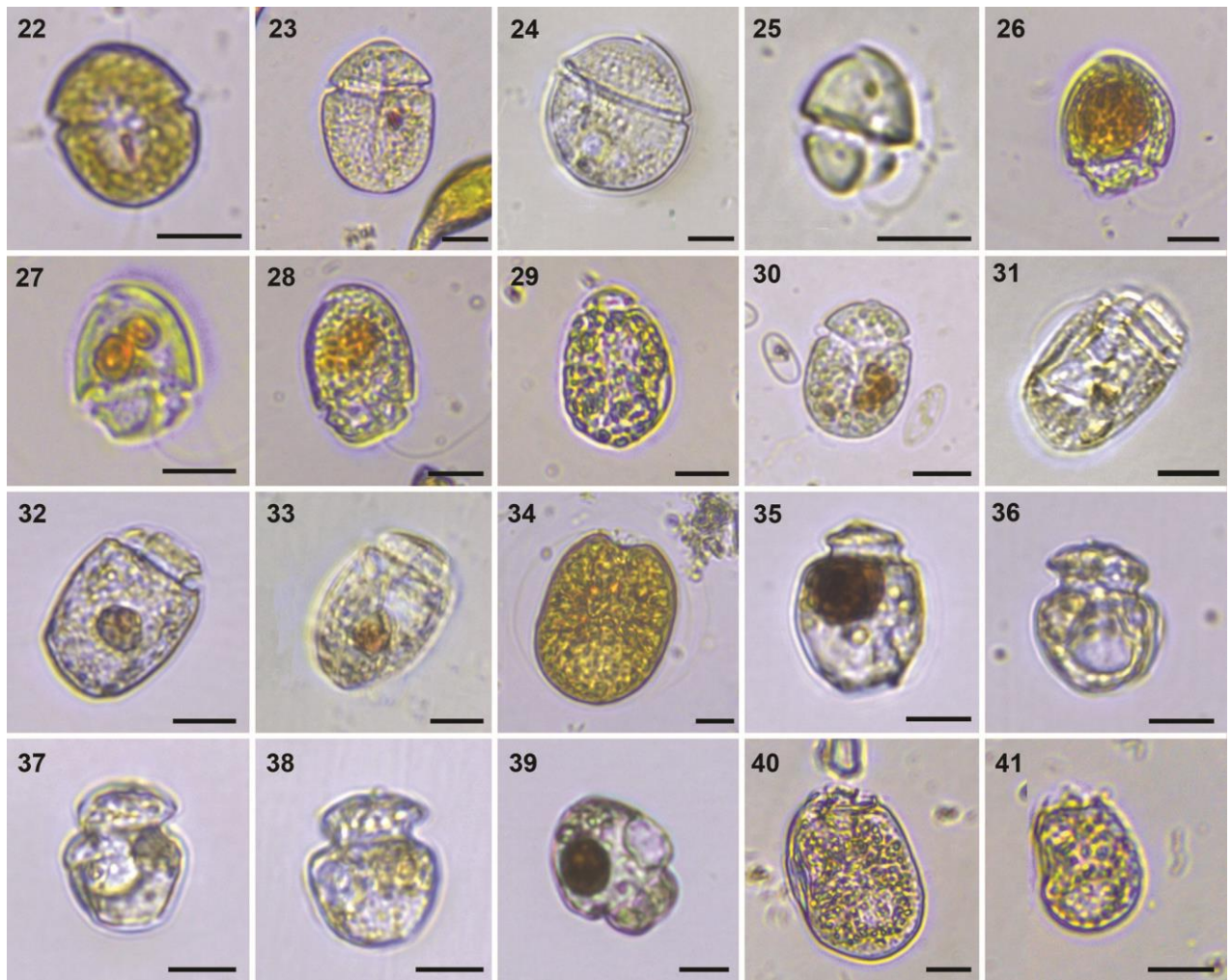


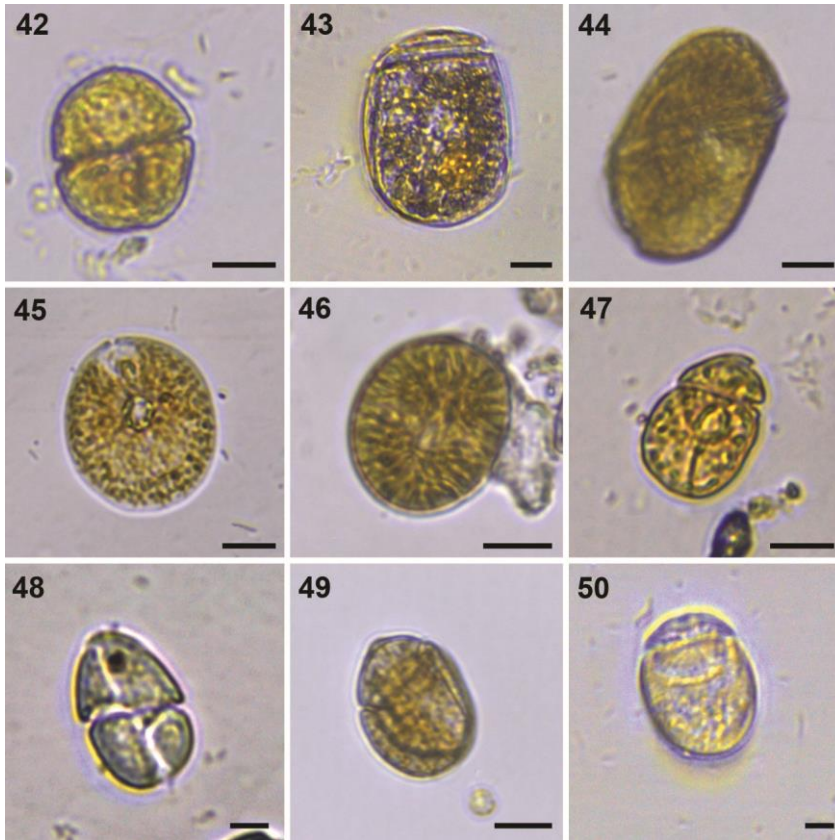
Fig. 1. Location of the sampled stations. Map of the North Sea area (German Bight) showing the sampling locations in the German Wadden Sea.



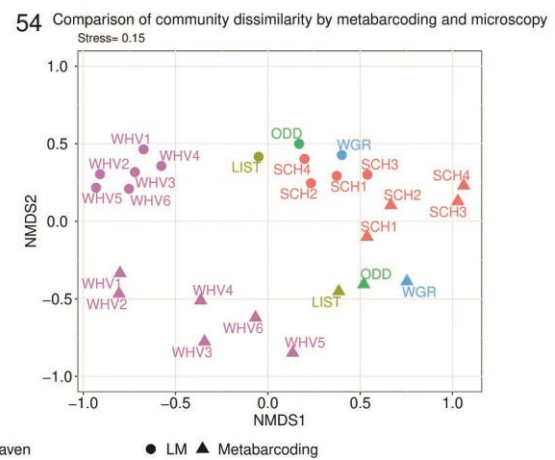
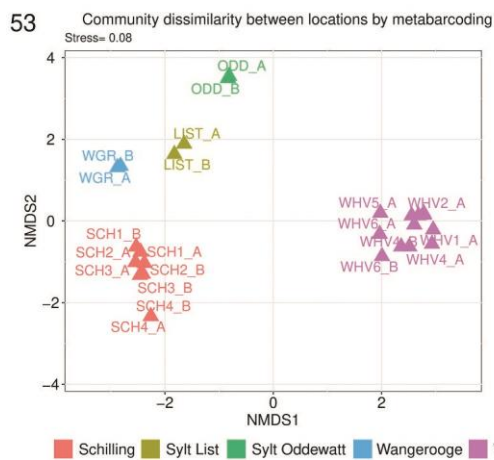
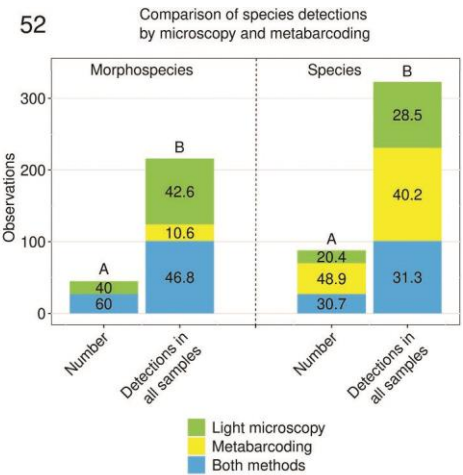
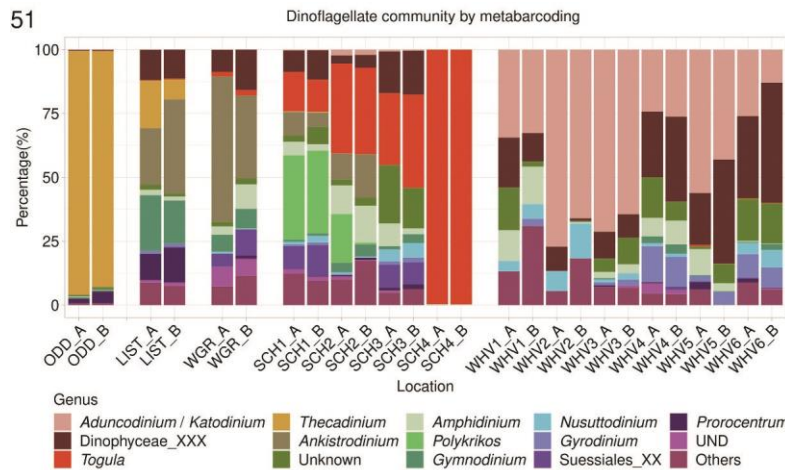
Figs 2–21. Light microscopy images of cells observed and isolated for molecular sequencing. **Fig. 2.** *Amfidiniopsis* sp. **Fig. 3.** *Amfidiniopsis dragescoi*. **Fig. 4.** *Amfidiniopsis* cf. *kofoidii*. **Fig. 5.** *Amfidiniopsis konovalovae*. **Fig. 6.** *Amfidiniopsis* sp. **Fig. 7.** *A. swedmarkii*. **Fig. 8.** *Amfidiniopsis hirsuta*. **Fig. 9.** *Amfidiniopsis* aff. *rotundata*. **Fig. 10.** cf. *Amfidiniopsis* gen. nov. spec 2¹. **Fig. 11.** cf. *Amfidiniopsis* gen. nov. spec 1¹. **Fig. 12.** *Amphidinium bipes* (AR07). **Fig. 13.** *Amphidinium corpulentum* (AR61). **Fig. 14.** *Amphidinium herdmanii* (AR37). **Fig. 15.** *Amphidinium incoloratum* (Ext. C). **Fig. 16.** *Amphidinium psittacus*. **Fig. 17.** *Amphidinium* sp. **Fig. 18.** *Amphidinium sensu lato*. **Fig. 19.** *Amphidinium sensu lato* 2. **Fig. 20.** *Apicoporus glaber*. **Fig. 21.** *Apicoporus* sp. (AR05). Codes in parentheses refer to the corresponding single-cell isolates and sequences. Scale bars = 10 μ m. ¹The genus has been recently described as *Aliferia* (Selina *et al.*, 2023)



Figs 22–41. Light microscopy images of cells observed and isolated for molecular sequencing. **Fig. 22.** *Durinskia* sp. (AR13). **Fig. 23.** *Gymnodinium venator*. **Fig. 24.** *Herdmania litoralis*. **Fig. 25.** ‘*Katodinium*’ *asymmetricum* (Ext. E). **Figs 26, 27.** ‘*Katodinium*’ sp. **Fig. 28.** ‘*Katodinium*’ spec 2 *sensu* Hoppenrath (2000d). **Fig. 29.** *Nusuttodinium poecilochroum*. **Fig. 30.** *Nusuttodinium* sp. **Figs 31, 32.** *Planodinium striatum* (AR23). **Fig. 33.** *Planodinium* sp. nov. **Fig. 34.** *Prorocentrum fukuyoi*. **Fig. 35.** *Roscoffia capitata* (AR16). **Figs 36, 37.** *Roscoffia minor* (AR18, AR19). **Fig. 38.** *Roscoffia* sp. nov. **Fig. 39.** Podolampadaceae gen. nov. **Fig. 40.** *Sinophysis* sp. **Fig. 41.** *Sinophysis* cf. *vespertilio*. Codes in parentheses refer to the corresponding single-cell isolates and sequences. Scale bars = 10µm.



Figs 42–50. Light microscopy images of cells observed and isolated for molecular sequencing. **Fig. 42.** *Spiniferodinium galeiforme*. **Fig. 43.** *Thecadinium* sp. **Fig. 44.** *Thecadinium yashimaense*. **Fig. 45.** *Testudodinium testudo*. **Fig. 46.** *Togula compacta* (Ext. D). **Fig. 47.** *Amphidiniella* sp. *sensu* Murray (2003).² **Fig. 48.** ‘*Gyrodinium*’ spec1 *sensu* Hoppenrath (2000d) (Ext. A). **Fig. 49.** Undetermined (AR06). **Fig. 50.** Undetermined (AR59). Codes in parentheses refer to the corresponding single-cell isolates and sequences. Scale bars = 10 μm . ²The species has been recently described as *Coutea sabulosa* (Chomérat *et al.*, 2023).



Figs 51–54. Structure of sand-dwelling communities. Successive samplings performed in the same location are indicated by numbers, as shown in Table S1, and replicates are indicated by A and B. SCH (Schilling), WGR (Wangerooge), LIST (Sylt List ferry pier), ODD (Sylt Oddewatt) and WHV (Wilhelmshaven). **Fig. 51.** Barplot of community composition, showing the relative abundance of ASVs, grouped at the genus level, obtained at each sample (in replicates A and B) from each location. The 14 most abundant genera are shown and the rest are grouped under the ‘Others’ category. UND = Undetermined Naked Dinoflagellates. Unknown = Dinoflagellate ASVs showing < 90% identity with any reference sequence. **Fig. 52.** Barplot comparing the number of species detected by light microscopy (LM), metabarcoding, and both methods along all samples analysed. Left, numbers only considering the morphospecies that were observed by microscopy in any sample and for which molecular sequences are available. Right, numbers also including species never observed by microscopy during the study, thus only detected by metabarcoding, and with > 98% similarity to any reference sequence. Numbers inside the bars represent the percentage (%). A columns correspond to the number of (morpho-)species observed. B columns correspond to the total detections of those species sample by sample. **Fig. 53.** Non-metric multidimensional scaling using Bray–Curtis distances, showing the dissimilarity between communities from each location based on metabarcoding-determined relative abundances. **Fig. 54.** Non-metric multidimensional scaling using Bray–Curtis distances, comparing the community dissimilarities determined by LM and metabarcoding (presence/absence) for each sample. Only morphospecies for which molecular sequences were available and ASVs with > 98% similarity to any reference sequence were used.

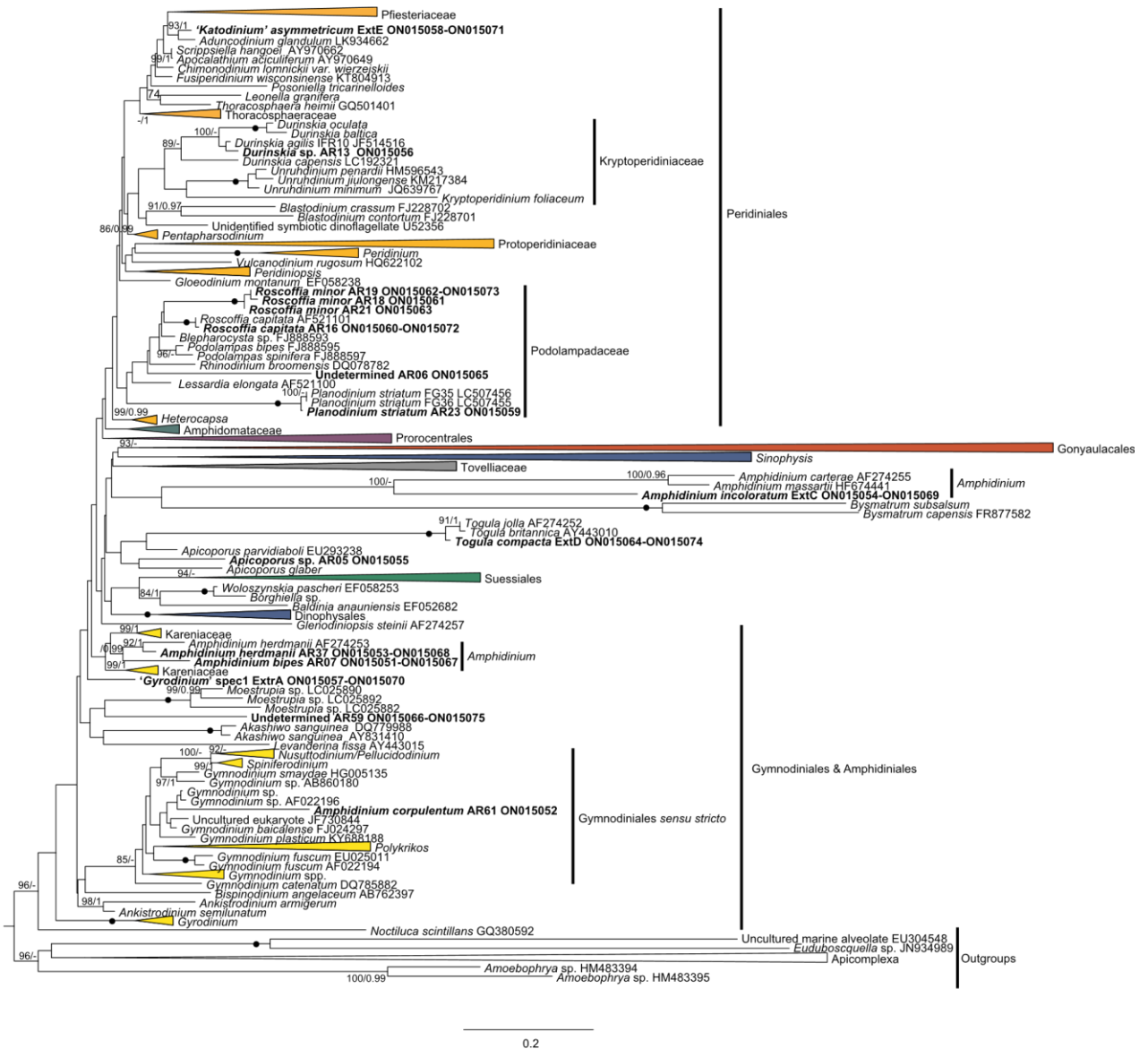


Fig. 55. Maximum-likelihood phylogenetic tree of concatenated SSU + ITS + LSU rRNA gene sequences of dinoflagellates representative of major phylogenetic groups. Sequences of Syndiniales and Apicomplexa served as outgroups. The different colours used for collapsed clades represent the different dinoflagellate orders. New sequences obtained in this study are shown in bold. The numbers in nodes represent the bootstrap values (%) and Bayesian posterior probability. Only values $\geq 80\%$ and ≥ 0.9 , respectively, are shown; black dots indicate maximum statistical support.

- Phylogenetic group
- Gonyaulales
 - Dinophysales
 - Suessiales
 - Gymnodinales & Amphidinales
 - Procoentrales
 - Amphidomataceae
 - Peridinales
 - Uncertain naked
 - Uncertain thecate
 - Outgroup

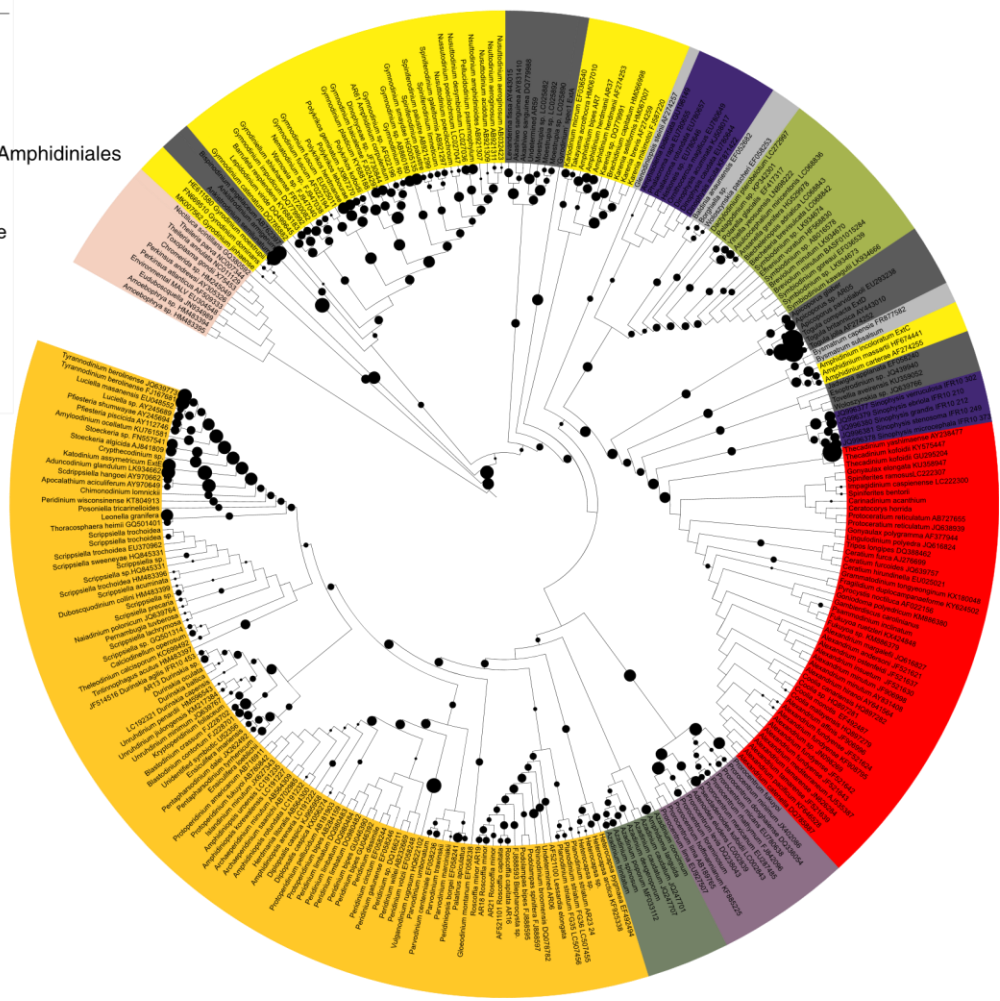


Fig. 56. Circular cladogram of the phylogenetic relationships of species belonging to major phylogenetic groups using SSU +ITS+LSU rRNA gene sequences and the inferred evolutionary position of ASVs obtained in this study, represented as black dots. The size of the dots reflects the read abundance of each ASV. Sequences are coloured at the order level. The ASVs resolved only at high taxonomic levels are positioned at deeper branches of the cladogram.